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SUBCELLULAR LOCALIZATION AND PROPERTIES OF ADENOSINE DIPHOSPHATASE ACTIVITY IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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Summary

Adenosine diphosphatase (ADPase) activities were studied in human polymorphonuclear leukocytes with a recently developed radio-assay. The neutrophils were homogenized in isotonic sucrose and subjected to analytical subcellular fractionation. The sucrose density gradient fractions were assayed for ADPase activity and for principal organelle marker enzymes. ADPase activity was distributed between the plasma membrane, specific granule and soluble fractions. The plasma membrane and specific granule activities had similar kinetic and inhibitor properties but the cytosolic enzyme was clearly different. Studies with the non-penetrating inhibitor diazotized sulphanilic acid and measurements of latent activity indicate that plasma membrane ADPase activity is located on the external aspect to the cell. Its possible role in inhibiting platelet aggregation is discussed.

Neutrophils were isolated from control subjects, patients with chronic granulocytic leukaemia and patients in the third trimester of pregnancy. The specific activities (mU/mg protein) of ADPase activity, in contrast to those of alkaline phosphatase, were similar in all three groups. This result, together with fractionation experiments and inhibition studies strongly suggests that ADPase activity is not attributable to neutrophil alkaline phosphatase.

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Introduction

It is well known that adenosine diphosphate (ADP) is an important agent responsible for aggregating platelets in damaged vascular tissue [1]. This a rapid process, occurring in all species studied [2], and ADP is the only naturally occurring nucleotide with this property. Further studies [3] have shown that breakdown products of ADP, such as adenosine monophosphate (AMP) and especially adenosine, inhibit the ADP-mediated platelet aggregation. ADP-degrading systems have been described in the vascular tree of a variety of species [4—7].

Normal human polymorphonuclear leukocytes are a rich source of alkaline phosphatase and the levels of activity vary quite considerably is neutrophil disorders [8]. Recently this alkaline phosphatase activity has been localized to a hitherto undescribed organelle (phosphasome) of the human neutrophil [9], the function of which is as yet unknown. We are attempting to establish the function of this organelle: ADPase is an alkaline phosphohydrolase which could contribute to the neutrophil alkaline phosphatase activity.

The present study determines the subcellular localisation of ADPase activity with analytical subcellular fractionation techniques and investigates certain properties of the enzyme and compares the activities in neutrophilic disorders known to show marked alterations in alkaline phosphatase activity with control values.

Materials and Methods

Preparation of polymorphonuclear leukocytes. Polymorphonuclear leukocytes were isolated as described previously [8]. 35 ml of blood were used from control subjects and pregnant women, whilst blood from leukaemic patients with a high leukocyte count was diluted with an equal volume of 0.15 mol/l NaCl before isolating the neutrophils by dextran sedimentation and Ficoll-Hypaque centrifugation [10]. The cells were pelleted in 4 ml 0.2 mol/l sucrose containing 1 mmol/l $Na_2 \cdot$ EDTA, pH 7.2 and 5000 U heparin/l, and disrupted with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.) by 30 strokes of a tight fitting (Type B) pestle. An aliquot of the homogenate was retained for analysis and a portion centrifuged at $800 \times g$ for 10 min in a Coolspin 4×100 ml swing-out rotor (MSE Scientific Instruments, Crawley, Sussex). The post-nuclear supernatant was removed, stored on ice and subjected to analytical subcellular fractionation and enzyme analysis as described previously [8].

Analytical subcellular fractionation. Approx. 5 ml of the post nuclear supernatant was layered onto a 28 ml sucrose density gradient extending linearly with respect to volume, from a density of 1.05 $g \cdot cm^{-3}$ to one of 1.28 $g \cdot cm^{-3}$ and resting on a 6 ml cushion of density 1.32 $g \cdot cm^{-3}$ in a Beaufay automatic zonal rotor. All gradient solutions contained 1 mmol/l $Na_2 \cdot$ EDTA, pH 7.2 and 5000 U heparin/l. The rotor was run at 35 000 rev per min for 35 min and, after slowing to 8000 rev per min, some 15 fractions were collected, weighed and their density determined as described previously [11].

