Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 652-659

MODULATION OF HUMAN ERYTHROCYTE Ca^{2+}/Mg^{2+} ATPase ACTIVITY BY PHOSPHOLIPASE A_2 AND PROTEASES. A COMPARISON WITH CALMODULIN.

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Received April 16,1980

Summary: The human erythrocyte membrane Ca^{2+}/Mg^{2+} ATPase responded to the presence of an acidic phospholipase A_2 and to low levels of trypsin (and chymotrypsin) in much the same way as it did to calmodulin isolated from human erythrocytes. The increased concentration of ATP hydrolyzed in 1 hour was similar to that observed when calmodulin had been added to a suspension of membranes during the assay. The observations reported here strongly suggest that activation of the Ca^{2+}/M^{2+} ATPase can proceed by introducing apparently distinct perturbations either to the protein or to phospholipid domains of the erythrocyte membrane.

INTRODUCTION

The level of Ca^{2+} within the human erythrocyte is now considered to be regulated by a Ca^{2+} stimulated/Mg^{2+} dependent ATPase (1). Following the original report of Bond and Clough (2) on the presence of an activator protein for this enzyme in human erythrocytes, and then the isolation and purification of this activator protein by Luthra et al. (3,4), considerable interest developed in establishment of the mechanism by which this protein controlled the activity of the ATPase.

In this communication our findings show that modulation of the Ca^{2+}/Mg^{2+} ATPase of human erythrocyte membranes also can be accomplished by exposure of the membrane to low levels of trypsin (and chymotrypsin) or a purified acidic phospholipase A_2. The results of phospholipase and protease attack suggest that ATPase modulation can result either from a

*Supported by a grant from the National Institutes of Health (AM-25547) and the National Science Foundation (PCM 77-08816).

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Abbreviations BHT - 2,6 ditert butyl 4 methylphenol

DEGS - diethylglycol succinate NPGB - p-nitrophenyl-p'-guanidino benzoate

0006-291X/80/100652-08$01.00/0

perturbation to membrane organization or possibly by a direct hydrolytic attack upon the ATPase. Similarly, calmodulin may elicit local perturbations resulting in stimulation of the rate of ATP hydrolysis.

EXPERIMENTAL

Materials

Disodium ATP, L-histidine, saponin, ouabain octahydrate and trypsin type III were obtained from Sigma Chemical Co. (St. Louis). Chymotrypsin (47 units/mg) was obtained from Worthington Biochemicals and acidic phospholipase A_2 (43 units/mg) was purified from whole venom of Agkistrodon halys blomhofii (unpublished observation). p-Nitrophenyl-p'-guanidino benzoate was obtained from ICN Pharmaceutical, Inc. (Cleveland).

General -- The techniques for collection of human blood, recovery of the erythrocytes, lysis of these cells in an isotonic (saponin) medium, with 2 mM EDTA, and the assay system for Ca^{2+}/Mg^{2+} ATPase are described in detail in recent publications (5,6).

Calmodulin Isolation -- Human erythrocyte calmodulin was isolated according to Jarrett and Penniston (7), or a slight modification of their procedure.

Assay of Ca^{2+}/Mg^{2+} ATPase -- One hundred μl aliquots of the membrane suspension plus 100 μl of varying concentrations of additives were mixed in to acid washed tubes. Then 0.5 ml of a solution containing the following composition was added: 43 μM $CaCl_2$, 3.6 mM $MgCl_2$, 80 mM KCl, 80 mM histidine, 1.13 mM ouabain and 2.46 mM ATP, pH 7.6. This mixutre was incubated at 44°C for 60 min. The reaction was terminated by immediately chilling the tubes in ice, then adding 1.0 ml cold 10% w/v trichloroacetic acid anf cortexing. The accumulated phosphate released by the enzyme hydrolysis of ATP was then assayed (8) and the resultant activity expressed as μmoles phosphate released/hr/mg membrane protein.

