Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1023-1030

THE ROLE OF DIPHOSPHATIDYLINOSITOL IN ERYTHROCYTE MEMBRANE SHAPE REGULATION[1]

Eugene E. Quist and Karen L. Reece

Department of Pharmacology
School of Medicine
University of Minnesota, Duluth
Duluth, Minnesota 55812

Received June 23,1980

SUMMARY

Rabbit erythrocyte ghosts were transformed from echinocytes to discocytes by 1 mM Mg-ATP at 25°C. This shape transformation was completely inhibited by either 0.5 mM neomycin or 10 μM Ca^{2+}. Either agent could also transform discocytic ghosts formed by preincubation with 1 mM Mg-ATP back to echinocytes. Under identical conditions, 10 μM Ca^{2+} or 0.5 mM neomycin inhibited the formation of ^{32}P labeled diphosphatidylinositol by 80% in the presence of 1 mM Mg-[γ-^{32}P]-ATP. Ca^{2+} and neomycin also decreased diphosphatidylinositol prelabeled with ^{32}P by 80%. The results of this study suggest that Mg-ATP induces an echinocytic-discocytic shape transformation by stimulating the formation of membrane diphosphatidylinositol through phosphorylation of a membrane substrate. Neomycin and Ca^{2+} may produce shape changes by decreasing the levels of diphosphatidylinositol. The effects of neomycin and Ca^{2+} on the phosphorylation of bands 2 and 3 proteins and other phospholipids were also determined.

INTRODUCTION

Human erythrocyte ghosts are transformed from echinocytic to discocytic or cup-shaped forms in the presence of Mg-ATP (1,2,3). Mg-ATP can also specifically induce folding of unsealed freeze-thawed erythrocyte membrane fragments (4,5). Birchmeier and Singer (6) proposed that Mg-ATP induced membrane shape changes were mediated by a Mg^{2+} kinase which phosphorylates a membrane site, possibly band 2 of spectrin. Mg-ATP dependent shape transformations in erythrocyte membranes are inhibited by low concentrations of Ca^{2+} (2,3,4). Ca^{2+} in the presence of A23187 converted discocytic ghosts, preincubated with Mg-ATP, to echinocytes and also stimulated dephosphorylation of erythrocyte membranes by 25% (3). It was suggested that Ca^{2+} may induce these shape changes by stimulating a membrane Ca^{2+} phosphatase activity which dephosphorylates the

1. The abbreviations used are: EGTA, ethylene-glycol bis (β-aminoethyl ether)-N; N'-tetraacetic acid; SDS, sodium dodecylsulfate; PA, phosphatidic acid; DPI, diphosphatidylinositol; TPI, triphosphatidylinositol.

0006-291X/80/151023-08$01.00/0

site phosphorylated by a Mg^{2+} kinase activity. Presently, the effect of Ca^{2+} on phosphorylation of spectrin is controversial (6,7,8) and therefore it is uncertain whether spectrin is the site of phosphorylation involved in initiating shape transformations. In addition, other workers have shown that relatively high concentrations of Ca^{2+} ($\geq$ .5 mM) can stimulate dephosphorylation of membrane diphosphatidylinositol and triphosphatidylinositol indicating the possibility that these phospholipids may be involved in shape regulation (9,10,11).

In this study, neomycin and Ca^{2+} were found to inhibit and reverse Mg-ATP dependent shape changes in rabbit erythrocyte ghosts. Therefore, the effects of both neomycin and Ca^{2+} on Mg-ATP dependent phosphorylation of membrane proteins and phospholipids were compared under conditions in which they affect shape to identify the phosphorylated site involved in regulating membrane shape.

MATERIALS AND METHODS

Materials. [γ-^{32}P]-ATP was obtained from New England Nuclear and A23187 was obtained from Calbiochem (California). L-α-phosphatidylinositol 4-monophosphate (DPI), L-α-phosphatidylinositol 4,5, diphosphate (TPI), L-α-phosphatidic acid (PA), and neomycin sulfate were obtained from Sigma (Missouri).

Preparation of Rabbit Erythrocyte Ghosts. Heparinized blood was obtained from male and female rabbits. Erythrocytes were separated and washed free of plasma and white cells in isotonic saline. Ghosts were prepared by washing the erythrocytes twice in 20 mM Tris HCl, pH 7.6 at 20,000 x g for 15 minutes at 5°C. Ghosts were used within one hour of preparation.