Organelle marker enzymes. The gradient fractions were assayed for marker

enzymes for the principal subcellular organelles. The enzymes assayed, with the organelle shown between parentheses were: alkaline phosphatase, EC 3.1.3.1 (phosphasome); 5′-nucleotidase, EC 3.1.3.5 (plasma membrane); myeloperoxidase, EC 1.11.1.7 (azurophil); lactate dehydrogenase, EC 1.1.1.27 (cytosol); particulate malate dehydrogenase, EC 1.1.1.37 (mitochondria) and neutral α-glucosidase, EC 3.2.1.20 (endoplasmic reticulum). Conditions for these assays have been described previously [9]. Unsaturated vitamin B-12 binding capacity (specific granule) was determined by the charcoal radioassay [12]. Protein was estimated by a modification of the method of Lowry et al. [13] with bovine serum albumin as standard.

Adenosine diphosphatase (EC 3.6.1.5) assay. The homogenates and gradient fractions were assayed for ADPase activity by a recently described method [14]. Under normal assay conditions 0.1 ml of suitable diluted enzyme was incubated in a mixture of 0.1 ml 0.05 mol/l Tris-HCl buffer, pH 7.5/0.05 ml 2 mmol/l $MgCl_2$/0.05 ml H_2O and 0.1 ml 1 mmol/l [β-^{32}P]ADP at 37°C for up to 60 min. ADPase activity was proportional to the concentration of enzyme used and the rate of reaction was linear up to 50% hydrolysis. In assays with inhibitors the water was replaced by 0.05 ml of the appropriate inhibitor solution, and the assay performed in the standard way. Latent ADPase activity [15] was determined in freshly isolated neutrophil preparations suspended in isotonic sucrose (0.3 mol/l) containing 1 mmol/l $Na_2 \cdot$ EDTA, pH 7.2 and 5000 U heparin/l. The enzyme was then assayed under standard conditions with appropriate buffered substrate in sucrose (0.3 mol/l) containing $Na_2 \cdot$ EDTA (1 mmol/l) pH 7.4 and heparin (5000 U/l) with (total activity) and without (free activity) Triton X-100 (100 mg/100 ml). The effect of diazotized sulphanilic acid on leukocyte ADPase activity was investigated using the method of Smolen and Weissmann [16].

Results

Fig. 1 shows the averaged sucrose density gradient distributions for ADPase activity and some of the principal organelle marker enzymes. ADPase activity has two peaks of particulate activity and a soluble component. This trimodal distribution was clearly seen in each individual experiment. There is some loss of resolution during the averaging procedure but the three peaks are clearly distinct. The peak at density 1.12 $g \cdot cm^{-3}$ corresponds with the plasma membrane marker 5′-nucleotidase, whilst that at 1.20 $g \cdot cm^{-3}$ corresponds to vitamin B-12 binding protein, the marker for the specific granule. The cytosolic component follows the soluble enzyme marker lactate dehydrogenase remaining in the sample layer.

In order to confirm the plasma membrane localization of part of the ADPase activity and to further contrast the subcellular localization of 5′-nucleotidase and alkaline phosphatase, subcellular fractionation experiments were carried out on neutrophils which had been suspended in isotonic sucrose containing 0.02 mg/ml digitonin and then washed in digitonin-free sucrose. Digitonin is a selective membrane perturbant which binds to the cholesterol in plasma membrane, selectively increasing the density of this organelle [17—19]. Intracellular organelles to which the digitonin is inaccessible would be unaffected by this

treatment. As seen in Fig. 2, the distribution of the plasma membrane marker 5′-nucleotidase is markedly affected by the digitonin treatment with most of the activity moving to a density of 1.16 g · cm^{-3}. For ADPase activity there is a loss of the plasma membrane component at density 1.12 g · cm^{-3} with a major peak at density 1.16 g · cm^{-3}. There also appears to be a decrease in the proportion of cytosolic activity. In contrast, the distribution of alkaline phosphatase is unaffected by the digitonin treatment.

Studies were carried out to determine whether ADPase activity is localised to the outer or inner aspects of the plasma membrane. Intact polymorphonuclear leukocytes were assayed for latent ADPase activity. The percent latent activity (±S.E.) was 77.4 ± 4.7% (n = 6) indicating that 23% of ADPase activity in the intact cell was accessible to substrate, i.e. approx. one-fifth of the activity is on the cell surface, i.e. approx. one-fifth of the activity is on the cell surface. Diazotized sulphanilic acid is a poorly permeant reagent which has been used to selectively inactive ecto-enzymes [16,20] (Fig. 3). In intact cells (A) ADPase activity was inhibited by this reagent to a maximum of approx. 25% whereas lactate dehydrogenase was unaffected. 5′-Nucleotidase, the plasma membrane marker enzyme, shows a progressive and almost complete inhibition by diazotized sulphanilic acid. With sonicated cells (B) the three enzymes were inhibited

