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A Ca^{2+}-STIMULATED ATPase ACTIVITY IN RABBIT NEUTROPHIL MEMBRANES

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Summary

An ATPase activity specifically stimulated by micromolar Ca^{2+} concentrations has been identified in association with rabbit neurophil membranes. These studies provide the basis of further characterization of the Ca^{2+}-ATPase activity with regard to neutrophil function.

It is generally accepted that divalent cations have an important role in the activation of cell function and of neutrophils, in particular [1—4]. There are now several observations consistent with the hypothesis that neutrophil activation by chemotactic factors involves (1) a graded displacement of prebound calcium and (2) a graded increase in membrane permeability to calcium [2,3,5—7]. Such cellular alterations result in an increased concentration of cytosolic exchangeable calcium which, in turn, may participate in other aspects of neutrophil activation, e.g. activation of the contractile apparatus that leads to cell movement. Regulation or reduction of the cytosolic exchangeable calcium levels following activation is another aspect of Ca^{2+} metabolism necessary for the reiterative response of the neutrophil to stimuli. Extrusion of cytosolic calcium via active transport across cell membranes is a regulatory mechanism which has been suggested by several investigators [2,3, 6—8]. This extrusion of cytosolic calcium is achieved by a calcium pump which is driven by the hydrolysis of ATP by the Ca^{2+}-stimulated ATPase. These studies were undertaken to demonstrate the existence of such an ATPase activity.

Rabbit neutrophils were obtained by collecting the peritoneal exudate from albino rabbits injected 14 h previously with 400 ml 0.1% glycogen in 0.9% NaCl. The cell suspension was centrifuged at 600 × *g* for 5 min and the

pelleted cells were washed with 150 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4, to hemolyze erythrocytes [9–11]. The neutrophils were then washed with 11.6% (w/v) sucrose, 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid), 1 mM EDTA, pH 7.4, and centrifuged at 1600 × *g* for 5 min. The cells were washed twice more and the final cell pellet was homogenized on ice in a T-line Laboratory Homogenizer with a teflon pestle (Talboys Engineering Corp., Emerson, NJ) at approximately 950 rev./min for 15 strokes after which treatment the majority of neutrophils were broken as determined by microscopic examination. Membranes were prepared from the homogenate by a slight modification of the method of Woodin and Wieneke [10,12]. Briefly, a postnuclear fraction was prepared by centrifugation of the cell homogenate at 2500 × *g* for 10 min. The supernatant was layered on a discontinuous gradient containing 5.9 ml of 30% (w/v) sucrose, 4 ml of 40% sucrose and 2 ml of 50% sucrose. All of the sucrose solutions contained 10 mM Hepes, pH 7.4, and 1 mM EDTA. Centrifugation was carried out at 130 000 × *g* for 1 h in a Beckman SW-27 rotor. The material at the interface between the 11.6% and 30% sucrose solutions (termed Band I), and between the 30% and 40% sucrose solutions (termed Band II) was collected. The bands were concentrated by adding 1 volume of 10 mM Hepes, pH 7.4, to lower the sucrose content and centrifuging the suspension at 105 000 × *g* for 1 h. The resulting pellets were resuspended in 1 ml of 10 mM Hepes, pH 7.1, and frozen in aliquots at –20°C. Electron microscopy of the pelleted material indicated the presence of membraneous sheets and vesicles in both Bands. We have shown previously that band II contains ATPase activity [10] which meets all the requirements (Na^+-activated, K^+-activated and ouabain-inhibited) of the commonly known (Na^+,K^+)-ATPase involved in the maintenance of the Na^+,K^+ concentration gradients across all plasma membranes [13]. Moreover, band II contains the binding site for the synthetic chemotactic peptides [14]. The (Na^+,K^+)-ATPase activity in band II was also found to be stimulated by the chemotactic factor formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe) [15].

