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Demonstration of plasma-membrane adenosine diphosphatase activity in rat lung

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The adenosine diphosphatase (ADPase) activity of rat lung has been investigated. Subcellular fractionation of lung tissue homogenates by sucrose density gradient centrifugation has shown the ADPase activity to be associated with the plasma membrane. ADPase was solubilised from the membranes and fractionated by ammonium sulphate precipitation to separate a specific, low-K_m ADPase from non-specific alkaline phosphatase activity. The solubilised ADPase has a K_m of 50 μM at pH 7.5 and appears to be distinct from ATPase.

Introduction

Adenosine diphosphate (ADPase) activity has been demonstrated in plasma [1,2], aorta [3–5] and in the microvasculature of the lung [5,6] and heart [5–7]. It has been shown that ADP released from damaged cells is involved in the initiation of platelet aggregation [8] which in turn releases ADP causing further aggregation to occur. It has therefore been suggested that the ADPase activity of the vascular wall is involved in limiting the degree of platelet aggregation. However, the enzyme responsible for this activity has not been isolated or characterised.

ADPase activity has been localised to the plasma membrane in cultured vascular smooth muscle and endothelial cells [9–11] and rat heart [7] and to mitochondria in rat liver [12]. It was later shown that the apparent mitochondrial activity was due largely to the concerted action of adenylate kinase and mitochondrial ATPase rather than to a distinct ADPase [13].

The lung is a highly vascular tissue which rapidly degrades circulating ADP [6] and tissue distribution studies [14] have shown that the lung possesses very high ADPase activity relative to a mitochondrial marker enzyme (succinate dehydrogenase), suggesting that lung tissue contains high levels of specific ADPase activity. However, little is known about the properties, subcellular localisation or identity of this activity in rat lung.

Methods

ADPase activity was assayed by the release of $^{32}PO_4$ from [β-^{32}P]ADP as previously described [15]. [^{32}P]ADP was synthesised enzymically from [γ-^{32}P]ATP, AMP and adenylate kinase [16]. The reaction mixture for the ADPase assay contained 50 mM Tris-HCl (pH 7.5), 0.33 mM $MgCl_2$ and 0.5 mM AD^{32}P in a final volume of 0.5 ml. Samples were incubated for 30 min at 37°C and the reaction terminated by addition of charcoal-celite [15]. ATPase and 5′-nucleotidase (AMPase) were assayed radiometrically [17,18]. Alkaline phosphatase was measured fluorimetrically with 4-methylumbelliferone phosphate [18]. Adenylate

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kinase activity was measured as previously described [16] under conditions identical to those used for the ADPase assay. Sub cellular fractionation of lung homogenates was performed on a continuous sucrose gradient in a Beaufay automatic zonal rotor [19]. Radioactive substrates were purchased from Amersham International Ltd. Amersham, U.K. Trypsin was purchased from Flow Laboratories. Collagenase (*Clostridium histolyticum*) was purchased from Sigma Chemical Co. Ltd. All other reagents were purchased either from Sigma Chemical Co Ltd. or BDH Chemical Co. Ltd. and were of the highest purity obtainable.

Results

Fig. 1 shows the density gradient distribution of the various phosphatases in rat lung. ADPase was localised to a peak of activity over the density range 1.14–1.18 g/cm^3, with modal and median densities at 1.15 g/cm^3, and ATPase had a similar distribution. No distinct peak of ADPase activity was observed in the region around 1.2 g/cm^3 which would indicate that the artifact observed in rat liver homogenates [12,13] is not significant in rat lung.

