Journal of Supramolecular Structure 9:269–274 (1978)
Normal and Abnormal Red Cell Membranes 259–264

Modulator Binding Protein Antagonizes Activation of (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} Transport of Red Blood Cell Membranes

Fred L. Larsen, Beat U. Raess, Thomas R. Hinds, and Frank F. Vincenzi

Department of Pharmacology, School of Medicine, University of Washington, Seattle, Washington 98195

Red blood cells contain a protein that activates membrane-bound (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} transport. The red blood cell activator protein is similar to a modulator protein that stimulates cyclic AMP phosphodiesterase. Wang and Desai [Journal of Biological Chemistry 252:4175–4184, 1977] described a modulator-binding protein that antagonizes the activation of cyclic AMP phosphodiesterase by modulator protein. In the present work, modulator-binding protein was shown to antagonize the activation of (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} transport by red blood cell activator protein. The results further demonstrate the similarity between the activator protein from human red blood cells and the modulator protein from bovine brain.

Key words: modulator-binding protein, (Ca^{2+} + Mg^{2+})-ATPase, calcium transport, red blood cell membrane, calcium-dependent regulator protein

The Ca^{2+} pump of the human red blood cell (RBC) membrane has been linked to membrane-bound (Ca^{2+} + Mg^{2+})-ATPase activity [1–3]. A proteinaceous activator of the (Ca^{2+} + Mg^{2+})-ATPase activity is present [4] and can be isolated [5] from RBC hemolysates. Hemolysates [6] as well as purified activator protein increase the rate of uptake of $^{45}Ca^{2+}$ into inside-out vesicles of RBC membranes [7]. Because of the close association of ATPase activity and transport [3], and because of reversible Ca^{2+}-dependent binding of the activator protein to RBC membranes [8, 9], it has been suggested that the activator protein acts as a "switch" for the Ca^{2+}pump.

RBC activator protein is similar, if not identical, to a modulator protein that has been reported to activate cyclic nucleotide phosphodiesterase [10] and adenylate cyclase [11]. Modulator protein, as it will be referred to here, is also known as calcium-dependent regulator (CDR) [11]. Modulator protein also activates the (Ca^{2+} + Mg^{2+})-ATPase activity of isolated RBC membranes [12, 13] and stimulates active Ca^{2+} uptake into inside-out vesicles [7], presumably by a similar mechanism. In addition, RBC activator protein

Received April 25, 1978; accepted June 22, 1978.

0091-7419/78/0902-0269$01.40 © 1978 Alan R. Liss, Inc.

activates phosphodiesterase [12]. Thus, the two proteins appear to be functionally similar. Modulator protein, and/or activator protein, or the family of Ca^{2+}-binding proteins they represent, may mediate a number of intracellular actions of Ca^{2+} [14], but elucidation of these remains to be determined.

Recently, Wang and co-workers described the isolation from bovine brain of a protein that antagonizes the activation of cyclic AMP phosphodiesterase by a modulator protein [15]. This protein was named modulator-binding protein (MBP) because it antagonizes the activation of phosphodiesterase by binding the modulator protein-Ca^{2+} complex, thereby antagonizing the activation of phosphodiesterase. Thus, MBP and phosphodiesterase can "compete" for the protein modulator–Ca^{2+} complex. The activity of phosphodiesterase in vitro thus depends on the relative amounts of MBP and phosphodiesterase (as well as protein modulator and Ca^{2+}) [15]. We reasoned that if RBC protein activator and the protein modulator are closely related or identical and if activation of (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} transport is analogous to phosphodiesterase activation, then modulator-binding protein should antagonize the activation of the ATPase and transport by RBC protein activator. Present results demonstrate that MBP antagonizes the activation of both (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} transport by RBC activator protein.

