

PURIFICATION AND CHARACTERIZATION OF CALMODULIN FROM BOVINE PLATELET. J. Kambayashi, M. Sakon, G. Kōsaki, K. Sobue* and S. Kakiuchi*. The Second Department of Surgery and *Institute of Higher Nervous Activity, Osaka University Medical School, Osaka, Japan.

Calmodulin, which was originally reported as a Ca^{2+} -dependent activator of phosphodiesterase, mediates a number of actions of Ca^{2+} as a second messenger for stimulus linked cellular responses in eukaryotic cells. In blood platelets, the availability of Ca^{2+} is a prerequisite for steps of platelet reaction leading to the formation of hemostatic plug. The presence of this protein in platelets has been reported. In the present study, calmodulin was purified from bovine platelets and was extensively characterized.

The identification of the protein was based on the activity to activate the brain calmodulin deficient phosphodiesterase. Calmodulin was purified 600 fold from bovine platelet pellet by a 3-step purification method consisted of trichloroacetic acid treatment, DEAE-cellulose column chromatography and phenothiazine affinity chromatography. The recovery rate was more than 70% and the purified protein was homogenous on polyacrylamide gel electrophoresis. The mobility of the purified material upon polyacrylamide gel electrophoresis was identical with that of brain calmodulin in the presence of either Ca^{2+} or EGTA. In the quantitative analysis of the phosphodiesterase activation, the behaviour of the purified protein was also identical with brain calmodulin.

From these strict characterization, it may be concluded that the purified protein is indistinguishable from brain calmodulin. The amount of the protein in bovine platelet was estimated to be 63 micrograms per wet gram, which is about one sixth of the amount in brain tissue.

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STUDIES ON CALCIUM-ACTIVATED NEUTRAL PROTEASE IN PLATELET. M. Sakon, H. Ohno, E. Kurokawa, J. Kambayashi and G. Kōsaki. The Second Department of Surgery, Osaka University Medical School, Osaka, Japan.

It has been reported that platelet aggregation or release of arachidonic acid in stimulated platelets is inhibited by protease inhibitors or by synthetic substrates for proteases, suggesting possible involvement of unknown proteases in the platelet reaction. The present study was initiated to identify such proteases in platelets.

Subcellular fractions of bovine platelets were obtained according to the method of Barber and Jamieson and caseinolytic activity was studied in each fraction at pH 8.0 in the presence or absence of Ca^{2+} , using fluorescamine method. A significant caseinolytic activity was detected in the soluble fraction but none in the fractions of plasma membrane or pellet. Then, this protease was partially purified from the soluble fraction by means of ammonium sulfate fractionation and subsequent column chromatographies on DEAE-Sephadex CL-6B and Sephadex G-150. The partially purified protease required Ca^{2+} strictly for its activity with the optimal concentration of 1 mM and pH optimum for the activity was 8.0, by which this enzyme was classified as a calcium-activated neutral protease. The molecular weight was estimated to be 135,000 by Andrew's method. The activity of the protease was strongly inhibited by sulfhydryl reagents but no enhancement of the activity was observed in the presence of cysteine or reduced glutathione. The inhibition of the activity by PMSF or DFP was observed only when the protease was preincubated at 25°C for 1 hour in the presence of Ca^{2+} .

From these observations, it may be suggested that the enzyme may possess calcium binding site independent of the catalytic site and that by changing the conformation of the enzyme Ca^{2+} may convert the enzyme from an inactive form to an active form. However, the physiological role of the enzyme in the platelet reaction has yet to be fully elucidated.

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INHIBITION OF PLATELET AGGREGATION AND RELEASE REACTION BY A CALMODULIN INTERACTING AGENT, N-(6-AMINOHEXYL)-5-CHLORO-1-NAPHTHALENE SULFONAMIDE. J. Suda and N. Aoki. Institute of Hematology, Jichi Medical School, Tochigi-Ken, Japan.

Platelet myosin light chain kinase is a calmodulin-dependent enzyme which requires calmodulin for its activity and is intimately related to actomyosin contracting system. Platelet actomyosin contraction may cause the release reaction of platelet. N-(6-aminohexyl)-5-chloro-naphthalene sulfonamide (W-7) is one of the calmodulin interacting agents, and selectively inhibits calmodulin activity. Since platelet membrane phospholipases which are responsible for the release of arachidonic acid from membrane phospholipids may also be calmodulin-dependent enzymes, the inhibitory effects of W-7 on platelet function were examined. W-7 in a concentration as low as 10 μM inhibited platelet aggregation and release reaction induced by ADP, collagen, epinephrine, thrombin, arachidonic acid and Ca^{2+} -ionophore A23187. When platelets were preincubated with W-7 before addition of an aggregating agent, the inhibition became more pronounced. In ADP-induced aggregation, only the second phase was inhibited. Arachidonate metabolites (PGG_2/H_2 and thromboxane A_2) failed to induce aggregation and release reaction of platelets preincubated with W-7. Furthermore, release of arachidonic acid from platelet membrane phospholipids induced by A23187 in the presence of indomethacin was inhibited by W-7. W-7 had no effect on glucose metabolism in platelets. The c-AMP levels in platelets were not significantly changed by incubation with W-7. These results suggest that W-7 inhibits directly phospholipase A_2 and/or phospholipase C in addition to myosin light chain kinase. These results may in turn indicate that calmodulin is involved in platelet aggregation and release reaction mechanism including membrane phospholipases and the target system of thromboxane A_2 (possibly actomyosin contracting system).

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PLATELET SHAPE CHANGE AND CYTOSKELETAL REORIENTATION DURING ADHESION AND SPREADING. J.C. Mattson. Department of Pathology, Michigan State University, East Lansing, MI.

Sequential morphologic and cytoskeletal changes which occur during platelet adhesion have been examined by transmission electron microscopy of detergent extracted whole mounts. Human platelets allowed to spread on formvar-coated grids for 5, 15, 30 or 60 min. were fixed in glutaraldehyde to which the non-ionic detergent Nonidet P40 had been added. Whole mounts examined after 5 min. of contact demonstrated "dendritic platelets" with centralized organelles and numerous elongated pseudopodia. The cytoskeletal organization was oriented around the pseudopodia with bundles of 60-120 \AA filaments extending into each pseudopod. At the base of many pseudopodia dense filamentous mats measuring from 0.2-0.5 μ were intimately associated with the filament bundles; filaments appeared to radiate from filament mats producing stellate configurations. An underlying trabecular network of 30-85 \AA filaments was present interconnecting the longer filaments. The microtubular coil usually associated with the centralized granulomere of aggregated platelets appeared to uncoil in adherent platelets. As adhesion progressed, platelets were seen to spread on the formvar surface. Web-like extensions of platelet cytoplasm filled in and connected pseudopodia eventually replacing these processes with large veils of extended cytoplasm. By 60 min. most platelets were completely spread and had circular, fan-shaped or polygonal configurations. Radially oriented filament bundles extending from dense filament mats remained visible in the cytoplasmic veils of early spreading platelets, but as spreading progressed reorganization of filaments and filament mats occurred. Filament mats, in more completely spread platelets, were present at the platelet margin and filament bundles were no longer radially oriented but assumed a circumferential organization surrounding the centralized platelet granules. These studies demonstrate that cytoskeletal reorganization is intimately associated with the platelet shape change.