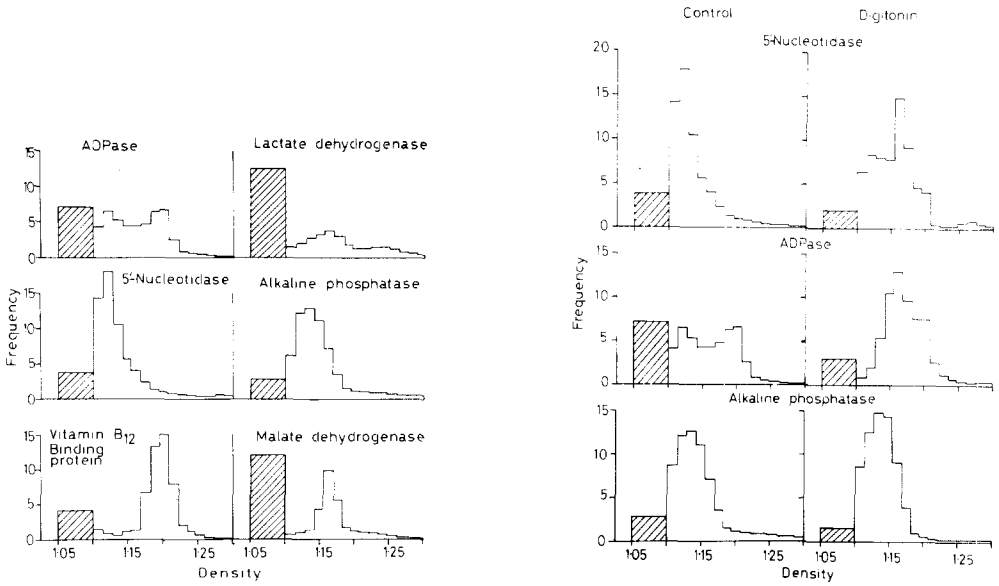

Fig. 1. Isopycnic centrifugation of post-nuclear supernatant prepared from neutrophil leukocyte homogenates. Results show mean of five experiments. Frequency is defined as the fraction of total recovered activity present in the gradient fraction divided by the density span covered. The activity present over the density span 1.05—1.10 represents enzyme remaining in the sample layer. Recovered activities range from 85—105%.

Fig. 2. Isopycnic centrifugation of post-nuclear supernatant from neutrophil leukocyte homogenate. Prior to homogenization the leukocytes were resuspended in 23 ml 0.34 mol/l sucrose containing no (control) or 0.02 mg/ml digitonin, centrifuged at 120 × g and washed in digitonin-free 0.2 mol/l sucrose. The cells were then homogenized and fractionated. Averaged control data taken from Fig. 1. Digitonin data are from a single representative experiment.

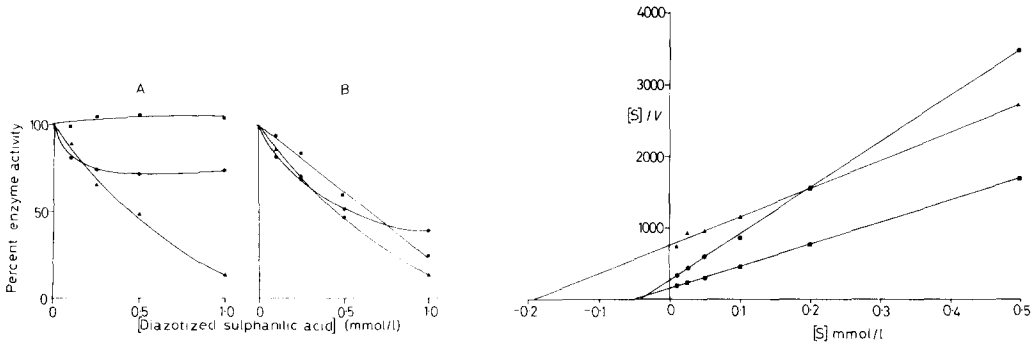

Fig. 3. A. Polymorphonuclear leukocytes, suspended in phopshate-buffered saline, were incubated for 15 min at 37°C with various concentrations of diazotized sulphanilic acid. The cells were then washed twice with ice-cold phosphate-buffered saline, sonicated for 30 s, and finally assayed for total lactate dehydrogenase, 5′-nucleotidase and ADPase. B. Polymorphonuclear leukocytes were resuspended in phosphate buffered saline, sonicated for 30 s, and then incubated for 15 min at 37°C with various concentrations of diazotized sulphanilic acid. The treated sonicates were then assayed for total lactate dehydrogenase, 5′-nucleotidase and ADPase. In both cases enzyme activities were expressed as percentages of those measured for untreated cells. Results are the mean of two duplicate estimations for two leukocyte preparations. (■), lactate dehydrogenase; (●), ADPase; (▲), 5′-nucleotidase.