ATPase activity was determined by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP (New England Nuclear) according to Seals et al. [16]. The basic medium for the assay of ATPase activity (100 μl final volume) contained 30 mM imidazole-HCl, pH 7.0, 0.2 M sucrose, 0.1 mM ouabain and 0.1 mM EGTA (ethyleneglycol bis-(β-aminoethylether)-*N*,*N*'-tetraacetate). The amounts of $MgCl_2$, $CaCl_2$ and Na_2ATP (neutralized) added to the assay medium to reach the desired concentrations of the metals in ionized form were calculated by solving the multiple equilibria equations that describe the dissociation of ATP and EGTA at pH 7.0, the association of Mg^{2+} and Ca^{2+} with the anions ATP^{4-}, ATP^{3-}, $EGTA^{4-}$, and $EGTA^{3-}$, and the balance of material for ATP, EGTA, Ca^{2+} and Mg^{2+}. The stability constants were taken from Sillen and Martell [17]. The final concentration of Mg^{2+} was maintained at 0.1 mM and of MgATP, 0.3 mM. The final Ca^{2+} concentrations are indicated in the accompanying figures. The enzyme assays were initiated by the addition of neutrophil membranes followed by incubation at 37°C for 10 min (unless otherwise indicated). Assays were terminated by the addition of 20 μl 6% SDS followed immediately by vigorous mixing. Blanks for the determina-

tion of background and nonenzymatic hydrolysis of ATP were comprised of salts, sucrose, ATP, membranes and SDS. The specific activity of [γ-^{32}P]ATP was generally 1 mCi/mmol or greater and under the described conditions the amount of $^{32}P_i$ produced never exceeded 5% of the ATP originally present in the medium thus warranting high ATP/ADP ratios throughout the assay. After termination of the assay, 60 μl of phosphate reagent made up of 4 N H_2SO_4, 4% (w/v) ammonium molybdate and 0.02 M silicotungstic acid was added to each assay tube and the $^{32}P_i$ was separated from [γ-^{32}P]ATP by extraction of the phosphomolybdate complex into 500 μl xylene/isobutanol (65 : 35, v/v). Aliquots of the xylene : isobutanol (200 μl) were quantitated by scintillation counting in ACS^{TM} (Amersham Corp.). Specific activity of the [γ-^{32}P]ATP used in each assay was determined by counting a 10-μl aliquot in 200 μl xylene : isobutanol in ACS^{TM}. Extraction of the phosphomolybdate complex was linear up to 10^{-7} mol of $^{32}P_i$ from the aqueous solution and up to 300 μg protein/ml. Protein concentration was determined by the method of Lowry et al. [18] using bovine serum albumin as standard.

The hydrolysis of [γ-^{32}P]ATP to ADP and $^{32}P_i$ catalyzed by membranes prepared from rabbit neutrophils was measured in the presence of 0.1 mM Mg^{2+}, 0.3 mM MgATP and also with the addition of Ca^{2+} in micromolar quantities. A representative time course of this hydrolysis in the presence and absence of 10 μM Ca^{2+} is shown in Fig. 1. Under the conditions employed in the assay, Ca^{2+} significantly stimulates the production of P_i above the background Mg^{2+}-ATPase activity in a linear fashion. Approximately 10 nmol of P_i per min per mg membrane protein are released in response to 10 μM Ca^{2+}. The stimulation of the ATP hydrolysis by Ca^{2+} cannot be mimicked by the addition of equivalent or excess amounts of Mg^{2+}.

The Ca^{2+}-stimulated ATPase activity could not be due to possible contamination of the neutrophil preparation by erythrocyte membranes. After the

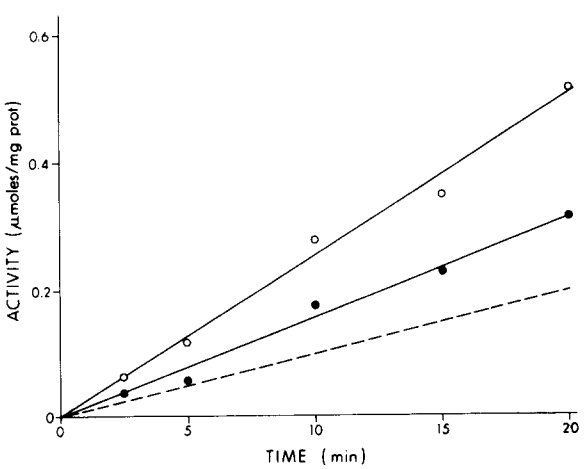

Fig. 1. Time course of ATPase activity in rabbit neutrophil membranes. The ATPase activity was assayed in the presence of 0.1 mM Mg^{2+}, 0.3 mM MgATP (●) and with the addition of 10 μM Ca^{2+} (○). The assay medium also contained 30 mM imidazole-HCl, pH 7.0, 200 mM sucrose, 0.1 mM EGTA and 0.1 mM ouabain. The incubation temperature was 37°C. The Ca^{2+}-stimulated ATPase activity (---) is the difference between the total ATPase activity in the presence of both Ca^{2+} and Mg^{2+}, and the background Mg^{2+}-ATPase activity.

erythrocytes were hemolyzed with NH_4Cl, the suspension was centrifuged for 5 min at 600 × *g*. At this speed and time of centrifugation most, if not all, cell membranes or ghosts will stay in the supernatant. In addition, contamination of the neutrophil preparation with other cell types did not exceed 10%.