Measurement of Mg^{2+} ATPase Activity -- The contribution to the total hydrolysis of ATP by Mg^{2+} ATPase was measured in parallel experiments described by Hanahan et al. (6). Under all circumstances where hydrolytic enzymes were used, the Mg^{2+} ATPase activity was less than 30 percent of the total maximum stimulated Ca^{2+}/Mg^{2+} ATPase activity. A slight elevation in Mg^{2+} ATPase activity was observed when the hydrolytic enzyme concentration was increased by 10-fold above that needed for maximum Ca^{2+}/Mg^{2+} ATPase activity. Since this activation had little effect upon the stimulatory portion of the Ca^{2+}/M^{2+} ATPase activity assays, the Mg^{2+} ATPase contribution was not subtracted from the overall Ca^{2+}/M^{2+} ATPase data; hence, these are not reported here.

Preincubation of Membranes with Trypsin -- Membranes were incubated in isotonic NaCl-His, pH 7.6, at 44°C for various time periods. Trypsin was added to this suspension such that the final concentration of enzyme was 0.7 μg/ml.

Inhibition of Trypsin Activity -- A stock solution of 10 mM p-nitrophenyl-p'-guanidino benzoate in acetonitrile-dimethyl formamide (9) was prepared and stored at 4°C until needed. In order to inhibit trypsin

activity a minimum of 200-fold molar excess NPGB was added to the membrane-trypsin incubation mixture. The maximum added volume was 20 μl in 0.7 ml of mixture. Control experiments did reveal a 5 percent loss in ATPase activity in the presence of this reagent or its solvents.

Fatty Acids Liberated from Acidic Phospholipase A_2 Treated Membranes -- Membranes suspended in assay medium containing the appropriate added proteins were incubated at 44°C for 70 min and then extracted at room temperature by adding 1.8 vol methanol followed by 1 vol petroleum ether (B.p. 30-60°) containing 0.05% (w/v) BHT. Then, 20 μg heptadecanoic acid were added as an internal standard. The petroleum ether phase was collected and the aqueous methanol phase was twice re-extracted with fresh petroleum ether and the latter combined with the first petroleum ether extract. This extract was concentrated to a small volume and applied to a 1000 micron preparative thin layer plate (Analtech Silica Gel G) and developed using a petroleum ether/diethyl ether/acetic acid (85:15:1, v/v) solvent system. Spots were visualized with rhodamine 6G spray. The migration distance of free fatty acids was determined by the use of a heptadecanoic acid standard chromatographed in a separate lane. The fatty acid band was scraped from the plate into screw top tubes. The fatty acids were esterified by refluxing the silica gel scrapings in 1 ml 0.5% (v/v) sulfuric acid in methanol at 70°C for 70 min, sample cooled and extracted with petroleum ether. The upper phase was withdrawn and analyzed by gas-liquid chromatography using a Varian 3700 gas chromatograph with 15% DEGS on Chromasorb W, 80/100 mesh, column at 185°C.

RESULTS AND DISCUSSION

The influence of various modifiers and their Ca^{2+}/Mg^{2+} ATPase activity are described in the following sections.

Acidic Phospholipase A_2 -- When membranes were incubated in the presence of purified phospholipase A_2 the rate of ATP hydrolysis increased with an increase in phospholipase concentration (Figure 1, curve a) and the response is similar to the activation observed when calmodulin was used (Figure 1, curve b).

The considerable change in Ca^{2+}/Mg^{2+} ATPase activity resulting from exposure to phospholipase A_2 was not reflected by extensive changes in membrane phospholipid composition. The fatty acids liberated by phospholipase A_2 which accounted for less than 1 percent of the total membrane fatty acid are shown in Table I. None were detected at zero time. No fatty acids were liberated from membranes exposed to trypsin or calmodulin.

In order to examine further the specificity of enzymatic perturbations influencing Ca^{2+}/Mg^{2+} ATPase activity, the membranes were incubated in the presence of constant phospholipase A_2 concentrations plus calmodulin. It was found that stimulation by phospholipase A_2 and