Fig. 4. [S]/v against [S] plots for ADPase with cytosol (▲), plasma membrane (■) and specific granule (●) fractions from the sucrose gradient as enzyme source. The assays were performed in the presence of 0.05 mol/l Tris-HCl, pH 7.5 and 0.1 mmol/l Mg^{2+} at 37°C. The line was fitted to the points using parameters calculated from direct linear plots [42]. V is expressed as μmol of product/min.

by diazotized sulphanilic acid to a similar extent. This result, together with the latency and subcellular fractionation experiments indicate that between 20 and 25% of the leukocyte ADPase activity is located to the plasma membrane as an ecto-enzyme.

TABLE I

KINETIC CONSTANTS, OPTIMAL Mg^{2+} CONCENTRATIONS AND THE EFFECT OF INHIBITORS ON PLASMA MEMBRANE, SPECIFIC GRANULE AND CYTOSOLIC ADPase OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES

Results show the mean percent inhibition compared with control of duplicate assays on three leukocyte preparations from different subjects. Optimal $MgCl_2$ concentration and apparent affinity constant for ADP are shown.

Inhibitor (mM)	Inhibition of ADPase activity (%)		
	Plasma membrane fraction	Specific granule fraction	Cytosol fraction
Sodium azide (10)	79	76	11
Sodium fluoride (10)	73	75	50
Oligomycin (0.1)	2	8	1
Ouabain (0.1)	1	8	0
Levamisole (1)	1	8	0
	Parameter (below) (mM)		
Optimal $MgCl_2$	0.2	0.2	0.5
Apparent K_m for ADP	0.05	0.04	0.19

TABLE II

PHOSPHATASE ACTIVITIES IN LEUKOCYTE HOMOGENATES

Results are shown as mean values ± S.E. with the number of preparations assayed in duplicate shown between parentheses. Enzyme activities are expressed in mU/mg protein. Alkaline phosphatase data taken from [8].

Enzyme	Patient groups		
	Chronic granulocytic leukaemia	Control	Pregnant
Alkaline phosphatase	0.447 ± 0.116 (9) *	3.55 ± 0.58 (9)	28.4 ± 4.9 (9) *
ADPase	0.24 ± 0.03 (5)	0.30 ± 0.02 (8)	0.46 ± 0.03 (7)

* Significant differences, determined by the Student's t-test, from control $P < 0.001$.

Kinetic studies were carried out on the gradient fractions corresponding to the three peaks of ADPase activity. In all fractions the pH optimum occurred at pH 7.5 but, whilst both particulate fractions showed maximal activity in the presence of 0.2 mmol/l $MgCl_2$, the soluble fractions required 0.5 mmol/l $MgCl_2$ for optimal activity. All three fractions showed linear reaction rates with respect to time and amount of fraction protein.

The results of kinetic studies on the three fractions are shown as $[S]/v$ against [S] plots in Fig. 4. Activity in all fractions clearly obeyed Michaelis-Menten kinetics; kinetic constants are shown in Table I. Inhibitor studies were carried out to establish further differences between the enzymes from the three localizations (Table I). It is clear that the ADPase activity from both particulate localizations are affected similarly by all the inhibitors used, whilst their effect on the cytosolic component is much less. These results indicate a similarity of the plasma membrane and specific granule ADPase activities, but suggest that the cytosolic activity is distinctive. Levamisole (1 mmol/l), a potent inhibitor of leukocyte alkaline phopshatase [8] had little effect on ADPase activity in any of the three fractions. These results, taken together, indicate that there are three distinct localizations for ADPase activity in neutrophils.