METHODS

RBC membrane ghosts were prepared [16] from outdated human cells and assayed at 37° for ATPase activity as previously described [13], with modifications noted herein. The (Ca^{2+} + Mg^{2+})-ATPase assay medium contains, in a final incubation volume of 3.0 ml, approximately 0.2 mg membrane protein; 18 mM–18 mM histidine–imidazole buffer, pH 7.1; 3 mM ATP; 3 mM $MgCl_2$; 80 mM NaCl; 15 mM KCl; 0.1 mM ouabain; 0.2 mM $CaCl_2$ and 0.1 mM EGTA. Mg^{2+}-ATPase activity was determined in tubes in which $CaCl_2$ was omitted. Purified RBC activator protein and/or MBP were included in some tubes as noted. Tubes were incubated for 60 or 90 min, and the reaction was terminated by addition of sodium dodecyl sulfate (SDS, final concentration 6.67%).

The activator protein from RBCs was isolated by a method developed in our laboratory. The method will be reported elsewhere [Jung, Hinds and Vincenzi, in preparation]. In brief, the RBC hemolysate from outdated human RBCs was subjected to DEAE-Sephadex and gel filtration (G-75) chromatography (apparent mol wt ~ 30,000). The resulting protein gives a single band on isoelectric focusing (pI = 4.0) and SDS-polyacrylamide gel electrophoresis (apparent mol wt = 20,000). MBP from bovine brain was a gift from Dr. Jerry Wang, University of Manitoba, Canada.

Inside-out RBC membrane vesicles were prepared from fresh RBCs according to the method of Steck and Kant [17], with minor modifications in order to remove phosphate. Purified inside-out vesicles were washed once in 10 mM Tris-glycylglycine buffer (pH 7.4) with 0.025 mM $MgCl_2$, once in 20 mM Tris-glycylglycine buffer (pH 7.4) with 0.05 mM $MgCl_2$, and once in 40 mM Tris-glycylglycine (pH 7.4) with 0.1 mM $MgCl_2$. Vesicles were stored in a small volume of 40 mM Tris-glycylclycine buffer and were used within two days.

The percent inside-out vesicle concentration was assessed by the previously described method [17] of measuring acetylcholinesterase activity in the absence and presence of Triton X-100. Our preparation typically contained 75–80% inside-out vesicles. $^{45}Ca^{2+}$ uptake experiments were conducted at 25°C in 25 mM Tris-glycylglycine (pH 7.4), 50.0 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM ATP, 0.1 mM $CaCl_2$ ($^{45}CaCl_2$ specific activity, 1.799

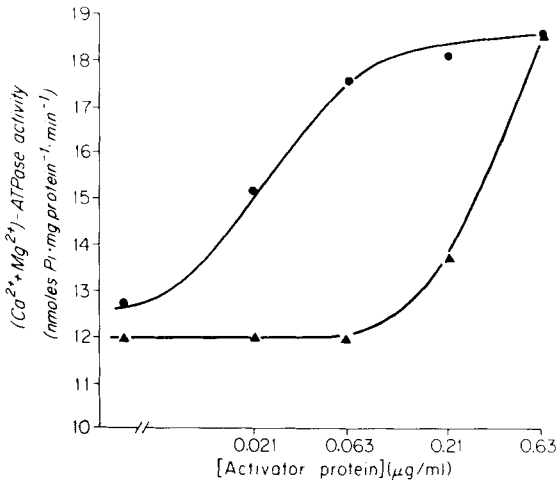

Fig. 1. Modulator-binding protein antagonism of $(Ca^{2+} + Mg^{2+})$-ATPase activation by RBC activator protein. ATPase activity was determined as described in Methods. In the absence of modulator-binding protein (●—●), the ATPase activity increased above "basal" (approximately 12.5 nmoles Pi·mg protein^{-1}·min^{-1}). In the presence of 1.67 μg/ml modulator-binding protein (▲—▲), the curve of activation was shifted.