Determination of Mg-ATP Dependent Ghost Shape Changes. The rate of Mg-ATP dependent shape changes in ghosts was determined by measuring the flow time of erythrocyte ghosts suspensions in Cannon-Manning semi-micro viscometers at 25°C as previously described (3). The medium contained in a final volume of 1 ml; 25 mM imidazole, pH 7.0, 1 mM EGTA, 10 µg A23187, 5 mM $MgCl_2$ and 1.2 mg of membrane protein. Shape changes were also monitored by phase-contrast microscopy at 1250x.

Phosphorylation and Dephosphorylation of Ghost Membrane Proteins and Lipids. Membranes were phosphorylated in medium (final volume 0.5 ml) containing 25 mM imidazole HCl, 1 mM EGTA, 5 mM $MgCl_2$, 5 µg A23187, 1 mM [γ-^{32}P]-ATP and 300 µg of membrane protein for 15 minutes at 25°C. Neomycin and $CaCl_2$ were varied in this medium and free Ca ion concentration was calculated as previously described (4). The reaction was stopped either of two ways to measure labeled lipids or proteins: (i) By the addition of 3 ml of cold 5% trichloroacetic acid, 0.5 mM Na_2ATP and 0.5 mM K_2HPO_4. The tubes were centrifuged at 4,000 rpm for 15 minutes at 5°C and the pellet was washed again with 3 ml of cold H_2O. Lipids were extracted from the pellets according to Alan and Michell (10) and separated by thin layer chromatography on Silica Gel 60 plates (E. Merck) according to Schacht (12). Labeled lipids were detected by autoradiography and identified using lipid standards. Labeled spots were scraped from the plates and counted in 10 ml of Aquasol. (ii) By the addition of 3 ml of cold 25 mM imidazole HCl, pH 7.0 and 1 mM EGTA. The tubes were centrifuged at 20,000 x g for 15 minutes at 5°C and the pellets were solubilized in 0.1 ml of 1% SDS, 0.2%-mercaptoethanol,

15% glycerol, and .002% bromophenol blue. Samples (50 λ) were applied to 1.5 mm thick 5% SDS polyacrylamide gels and proteins were separated by electrophoresis according to Fairbanks et al. (13) at 10°C and pH 7.4. The gels were stained for 10 to 15 minutes in staining solution (13) and bands 2 and 3 were cut from gels. Gel slices were dissolved with 0.2 ml of $HClO_4$ and 0.4 ml of H_2O_2 in glass scintillation vials at 70°C and counted in 10 ml of Aquasol.

To study the effects of neomycin and Ca^{2+} on prephosphorylated ghost proteins and lipids, ghosts were preincubated for 15 minutes at 25°C under the phosphorylation conditions above except that 0.2 mM rather than 1.0 mM [γ-^{32}P]-ATP was used. After this preincubation, 2 mM cold Na_2 ATP was added to all tubes to reduce further phosphorylation and 10 μM Ca^{2+} or 0.5 mM neomycin were added as indicated. The tubes were further incubated for 15 minutes at 25°C and the reaction was stopped and analyzed for labeled proteins and lipids as above.

RESULTS

Inhibition and Reversal of Mg-ATP Induced Shape Changes by Neomycin and Ca^{2+}.

Previously, it was reported that the rate of Mg-ATP dependent echinocytic-discocytic shape transformations can be readily determined by measuring the specific viscosity of ghost suspensions (3). In the presence of 1 mM Mg-ATP, the specific viscosity of suspensions of rabbit ghosts decreases and levels off after 10-15 minutes at 25°C (Fig. 1). This viscosity decrease is associated with a complete echinocytic to discocytic shape transformation. The viscosity or shape changes induced by Mg-ATP were half-maximally inhibited by 0.3 mM neomycin and completely inhibited by 0.5 mM neomycin. Although not shown, 10 μM Ca^{2+} completely inhibited Mg-ATP induced viscosity or shape changes of rabbit ghosts.

To determine if Ca^{2+} and neomycin can reverse Mg-ATP dependent shape changes, ghosts were first preincubated for 15 minutes with 1 mM Mg-ATP to produce a maximal decrease in viscosity. After this time, the effect of added neomycin and Ca^{2+} on the viscosity was determined. Ca^{2+} or neomycin increased the viscosity of these ghosts to the levels observed in ghosts not preincubated with ATP within 15 minutes (Table 1). The increase in viscosity is correlated with a complete discocytic-echinocytic shape transformation. These studies were carried out at low ionic strength ($\sim$ 40 mM) to reduce the rate of resealing in order that neomycin and Ca^{2+} could penetrate to the inside of the preincubated ghosts. For instance, it was found that 0.5 to 1.0 mM neomycin had no

