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PURIFICATION AND CHARACTERIZATION OF A CALCIUM BINDING PROTEIN WITH "SYNEXIN-LIKE" ACTIVITY FROM HUMAN BLOOD PLATELETS

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Summary: A calcium binding protein of M_r=54,000 has been isolated from human blood platelets. This protein has been shown to enhance Ca^{2+}-induced aggregation of phosphatidylserine liposomes, suggesting that it may be a member of a recently recognized class of binding proteins known to interact with phospholipids and the membrane in a Ca^{2+} dependent manner. Because of the "synexin-like" activity of this protein it may be involved in Ca^{2+} regulated platelet secretion as well as cell motility.

Calcium plays an important role in the binding of some agonists to platelet receptors but also supports a wide variety of intracellular processes in the course of platelet activation. Physiological responses to platelet activation are often preceded by an elevation of free calcium. This calcium signal is generally believed to be mediated by calcium binding proteins, some of which appear to be membrane associated and therefore, the investigation of such platelet calcium binding proteins may lead to a better understanding of platelet stimulation-response phenomena (1).

Gogstad et al examined platelets for their calcium-binding protein content by means of crossed immunoelectrophoresis of solubilized platelets against antibodies to whole platelets followed by incubation of the immunoplates with $^{45}Ca^{2+}$ and found three immunoprecipitates to be labelled with $^{45}Ca^{2+}$ (2). Two of the proteins were identified as glycoproteins IIb and IIIa whereas the third, weakly labelled antigen was designated G-18 whose function has not been characterized. By using the western blotting technique, we are able to find at least three heat stable calcium binding proteins within platelets and we

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ABBREVIATIONS
ABP, actin binding protein; SDS, Sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, Ethylene diamine tetraacetic acid; EGTA, Ethylene Glycol bis (2-Amino ethyl ether) N,N'-tetraacetic acid; PIPES, piperazine-N,N'-bis (2-ethanesulfonic acid); DTT, dithiothreitol; PS, phosphatidyl serine; TCA, trichloroacetic acid.

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have pursued the investigation of one of these with the highest specific Ca^{2+} binding activity. Its purification on hydroxyapatite resulted in the isolation of a single homogenous protein of M_r=54,000. This protein does not appear to be Gp IIb or GpIIIa or related to the calmodulin family of Ca^{2+}-binding proteins (3,4). The preliminary findings, described here, suggest that it may have synexin-like activity (5). Therefore, this newly described Ca^{2+}-binding protein from human blood platelets might belong to the recently described Ca^{2+}/phospholipid class of Ca^{2+}-binding proteins (6). It may play a role in Ca^{2+} regulated platelet secretion and/or cytoskeleton-membrane interactions.

Methods and Materials

1. Preparation of platelet lysate.

Human platelet concentrates were received approximately three hours after the blood was drawn at the Greater New York Blood Center. Platelets were prepared as previously described by Rosenberg et al (7). Washed platelets were then resuspended 1:1, vol:vol in platelet wash solution. 9 volumes of solution 80 (1% Triton X-100, 40mM KCl, 10mM imidazole-chloride, 10 mM EGTA, 2mM NaN_3 pH 7.0) were added to 1 volume of platelet suspension. After 12 minutes on ice, the mixture was centrifuged at 3000xg for 5 min to sediment the triton insoluble cytoskeleton. The supernatants were transferred and saved for further use.

2. Isolation of calcium binding protein from platelet lysate.

20 ml of platelet lysate were dialyzed overnight against 10 mM Tris-HCl, pH 7.5 and centrifuged at 25,000xg for 15 min to clarify any precipitate. The supernatant was then mixed with 20 ml packed DEAE-cellulose (DE-52) equilibrated with 10mM Tris-HCl, pH 7.5. The mixture was stirred for 30 min on ice. The slurry was poured into a column and washed with 100ml of 10mM Tris-HCl, pH 7.5. The protein was eluted with a linear gradient made from equal volumes of 10mM Tris-HCl (pH 7.5) and 10mM Tris-HCl pH 7.5), 0.45 M NaCl. Fractions (3ml) were assayed for protein concentration and calcium binding activity. For further purification, the calcium binding peak fractions from DEAE-cellulose chromotography were pooled and dialyzed extensively against 10mM potassium phosphate (pH 7.5) and 0.5 mM DTT and applied to a hydroxyapatite column previously equilibrated with 10mM potassium phosphate (pH 7.5), 0.5 mM DTT. The proteins were eluted with 100 ml of a linear potassium phosphate (pH 7.5) gradient from 10mM-300mM containing 0.5 mM DTT.