$\times 10^6$ cpm/μmole), and about 0.2 mg inside-out vesicle protein in a total volume of 0.6 ml. Vesicles were preincubated for 30 min prior to addition of ATP. Beginning 10 min after the addition of ATP, 100 μl samples were removed at 20-min intervals (total 5 samples) and quenched in 1.5 ml of ice-cold 40 mM tris-glycylglycine (pH 7.4) and 0.1 mM $MgCl_2$. Inside-out vesicles were collected by vacuum filtration on Amicon microporous filters (0.45 μm) and were washed once with 1.5 ml of the Tris-glycylglycine buffer. Filters were dissolved in 10 ml of Aquasol and counted for $^{45}Ca^{2+}$ activity. Transport rates were determined by linear regression of the five data points.

RESULTS

As previously reported, $(Ca^{2+} + Mg^{2+})$-ATPase activity of RBC membranes is stimulated by activator protein [12, 13]. Average values for maximal activation are usually about twofold or threefold above the "basal" activity (in the absence of added activator). Data in Figure 1 were obtained from membranes in which the magnitude of activation was somewhat less than aveage. As a function of activator protein concentration, $(Ca^{2+} + Mg^{2+})$-ATPase activity increased from approximately 12 to 19 pmoles Pi·mg protein^{-1}·min^{-1} (Fig. 1, solid circles). Half-maximal activation was obtained at an activator concentration of approximately 0.027 μg/ml; 1.35×10^{-9}M assuming a molecular weight of 20,000 or 0.40 μg activator·mg membrane protein^{-1}. MBP antagonized activation of $(Ca^{2+} + Mg^{2+})$-ATPase by activator. In the presence of modulator-binding protein (1.67 μg/ml, 1.75×10^{-8}M, assuming mol wt 95,000), the curve of ATPase activation was shifted approximately tenfold in an apparently competitive fashion (1.41×10^{-8}M activator for ½ maximal).

MBP inhibited the activated $(Ca^{2+} + Mg^{2+})$-ATPase in a concentration-dependent fashion. Figure 2 shows $(Ca^{2+} + Mg^{2+})$-ATPase activity of a different membrane prepara-

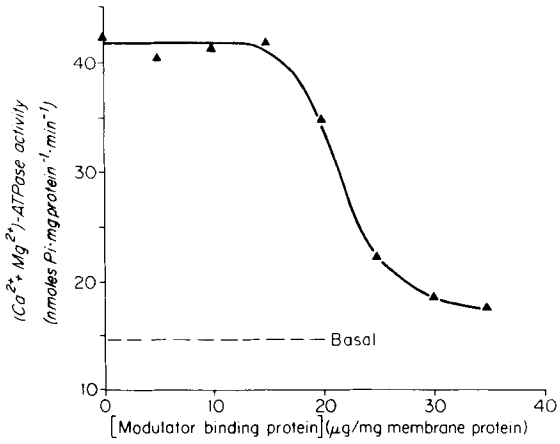

Fig. 2. Inhibition of activated (Ca^{2+} + Mg^{2+})-ATPase by modulator-binding protein. ATPase activity was assayed as described in Methods, except that all tubes contained 0.18 μg/ml of activator protein. "Basal" indicates activity present in the same membranes without activator. Addition of modulator-binding protein produced a dose-dependent inhibition of the activated (Ca^{2+} + Mg^{2+})-ATPase. As in Figure 1, modulator-binding protein did not inhibit the basal (Ca^{2+} + Mg^{2+})-ATPase activity.