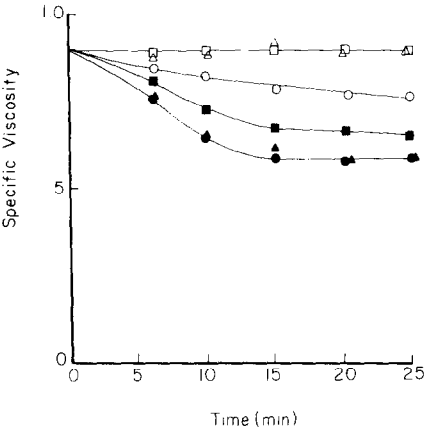

FIGURE 1: Inhibition of Mg-ATP dependent viscosity changes of erythrocyte ghost suspensions by neomycin. Viscosity was determined at 25°C in the presence of 1 mM ATP and the following concentrations of neomycin: 0 (●), 0.1 mM (▲), 0.2 mM (■), 0.3 mM (○) and 0.5 mM (△). Viscosity was also determined in the absence of ATP (□).

effect on the shape of intact rabbit erythrocytes in isotonic saline indicating that neomycin must penetrate to the cytoplasmic membrane surface to affect shape (not shown).

The Effect of Neomycin and Ca^{2+} on the Phosphorylation of Ghost Proteins. In agreement with others (8,14), the only two erythrocyte membrane proteins highly phosphorylated in the presence of Mg^{2+} and [γ -^{32}P]-ATP under conditions similar to those used here were band 2 of spectrin and band 3. Ca^{2+} slightly inhibited phosphorylation of bands 2 and 3 and neomycin stimulated phosphoryla-

TABLE I

THE EFFECT OF NEOMYCIN AND Ca^{2+} ON THE SPECIFIC VISCOSITY OF GHOSTS PREINCUBATED WITH 1 mM Mg-ATP

Additions	Specific Viscosity
none	0.58
0.5 mM Neomycin	0.92
10 μM Ca^{2+}	0.89

TABLE II

THE EFFECT OF NEOMYCIN AND Ca^{2+} on Mg^{2+}-DEPENDENT ^{32}P LABELING OF ERYTHROCYTE MEMBRANE PROTEINS

Additions	CPM/Gel Slice[c]	
	Band 2	Band 3
I. During Ghost Phosphorylation[a]		
none	620	381
0.5 mM Neomycin	2183	1310
10 μM Ca^{2+}	585	357
II. After Ghost Phosphorylation[b]		
none	2615	1600
0.5 mM Neomycin	2688	1780
10 μM Ca^{2+}	2415	1575

[a] Ghosts were phosphorylated for 15 minutes at 25°C with above additions.

[b] Ghosts were prephosphorylated for 15 minutes at 25°C, and then further incubated for 15 minutes at 25°C with above additions (See Methods for other conditions).

[c] Results are representative of at least three separate experiments.

tion of bands 2 and 3 approximately 3.5 fold (Table II). The slight inhibition of phosphorylation of bands 2 and 3 by Ca^{2+} is probably an indirect effect (i.e. due to a slight reduction in [γ-^{32}P]-ATP concentration resulting from stimulation of Ca+Mg-ATPase activity in these membranes). Ca^{2+} or neomycin were found not to have any effect on ^{32}P labeling of proteins prephosphorylated with Mg^{2+}-[γ-^{32}P]-ATP.

The Effect of Neomycin and Ca^{2+} on the ^{32}P Labeling of Ghost Lipids. The only lipids labeled with ^{32}P by 1 mM Mg^{2+}-[γ-^{32}P]-ATP were PA, DPI and TPI, as previously found (10,15). Neomycin or Ca^{2+} inhibited ^{32}P labeling of DPI by 75 to 80% at concentrations which inhibit Mg^{2+} dependent shape changes (Table III). On the other hand, Ca^{2+} stimulated phosphorylation of PA two-fold and had no effect on the phosphorylation of TPI. Neomycin had no effect

TABLE III

THE EFFECT OF NEOMYCIN AND Ca^{2+} on Mg^{2+}-DEPENDENT ^{32}P LABELING OF ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS

Additions	CPM/Incubation[c]		
	PA	DPI	TPI
I. During Ghost Phosphorylation[a]			
none	580	840	386
0.5 mM Neomycin	570	219	1870
10 μM Ca^{2+}	1180	212	420
II. After Ghost Phosphorylation[b]			
none	1276	1848	849
0.5 mM Neomycin	1166	466	1501
10 μM Ca^{2+}	1265	369	899

[a]Ghosts were phosphorylated for 15 minutes at 25°C with above additions.

[b]Ghosts were prephosphorylated for 15 minutes at 25°C, and then further incubated for 15 minutes at 25°C with above additions (See Methods for other conditions).