3. Calcium binding assay.

Calcium binding activity was measured by several methods. For column fractions, dot blotting was the most rapid and convenient. 100 ul fractions were applied to a nitrocellulose membrane using a Bio-Dot microfiltration apparatus. After binding of protein to the nitrocellulose membrane, the membrane was treated with $^{45}Ca^{2+}$ according to Maruyama et al (8). The membrane was first soaked in a solution containing 60mM KCl,5mM $MgCl_2$, 10mM imidazole-HCl, pH 7.0. The buffer was exchanged two times in an hour. The membrane was then incubated in the same buffer containing 1 uCi/100 ml $^{45}CaCl_2$ for 20 min. and rinsed with distilled water for 5 min. Excess water was absorbed between two sheets of Whatman No. 1 filter paper and the membrane was dried at room temperature overnight. Autoradiographs of $^{45}Ca^{2+}$-labelled proteins on the nitrocellulose membrane were obtained by exposure of the dried

membrane to Kodak XAR-5 X-ray film for 24 hr. In some experiments, calcium binding proteins were separated by native (non-denaturing) gel electrophoresis and electrophoretically transferred to nitrocellulose membrane according to the method of Towbin (9). The calcium binding activity was measured as described above.

4. Ca^{2+}-induced aggregation of phosphatidylserine (PS) liposomes.

The effect of the 54 kDa protein on Ca^{2+}-induced aggregation of phosphatidylserine liposomes was tested according to Morris et al (10). 4 mg bovine brain PS in chloroform-methanol was dried under nitrogen stream. 1.5 ml of K^+-HEPES buffer (10 mM KCl, 10mM HEPES, pH 7.4) were added to dried lipid and sonicated under nitrogen (15 watt, 5 min.). Incompletely dispersed lipid was removed by centrifuging at 12,000 g for 10 min., and the suspensions were kept at 25°C for Ca^{2+}-induced aggregation assay.

In order to assay the effect of the 54 kDa protein on Ca^{2+}-induced PS liposome aggregation, 0.1 ml of 150 mM KCl, 54 kDa protein in 0.35 ml of 40 mM HEPES buffer (pH 7.4) and 0.05 ml of PS liposome suspension were mixed in a 10mm light path, 0.5 ml cuvette. After recording a steady base line at 320 nm, the aggregation was started by the addition of 10 ul of 50 mM $CaCl_2$. The controls were made with all solutions but no 54 kDa protein was added to 40 mM HEPES solutions. The O.D. changes at 320 mu were recorded for 10 min.

Results

1. Purification of calcium binding proteins.

When platelet lysate was applied to a DEAE cellulose column equilibrated with 10mM Tris-HCl and then eluted with a 0-0.45M NaCl linear gradient in 10 mM Tris-HCl, pH 7.5, two or sometimes three peaks, detected by 280nm absorption, were found in the protein elution profile. When the resultant fractions were assayed for Ca^{2+} binding activity by the dot blotting technique and the autoradiographs of $^{45}Ca^{2+}$ labelled protein were scanned with an LKB ultroscan XL Laser Densitometer, three calcium binding peaks were found in the elution profile (Fig. 1). After comparing the protein elution profile and Ca^{2+}-binding profile, it was found that the first peak of the protein elution profile had the highest specific Ca^{2+}-binding activity. After analyzing all fractions by SDS-PAGE it was found that peak 1 contained only two major protein bands and it became of interest, therefore, to determine which protein in the peak 1 fractions was responsible for calcium binding.