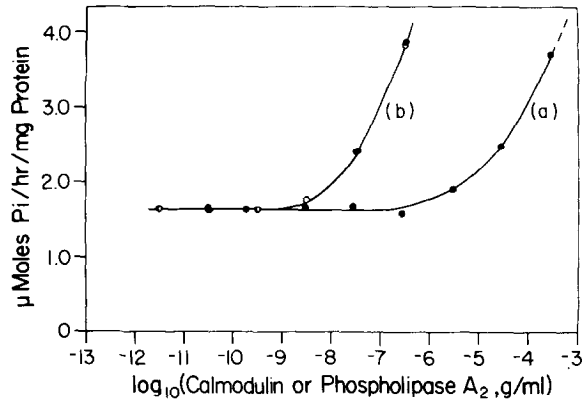


Figure 1. Composite dose response of several experiments using membranes from the same preparation as functions of acidic phospholipase A_2 (curve a) and calmodulin (curve b). Incubation was done in the presence of 43 μM Ca^{2+}.

calmodulin is additive at low calmodulin concentrations. In the converse experiments, additivity also was found at low phospholipase A_2 concentrations (data not shown). These data suggested that both added molecules influence the endogenous Ca^{2+}/Mg^{2+} ATPase rather than expose additional ATPase activity.

Proteases -- The influence of trypsin on membrane Ca^{2+}/Mg^{2+} ATPase activity is illustrated in Figure 2. Curve 2a represents a control membrane

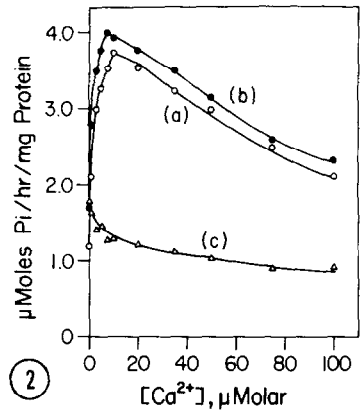
TABLE I

Fatty Acids Isolated from Acidic Phospholipase A_2-Activated Erythrocyte Membranes

Fatty Acid Species	Percent Yield*	Ratio: $\frac{\text{Fatty Acid Liberated}^{\dagger}}{\text{Total Fatty Acid}}$
16:0	15.9	0.65
18:0	4.3	0.27
18:1	22.2	1.30
18:2	22.3	2.12
20:4	35.1	2.58

*Percent yield is defined as the relative amount of material extracted.

†The ratio column represents the decimal equivalent of the fatty acids found in the extract with respect to that fatty acid species present in the lipids of the erythrocyte membrane. Data for total fatty acid obtained from that reported by Nelson (11).



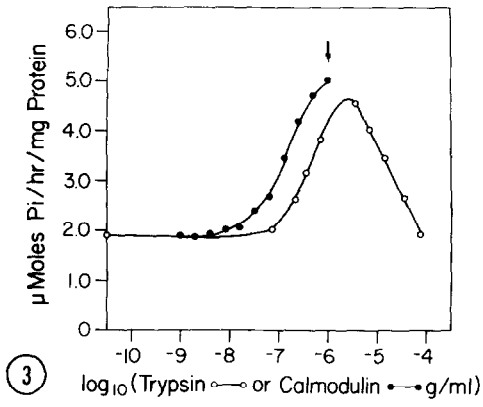


Figure 2. A comparison of the Ca^{2+}/Mg^{2+} ATPase activity as affected by calmodulin or trypsin in the presence of Ca^{2+}. Curve a (o-o) is the activity of membrane ATPase in the presence of 0.10 μg/ml calmodulin protein; curve b (•-•) in the presence of 0.71 μg/ml trypsin; curve c (△-△) the activity in the presence of 71.0 μg/ml of trypsin.

Figure 3. The response of Ca^{2+}/Mg^{2+} ATPase activity to varying concentrations of trypsin (o-o) or calmodulin (•-•) in the presence of 43.0 μM Ca^{2+}. Proteins were added separately to the assay medium, followed by the addition of the membrane suspension.

preparation in which calmodulin was incubated with membranes in the presence of varying calcium concentrations and the ATPase activity monitored. Curve 2b shows the results of the addition of trypsin to membranes at a level of 0.71 μg/ml incubation mixture. The latter data illustrated the similarity in response of these two reagents. However, at one hundred-fold increase in trypsin, 71.0 μg/ml (curve c), there was a distinct difference with a substantial decrease in ATPase activity with respect to $[Ca^{2+}]$.