AMPase was broadly distributed throughout the gradient with a median density of 1.19 g/cm^3. The particulate non-specific alkaline phosphatase

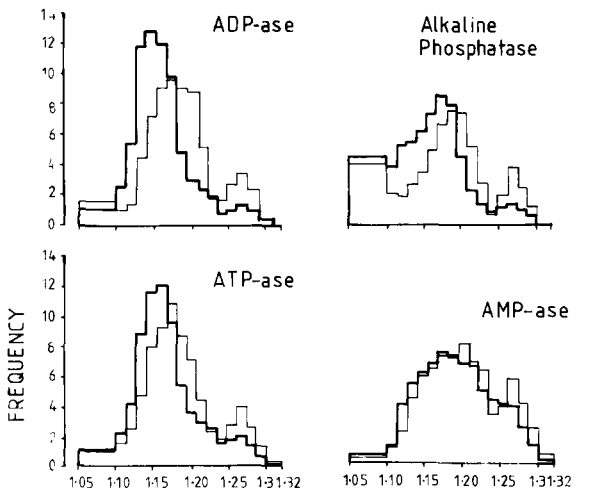

Fig. 1. Analytical subcellular fractionation of rat lung homogenate. ————, without digitonin; ————, with digitonin.

activity was located in the density region 1.12–1.19 g/cm^3 with a profile similar to that of AMPase. A considerable proportion of alkaline phosphatase activity appeared to be soluble (1.05–1.10 g/cm^3). In contrast little AMPase, ADPase or ATPase was found in this region. Homogenisation in the presence of digitonin (1 mg/ml) shifted the peak of ADPase activity to a modal density of 1.18 g/cm^3 indicative of a plasma membrane bound enzyme [19]. Mitochondrial marker enzymes do not display a digitonin-induced shift [22,23] indicating that the ADPase is a plasma membrane bound enzyme and is not due to the concerted action of mitochondrial ATPase and adenylate kinase [13]. ATPase and non-specific alkaline phosphatase displayed a similar shift in the presence of digitonin.

The pH profile of ADPase in lung homogenate displayed a broad optimum over the range 6.0–9.5 encompassing the pH optima obtained for ATPase (pH 8.0), AMPase (pH 6.5–7.5) and alkaline phosphatase (pH 9.5). Together with the data from subcellular fractionation, this suggested the possibility that non-specific alkaline phosphatase contributed to the measured ADPase activity. We therefore attempted to separate the various activities in order to determine whether the ADPase activity was due to a distinct enzyme.

Rat lung membranes were prepared by homogenisation of finely chopped tissue in a Waring blendor at 4°C for 2 min in 50 mM Tris-HCl (pH 8.5), containing 0.3 mM Mg Cl_2 (4.5 g tissue/ml). The homogenate was centrifuged at 34000 × *g* for 30 min and the pelleted membranes resuspended in homogenisation buffer. The membranes were washed by recentrifugation as above and resuspended in homogenisation buffer. The membranes were then treated with 0.075 mg · ml^{-1} trypsin/0.075 mg · ml^{-1} collagenase for 1 h at 37°C and the mixture was then extracted with 0.5% deoxycholate for 30 min at 4°C. The extract was centrifuged for 45 min at 34000 × *g*. The resultant supernatant contained 18% of the original ADPase activity of the membranes with a total recovery of approx. 50% of the original ADPase activity. A number of detergents were tested for their extraction properties without prior treatment with collagenase/trypsin and all detergents caused between 70 and 95% loss of activity. The extraction of activity was substantially enhanced by prior

treatment of the membranes with trypsin/collagenase.

The deoxycholate extract was fractionated with ammonium sulphate as shown in Fig. 2. Virtually all of the non-specific phosphatase and AMPase activities were precipitated by 25% ammonium sulphate. This fraction also contained some ADPase activity. However, the major proportion of the solubilized ADPase activity (40%) precipitated between 55 and 75% saturation. This fraction had ADPase and ATPase activities of 87 and 96 nmol/min per mg protein, respectively, and very low levels of AMPase (8.1 nmol/min per mg protein) and non-specific phosphatase (2.3 nmol/min per mg protein) activity. These specific activities were determined using 1 mM substrate at pH 7.4 in each case.

In order to determine whether an adenylate kinase/ATPase type artifactual ADPase activity was present in this fraction, the adenylate kinase activity was measured by the formation of ATP from 0.6 mM ADP. It was found that the steady-state concentration of ATP produced by the 55–75% fraction was 38 μM. It can be seen from Fig. 3B that the ATPase present in this fraction hydrolyses 38 μM ATP at a rate of 2.9 nmolmin per mg protein. The ADPase activity of this fraction under identical conditions was 37.5 nmol/min per mg protein. The adenylate kinase/ATPase type artifact could therefore only account for up to 7% of the total ADPase activity measured. This strongly suggests that the ADPase activity in the 55–75% fraction is not due to the concerted action of adenylate kinase and ATPase.