The fractions in peak 1 were pooled and dialyzed against 10mM potassium phosphate, pH 7.5, 0.5 mM DTT and chromatography was performed with a hydroxyapatite column as described in Methods. Figs. 2,3 show that the two proteins in the starting material could be separated easily. The higher molecular weight major protein (116 kDa) was eluted first and the fractions in the second peak contained a 54 kDAa major protein which was eluted from the hydroxyapatite column at about 0.15 M phosphate. When the calcium binding activities were measured by dot-blotting, it was found that only one calcium binding peak could be detected whose distribution corresponded well with the distribution of the 54 kDa protein in the elution profile (Fig. 3, fraction

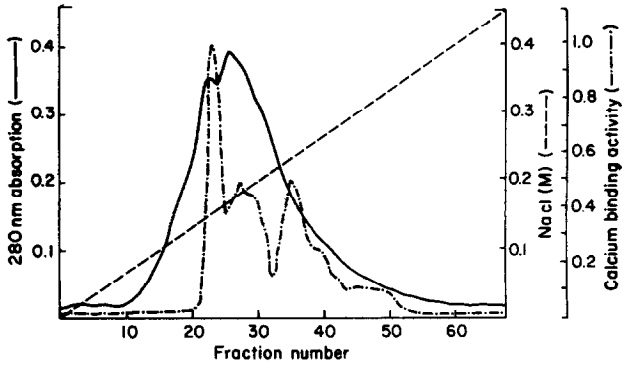
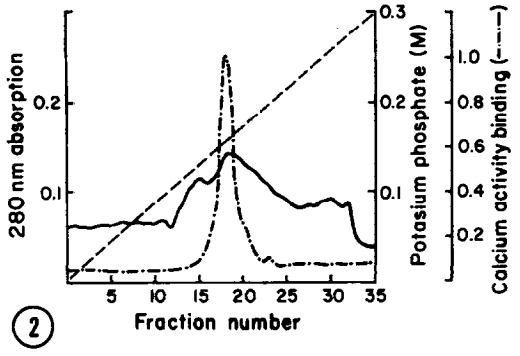


Fig. 1 Chromatography of platelet lysate on DEAE-cellulose. Platelet lysate was chromatographed on DEAE cellulose and the resultant fractions were assayed for Ca^{2+} binding activity (—·—·—) and protein concentration (— — —).

18). From this result, it is apparent that the 54 kDa protein is the major calcium binding protein in peak one of the DEAE-cellulose column fractions.

2. Calcium binding activity.

To make sure that the 54 kDa protein is responsible for the calcium binding activity in all steps of the purification, the DEAE-cellulose column first peak fractions were pooled and concentrated with 100% glycerol and separated by native 5.5% acrylamide gel electrophoresis. The proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was then incubated with $^{45}Ca^{2+}$ to detect calcium binding proteins as radioactive bands by autoradiography.



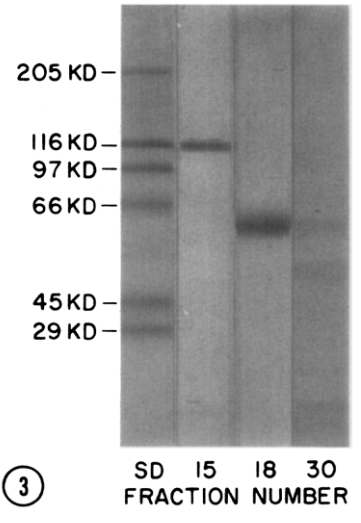


Fig. 2 Hydroxyapatite chromatography of Ca^{2+}-binding protein. Peak 1 (fractions 22-25) from DEAE cellulose chromatography were pooled and dialyzed extensively against 10 mM tris-HCl pH 7.5, 0.5 mM DTT and chromatographed on a hydroxyapatite column. Fractions were analyzed for Ca^{2+}-binding activity (—·—·) and protein concentration (— — —).

Fig. 3 SDS-PAGE of fractions from hydroxyapatite chromatography shown in Fig. 2.