The nature of the sensitivity of the Ca^{2+}/Mg^{2+} ATPase to trypsin was further examined by varying the trypsin concentration and incubating the membrane suspension at a single Ca^{2+} concentration. The results are shown in Figure 3. As the concentration of trypsin is raised, the ATPase activity of the membranes undergoes a sigmoidal increase. A reduction of the ATPase activity is caused by adding more trypsin to the incubation medium. These data are in accord with the results illustrated in Figure 2, where the higher trypsin concentration lowered the ATPase activity. In comparison (Figure 3) the calmodulin treated Ca^{2+}/Mg^{2+} ATPase activity is similar over the three decade stimulation in ATPase activity, e.g., concentration at half maximum Ca^{2+}/Mg^{2+} ATPase activity, calmodulin 0.85 μg/ml vs trypsin 1.0 μg/ml.

TABLE II

The Effect of Protease Addition to Membranes: The Influence of Inhibited Enzyme and Pre-incubation Proteolysis of Membranes upon Ca^{2+}/Mg^{2+} ATPase

Condition	Activity (μMoles P_i/hr/mg Protein)
Control (without added NPGB)	1.85
Control (in presence of NPGB)	1.83
Membranes plus NPGB-trypsin	1.82
Membranes plus calmodulin (in presence of NPGB)	3.79
Membranes plus calmodulin and NPGB-trypsin	3.71
Membranes plus trypsin (5 minute preincubation)	3.02

Trypsin was inhibited with NPGB at the completion of the preincubation (see Methods). Membranes were washed three times with isotonic sodium chloride-histidine buffer, then reconstituted to their original 10% suspension. ATPase assay was done as described in the Methods section. NPGB in the assay medium was 47 μmoles/ml. NPGB-trypsin was prepared prior to the addition of membranes. The final concentration of NPGB-trypsin in the assay was 3.5×10^{-7} g/ml. This was also the concentration of enzyme used during predigestion of the 10% membrane suspension.

Similar experiments using chymotrypsin revealed a similar pattern in stimulating the ATPase (data not shown).

Other experiments illustrating the effectiveness of trypsin are summarized in Table II. It is shown that inhibited trypsin does not activate the ATPase suggesting proteolysis of membrane proteins and/or the Ca^{2+}/Mg^{2+} ATPase is responsible for the stimulated activity previously observed. That NPGB did not inhibit the ATPase is apparent when a comparison of the controls in Table II is made.

This study shows for the first time the unexpected similarity of calmodulin action toward Ca^{2+}/Mg^{2+} ATPase and that of certain perturbant molecules such as proteases and acidic phospholipase A_2. It has been demonstrated that the influence can be affected by several pathways involving the lipid or protein moieties. In the former, purified acidic phospholipase A_2 altered the lipid moiety by hydrolyzing a small percentage of the fatty acid chains from their parent molecules. Presumably these perturbations were significant and influenced the ATPase activity. If the ATPase is intimately associated with structural elements

of the membrane, then perturbations of the protein cytoskeleton could likewise affect changes in the ATPase activities. In this regard, analysis of membrane proteins by polyacrylamide gel electrophoresis following trypsin exposure (data not shown) revealed membrane proteins are systematically hydrolyzed and that the initiation of Ca^{2+}/Mg^{2+} ATPase stimulation roughly coincides with the completion of membrane actin and spectrin to lower molecular weight fragments.

This observation does not preclude the possibility of a direct influence of the protease on the Ca^{2+}/Mg^{2+} ATPase itself. Of interest, Stewart and MacLennan (10) reported that Ca^{2+}-ATPase from sarcoplasmic reticulum exposed to trypsin may have been cleaved and as suggested by the authors this influence resulted in an enhanced ATPase activity. Still another possibility is that minute quantities of calmodulin-like fragments may be recruited from membrane proteins during exposure of the membrane to proteolytic enzymes. The fragments would activate the ATPase and this process lead to activation. This interpretation is not consistent with the phospholipase A_2 results where proteolytic activity was not present, nor is it consistent with the last data in Table II where membranes were washed three times following trypsinization. This procedure should have caused a decrease in the stimulated activity due to dissociation of the activating fragment. Of course it may be argued that the activating fragments remain tightly bound during the washes.

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