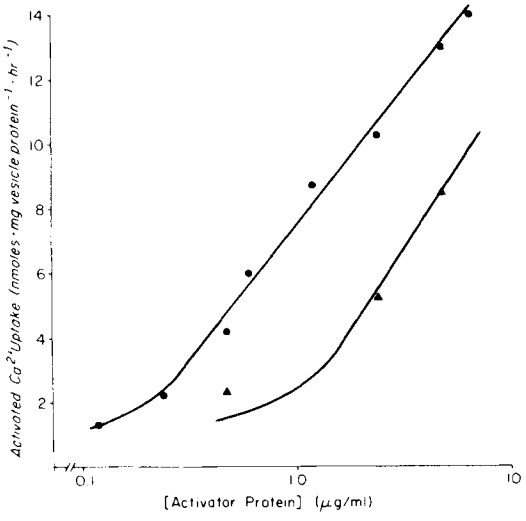

Fig. 3. Modulator-binding protein antagonism of Ca^{2+} transport activation by RBC activator protein. Activator stimulated uptake of $^{45}Ca^{2+}$ into inside-out vesicles of RBC membrane was measured as described in Methods. Both in the absence (●—●) and presence (▲—▲) of modulator-binding protein (3.25 μg/ml), $^{45}Ca^{2+}$ uptake was increased as a function of the concentration of activator protein. The curve was shifted to higher activator concentrations in the presence of modulator-binding protein.

tion as a function of MBP concentration in the presence of activator (1.02×10^{-8}M). The molar ratio of activator/MBP ranged from 2.63 (8% inhibition) to 0.38 (88% inhibition). Even at the highest concentration, MBP did not inhibit the activity below the basal level.

The Ca^{2+}pump normally transports Ca^{2+} from inside the cell to outside and Ca^{2+}, Mg^{2+}, and ATP are required at the inner membrane surface [3]. Because the transport is directional, and because the activator protein interacts with the inner membrane surface [9], it is convenient to use inside-out vesicles of RBC membrane for such studies. As reported previously [7], Ca^{2+} impermeable inside-out vesicles of RBC membrane actively take up Ca^{2+}. In the absence of added activator the vesicles took up 1.75 nmoles Ca^{2+}·mg vesicle protein^{-1}·h^{-1}. The rate of active transport of Ca^{2+} across the RBC membrane was increased by the activator protein. Activator-stimulated uptake (Fig. 3) was taken as the uptake rate above "basal" occurring in the presence of added activator protein. In the presence of activator (6.5 μg/ml) activated uptake was 14.95 nmoles Ca^{2+}·mg vesicle protein^{-1}·hr^{-1}, which corresponds to an eightfold activation. The stimulatory effect was antagonized by modulator-binding protein in an apparently competitive fashion (Fig. 3), although saturation was not obtained. A double reciprocal plot of these data yielded an apparent K_d for activator of 4.16×10^{-8}M. Even considering the influence of temperature, the rate of transport into inside-out vesicles is considerably less than the rates reported for RBCs or right-side out resealed RBC ghosts [18]. The specific activity of the (Ca^{2+} + Mg^{2+})-ATPase of inside-out vesicles is also markedly reduced. At 37° in the presence of 1 μg/ml of activator protein, inside-out vesicles exhibited an ATPase activity of 0.25 nmoles Pi·mg protein $^{-1}$·min. By contrast, isolated RBC membranes usually exhibit ATPase activities in the range of about 30–40 nmoles Pi·mg protein^{-1}·min^{-1} (activator present).

DISCUSSION

The present results demonstrate that MBP from bovine brain can antagonize the activation of membrane-bound (Ca^{2+} + Mg^{2+})-ATPase by RBC activator protein. It was also shown that MBP antagonizes activation of Ca^{2+} transport by the activator protein. From the work of Wang and Desai, it is known that MBP antagonizes activation of phosphodiesterase by protein modulator [15]. Given the similarities between modulator protein and RBC activator protein [12, 13], it seems likely that a similar mechanism of antagonism is operative in each case. Modulator protein activates phosphodiesterase by binding Ca^{2+} and then binding to the enzyme. Wang and Desai [15] showed that the modulator protein–Ca^{2+} complex can also bind to MBP. The result is that activation of phosphodiesterase can be competitively antagonized by modulator-binding protein, depending on the relative amounts of the two proteins. Similar findings were obtained in this study. Thus, modulator-binding protein appears to antagonize RBC activator protein competitively, both in the activation of ATPase and in transport. The model described by Wang and Desai would appear to be applicable and sufficient to interpret the present results.