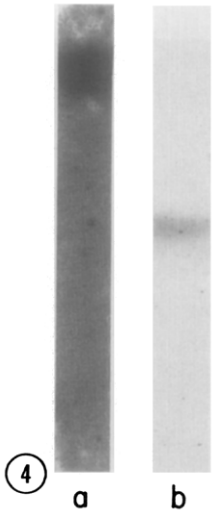
It was found that 5.5% native acrylamide gel electrophoresis could separate the proteins in peak one fractions into at least 4 bands when stained with coomassie blue. Nevertheless, only one band near the top of the gel could be seen in the autoradiograph after treatment with $^{45}Ca^{2+}$ (Fig. 4a). If the band which binds calcium was cut out and extracted with 10mM Tris-HCl pH 7.5, and the extracted proteins precipitated with 10% TCA and then analyzed with SDS-PAGE, it was found that the only protein that could be seen in the gel was the 54 kDa protein (Fig. 4b). This experiment gave further indication that it was the 54 kDa protein which was responsible for the calcium binding activity.

3. Ca^{2+}-induced aggregation of PS liposomes.

When Ca^{2+} was added to the PS liposome suspension in the absence of 54 kDa protein, the liposomes aggregated immediately and E_{320} was increased from 0.09 to 0.26. The E_{320} then increased gradually and reached 0.44 within 10 min. (Fig. 5, line a). When the 54 kDa protein was incorporated in the reaction mixture, the addition of Ca^{2+} enhanced calcium-induced membrane aggregation. The E_{320} reached 1.09 within 10 min. (Fig. 5, line b). From these data, it was concluded that the 54 kDa protein could enhance calcium-induced PS liposome aggregation by approximately threefold.

Discussion

A change in platelet intracellular free calcium ion concentration requires the cooperation of various factors and calcium binding proteins are considered



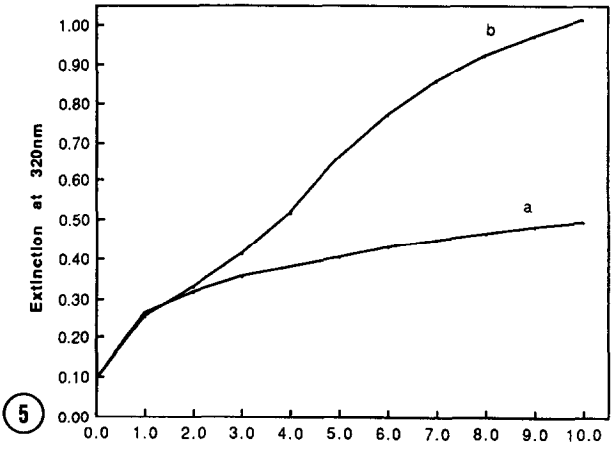


Fig. 4 Autoradiography of $^{45}Ca^{2+}$-binding protein from a) DEAE-cellulose chromatography peak I fractions run on a native gel and electrophoretically transferred to nitrocellulose. Only one Ca^{++} binding peak is observed. Lane (b) is the SDS-PAGE of the eluted band from gel (a).

Fig. 5 Time-course of Ca^{2+}-induced aggregation of PS liposomes in the absence (a) and presence (b) of 54 kDa protein. The final $CaCl_2$ concentration was 1mM and that of 54kDa protein was 6 ug/ml.

to be one of these factors. In this paper, we have described a method to purify a 54 kDa calcium binding protein and have verified its calcium binding activity. We have conducted several experiments to identify the properties of this protein and are satisfied that it does not resemble calmodulin or calmodulin binding proteins.

During the past several years, groups interested in the role of Ca^{2+} in secretion and in the control of membrane-cytoskeleton interactions have identified Ca^{2+}-binding proteins which seem to form a group distinct from the calmodulin "superfamily" of Ca^{2+}-binding proteins (6). These proteins are widely distributed and appear to be Ca^{2}+ dependent phospholipid and membrane binding proteins (11). Their binding to membranes is resistant to treatment with non-ionic detergents, suggesting that they may also interact with cytoskeletal proteins. The calpactins and synexins are two such groups of proteins (11). The protein which we have isolated and partially characterized in this study may share some of its properties with this group because our results have shown that the 54 kDa protein enhances Ca^{2+}-induced PS liposome aggregation. This "synexin-like" activity could be involved in Ca^{2+} regulated platelet secretion during exocytosis as suggested by Creutz et al in their research on the identification and purification of an adrenal medullary synexin (5). Further characterization of this newly described Ca^{2+}-binding protein from human platelets and its interaction with the platelet membrane and cytoskeleton is now in progress in order to define its role in regulating platelet function.

Acknowledgments

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