The distribution of the Ca^{2+}-stimulated ATPase activity between the two isolated membrane bands is shown in Table I. The specific activity of each is

TABLE I

ATPase ACTIVITY OF RABBIT NEUTROPHIL MEMBRANES

The activity is reported as μmol P_i per mg protein per hour. The assay medium contained 0.1 mM Mg^{2+}, 0.3 mM MgATP, 30 mM imidazole-HCl, pH 7.0, 200 mM sucrose, 0.1 mM EGTA, 0.1 mM ouabain and, when present, 10 μM Ca^{2+}. Samples were incubated for 10 min at 37° C. Ca^{2+}-stimulated ATPase activity refers to the difference between total ATPase activity measured with both Mg^{2+} and Ca^{2+} in the medium (column 2) and background Mg^{2+}-ATPase activity measured with Mg^{2+} alone (column 1). Means are given with the standard error of the mean. The number in parentheses refers to the number of membrane preparations.

Membrane fraction	Activity		Ca^{2+}-stimulated activity
	Mg^{2+} (1)	Mg^{2+} + 10 μM Ca^{2+} (2)	
Band I	0.96 ± 0.07 (4)	1.38 ± 0.06 (4)	0.42
	$P^* < 0.01$		
Band II	0.94 ± 0.05 (7)	1.30 ± 0.05 (7)	0.36
	$P < 0.01$		

*Using column 2 is significantly different from column 1. Student's *t*-test for unpaired samples.

approximately 0.4 μmol per mg protein per hour. This value compares well with that reported for the Ca^{2+}-ATPase in human erythrocytes, 0.7 μmol per mg protein per hour at 100 μM Ca^{2+} [19,20], although the value reported for the Ca^{2+}-ATPase associated with the macrophage plasma membrane is 30 times greater or 12 μmol per mg protein per hour at 10 μM Ca^{2+}. The apparent discrepancy may be due to differences in cell type, membrane isolation, or the fact that the Ca^{2+}-ATPase activity in the macrophage membranes was measured in the absence of Mg^{2+}. The background ATPase activity (the activity in the presence of Mg^{2+} alone) in the neutrophil membranes is reduced to about 0.14 μmol per mg protein per hour upon addition of 4 mM EDTA to the assay medium which, in turn, effects the removal of Mg^{2+}. This indicates that the removal of Mg^{2+} is responsible for almost a 7-fold decrease in the background ATPase activity. It is not clear whether Ca^{2+} can substitute for Mg^{2+} in the stimulation of the Mg^{2+}-dependent ATPase activity and, in order to avoid confusion of the Ca^{2+}-specific ATPase activity with the Mg^{2+}-dependent background ATPase activity, we employed assay conditions under which the stimulation by Mg^{2+} was minimal.

The Ca^{2+}-stimulated ATPase activities measured in the two membrane fractions apparently represent the same (or equivalent) enzyme(s) since both preparations exhibit identical kinetic properties. Both Band I and Band II ATPase activities are stimulated to the same extent by Ca^{2+} concentrations ranging from 1 to 10 μM and exhibit a K_m for Ca^{2+} of 3.3 μM. The data obtained from both fractions in three preparations were combined and linear regression analysis yielded a Lineweaver-Burk plot (correlation coefficient 0.98), from which K_m could be calculated.

We are presently investigating the cellular function(s) associated with the Ca^{2+}-stimulated ATPase activity in rabbit neutrophil membranes. Two likely

possibilities are (1) the transport of Ca^{2+} across the membranes in a manner analogous to the ATP-driven Ca^{2+} extrusion mechanism described in several systems including erythrocyte ghosts [19,20] and cardiac sarcolemma, or (2) activation of the contractile apparatus which is involved in neutrophil locomotion and phagocytosis (Shibata et al. [23,24] and Ref. 25). With regard to the latter possibility, some filamentous forms presumably representing actomyosin are evident in the electronmicrographs of the membrane fractions.

We have demonstrated the existence of a heretofore unidentified ATPase activity associated with neutrophil membranes that is stimulated specifically by micromolar Ca^{2+} concentrations. Our continuing studies will characterize the Ca^{2+}-ATPase activity further in an attempt to define its role in the response of neutrophils to various chemotactic and phagocytic stimuli.

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