[c]Results are representative of at least five separate experiments.

on the phosphorylation of PA but stimulated the phosphorylation of TPI 4 to 5 fold.

If membranes were prephosphorylated with Mg^{2+}-[γ-^{32}P]-ATP, both neomycin and Ca^{2+} decreased ^{32}P labeled DPI by approximately 80% (Table III). Neither neomycin or Ca^{2+} had any effect on prephosphorylated PA whereas neomycin increased ^{32}P labeling of TPI. Under these conditions, 10 μM Ca^{2+} did not effect ^{32}P labeled TPI.

DISCUSSION

In this study, the hypothesis that erythrocyte membrane shape is regulated by opposing Mg^{2+} kinase and Ca^{2+} phosphatase activities which phosphorylate and dephosphorylate a cytoplasmic membrane site, respectively, was further investigated (3,6). To determine the identity of this site, the effects of neomycin

and Ca^{2+} on Mg-[γ-^{32}P]-ATP dependent phosphorylation of ghost proteins and phospholipids were compared under conditions in which these agents can inhibit and reverse Mg-ATP dependent shape transformations (Table 1). The results found here suggest that neither band 2 or 3 proteins is the shape regulating site. For instance, Ca^{2+} did not significantly inhibit phosphorylation of these proteins whereas neomycin actually stimulated phosphorylation of these proteins 3.5 fold (Table II). It would be expected that these agents would inhibit phosphorylation of these site(s) to account for their inhibition of Mg-ATP dependent shape transformations. However, both 0.5 mM neomycin and 10 μM Ca^{2+} inhibited formation of ^{32}P labeled DPI and also stimulated a decrease in DPI prelabeled with Mg^{2+}-[γ-^{32}P]-ATP (Table III). The effect of these agents on DPI corresponds closely to their effects on Mg-ATP dependent shape transformations and therefore suggests that DPI plays an important role in membrane shape regulation. The other two phospholipids labeled with ^{32}P probably are not directly involved in shape regulation because Ca^{2+} stimulated phosphorylation of PA as previously reported (10) and neomycin strongly stimulated the phosphorylation of TPI. The presence of Ca^{2+} stimulated phosphomonoesterase and phosphodiesterase activities have been found in rabbit erythrocyte ghosts and therefore Ca^{2+} could inhibit Mg-ATP dependent shape transformations by breaking down DPI by one or both of these enzymes. On the other hand, neomycin decreased the amount of prelabeled DPI and simultaneously increased labeling of TPI (Table III). Therefore, neomycin may inhibit Mg-ATP dependent shape transformations by stimulating a Mg-kinase activity which further phosphorylates DPI to TPI.

ACKNOWLEDGEMENTS

This work was supported by grants from the Minnesota Medical Foundation #DMRF-43-79 and the Graduate School of the University of Minnesota, 475-0325-4909-02. I would also like to thank Leslie Johnson for preparing the manuscript.

REFERENCES

1. Palek, J., Stewart, G. and Lionetti, F.J. (1974) Blood 44: 583-597.
2. Sheetz, M.P. and Singer, S.J. (1977) J. Cell. Biol. 73: 647-659.
3. Quist, E.E. (1980) Biochem. Biophys. Res. Commun. 92: 631-637.
4. Quist, E.E. and Roufogalis, B.D. (1976) Biochem, Biophys. Res. Commun. 72: 673-679.
5. Quist, E.E. (1980) Arch. Biochem. Biophys. 203: 123-133.
6. Birchmeirer, W. and Singer, S.J. (1977) J. Cell. Biol. 73: 647-659.
7. Anderson, J.M. and Tyler, J.M. (1980) J. Biol. Chem. 255: 1259-1265.
8. Avruch, J. and Fairbanks, G. (1974) Biochemistry 27: 5507-5514.
9. Lang, V., Pryhitka, G. and Buckley, J.T. (1977) Can. J. Biochem. 55: 1007-1012.
10. Allan, D. and Michell, R.H. (1978) Biochim. Biophys. Acta. 508: 277-286.
11. Garrett, N.E., Garrett, R.J.S., Talwalkar, R.T. and Lester, R.L. (1976) J. Cell. Physiol. 87: 63-70.
12. Schacht, J. (1976) J. Neurochem. 27: 1119-1124.
13. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10: 2606-2617.
14. Vickers, J.D., Brierly, J. and Rathbone, M.P. (1979) J. Membrane Biol. 49: 123-138.
15. Santiago-Calvo, E., Mule', S., Redman, C.M., Hokin, M.R. and Hokin, L.E. (1964) Biochim. Biophys. Acta. 84: 550-562.