Table II compares the specific activity of both ADPase activity and alkaline phosphatase in neutrophil homogenates from normal subjects, women in the third trimester of pregnancy and patients with chronic granulocytic leukaemia. Alkaline phosphatase activity varied considerably in these three patient groups with reduced levels in the leukaemic patients and increased activity in pregnancy. In contrast, the specific activity of ADPase is similar in all three patient groups. This absence of a parallelism between the two activities is further evidence that they are due to two distinct enzymes.

Discussion

The present study has demonstrated that human polymorphonuclear leukocytes have a significant amount of ADPase activity which can be reliably measured and its activity determined in leukocyte disorders. This activity has been found to have a neutral pH optimum (pH 7.5) and is inactive in the absence of Mg^{2+} [14]. Analytical subcellular fractionation studies have shown that

ADPase activity was localized to three sites within the cell. There are two peaks of particulate activity, located to the plasma membrane and specific granule, and a soluble component. This distribution is confirmed by recent cytochemical studies [21]. Kinetic studies on ADPase activity from these three localizations indicated that whilst the plasma membrane and specific granule activities had similar kinetic and inhibitor properties, cytosolic ADPase has a distinct activity. This difference in properties between the cytosolic and particulate activities is of interest. It suggests that the cytosolic activity is not merely organelle-bound enzyme which has been released during the fractionation procedure and conversely that the particulate activity is not adsorbed soluble enzyme. Purification of the individual enzyme activities from the three subcellular locations would however be necessary to definitively show that they represented distinct enzyme proteins.

Further studies on the plasma membrane ADPase, which forms approx. 20% of the total cell activity, show it to be localized on the external aspect of the cell. This is a similar location to 5′-nucleotidase [10] and thus the consecutive action of these two enzymes would have the dual beneficial effect on platelet aggregation of degrading the ADP and forming adenosine monophosphate and adenosine which themselves inhibit the ADP-platelet aggregation reaction. It is not certain whether plasma membrane 5′-nucleotidase and ADPase are distinct enzyme proteins and this will have to await the purification of these enzymes from human neutrophils. Thus, as in the arterial wall [5], these coupled leukocyte enzyme activities could play an important platelet anti-aggregatory action. Several reports have noted ADPase activity in plasma [22—25] and this activity may in part be derived from leukocytes. Enzymes from both sources have similar pH optima, are markedly activated by Mg^{2+} ions and have K_m values in the range 10^{-4} to 10^{-5} mol/l for ADP.

The effect of digitonin on the leukocytes is of particular interest. Previous studies have homogenized the cells or their fractions in isotonic sucrose containing digitonin [18,26] causing this membrane perturbant to have complex actions on various organelles. In the present study the undisrupted cells were suspended in isotonic medium containing low concentrations of digitonin and immediately centrifuged and washed in digitonin-free medium. In this manner digitonin has the unique effect of selectively increasing the equilibrium density of the plasma membrane.

The local release of ADPase activity by activated neutrophils would counteract any tendency of platelets to aggregate at the site of the inflammatory response. Neutrophils release platelet-aggregating prostaglandin derivatives [27—31] and thromboplastins [32—35] during phagocytosis. In addition they also cause endothelial cell damage [36]. ADPase activity would tend to inhibit these potentially unfavourably reactions to infection. Further studies are clearly necessary to investigate the inter-relationships of these events.

ADPase is thus a further enzyme located to the specific granule in addition to lactoferrin [37,38], collagenase [39], lysozyme [12,37—39], vitamin B-12 binding protein [12] and neutral α-glucosidase and γ-glutamyl transferase [9] described previously.

Approx. 30—40% of neutrophil ADPase activity is located at the cytosol. This activity differs from the particulate enzyme in that it has a much higher

K_m value (0.19 mmol/l) for ADP, requires high Mg^{2+} concentrations for optimum activity and is generally more resistant to a range of inhibitors. The role of cytosolic ADPase is, as yet, unclear, but would seem to be implicated in nucleotide metabolism.

The subcellular distribution studies of ADPase showed no activity corresponding to particulate alkaline phosphatase, and this is strong evidence that ADPase is not located to the phosphasome. This is confirmed by the lack of parallelism between the specific activity of ADPase and alkaline phosphatase in leukocyte disorders. Levamisole, a specific alkaline phosphatase inhibitor [8] also had no effect on leukocyte ADPase activity.

Studies to date have clearly shown that neutrophil alkaline phosphatase is not implicated in the hydrolysis of AMP [8], ATP [40] or cyclic AMP [41] as well as ADP. Further experiments are therefore necessary before the role of this enigmatic enzyme is settled.

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