Although MBP did antagonize the effects of activator protein, it did not inhibit "basal" (Ca^{2+} + Mg^{2+})-ATPase activity. This probably means that the basal ATPase activity is not due to endogenous activator protein bound to the membrane. The basal (Ca^{2+} + Mg^{2+})-ATPase activity that is present in the absence of activator (or presence of sufficient MBP) may represent the activity of an enzyme not related to Ca^{2+} transport. The apparent K_d for activator protein determined by uptake experiments is considerably larger than the same value obtained from ATPase activities in isolated RBC membranes.

From this and the relatively low rate of transport in inside-out vesicles, we assume that some damage to the membrane and/or transport system occurs under the rather drastic conditions necessary to prepare inside-out vesicles. In spite of these limitations, active uptake of Ca^{2+} does occur, and the rate of uptake is increased as a function of activator protein concentration.

The present results provide another illustration of the similarities between RBC activator protein and protein modulator. It is not yet known whether RBC activator and protein modulator are identical. It is also not known whether modulator-binding protein exists in the RBC. If activator protein and/or similar proteins stimulate plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} transport, then, where present, they may influence Ca^{2+} transport as well as cyclic AMP metabolism.

ACKNOWLEDGMENTS

Dr. Jerry Wang of the University of Manitoba, Canada, kindly supplied the modulator-binding protein. This work was supported in part by US Public Health Service grants AM 16436 and GM 00109 and by a grant from the Cystic Fibrosis Foundation. B.U.R. was supported by a National Research Service Award from the National Institutes of Health (GM 07270).

REFERENCES

1. Schatzmann HJ, Vincenzi FF (1969): J Physiol (Lond) 201:369–395.
2. Olson EJ, Cazort RJ (1969): J Gen Physiol 53:311–322.
3. Schatzmann HJ (1975): In Bronner F, Kleinzeller A (eds): "Current Topics in Membranes and Transport," Vol. 6, New York: Academic, pp 125–168.
4. Bond GH, Clough DL (1973): Biochim Biophys Acta 323:592–599.
5. Luthra MG, Hildenbrandt GR, Hanahan DJ (1976): Biochim Biophys Acta 419:164–179.
6. Macintyre JD, Green JW (1977): Fed Proc (Abstr) 36:271.
7. Hinds TR, Larsen FL, Vincenzi FF (1978): Biochem Biophys Res Commun 81:455–461.
8. Farrance ML, Vincenzi FF (1977): Biochim Biophys Acta 471:59–66.
9. Vincenzi FF, Farrance ML (1977): J Supramol Struct 7:301–306.
10. Cheung WY (1970): J Biol Chem 246:2859–2869.
11. Brostrom MA, Brostrom CO, Breckenridge BM, Wolff DJ (1975): Proc Natl Acad Sci USA 72:64–68.
12. Jarrett HW, Penniston JT (1977): Biochem Biophys Res Commun 77:1210–1216.
13. Gopinath RM, Vincenzi FF (1977): Biochem Biophys Res Commun 77:1203–1209.
14. Vanaman TC, Sharief F, Watterson DM (1977): In Wasserman RH, Carradino RA, Carafoli E, Kretsinger RH, MacLennan DH, Siegal FL (eds): "Calcium-Binding Proteins and Calcium Function." New York: North-Holland, pp 107–116.
15. Wang JH, Desai R (1977): J Biol Chem 252:4175–4184.
16. Farrance ML, Vincenzi FF (1977): Biochim Biophys Acta 471:49–58.
17. Steck TL, Kant JA (1974): In Fleischer S, Packer L (eds): "Methods in Enzymology," Vol. 31, New York: Academic, pp 172–180.
18. Larsen FL, Hinds TR, Vincenzi FF (1978): J Membrane Biol 41:361–376.