Fig. 3A shows that the apparent K_m for the ADPase in the 55–75% fraction (50 μM) was 3-fold lower than that of the original homogenate (150 μM) possibly due to the removal of the low specificity alkaline phosphatase. The apparent K_m for ATPase in this fraction (350 μM) was also lowered 2-fold compared to the original homogenate (800 μM) (Fig. 3B). Interestingly, the V_{max} for ADPase increased 3.3-fold over that of the

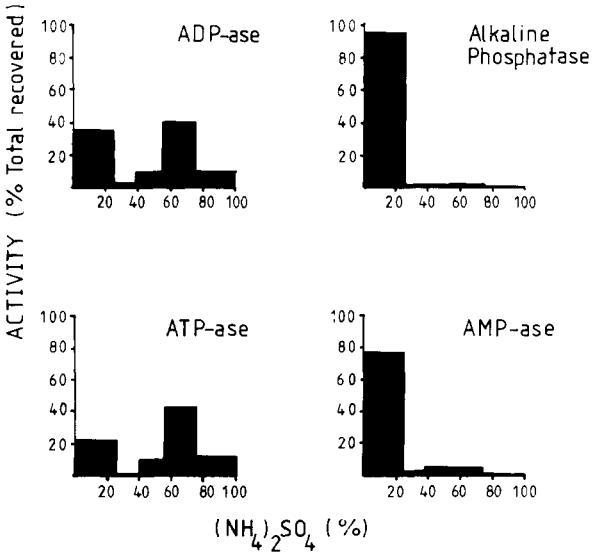

Fig. 2. Ammonium sulfate fractionation of the deoxycholate-solubilised phosphatases of rat lung.

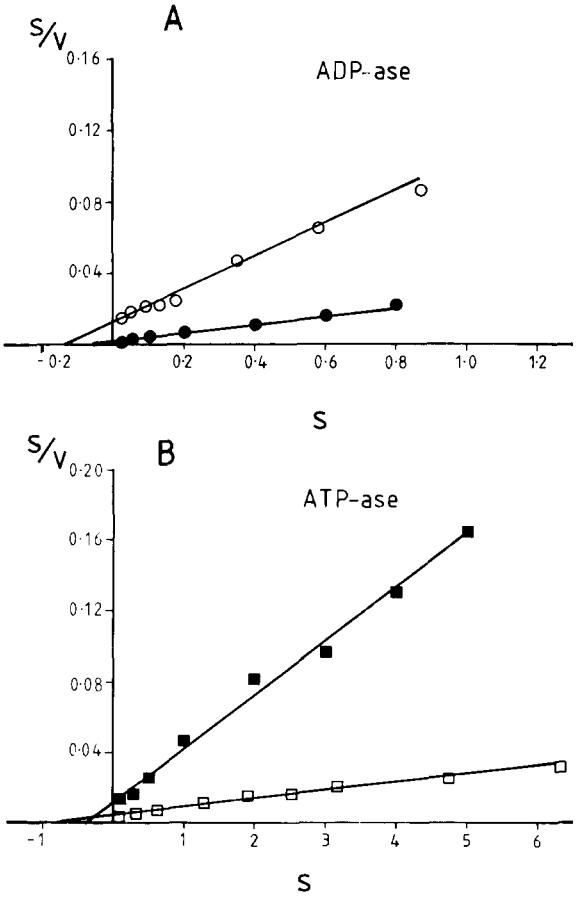

Fig. 3. Kinetics of ATPase and ADPase activities of rat lung homogenate and 55–70% salt fraction. (A) ADPase: ○, homogenate; ●, 55–75% fraction. (B) ATPase: □, homogenate; ■, 55–75% fraction. Lines were fitted to the points by the direct linear plot method [20].

homogenate whereas the ATPase activity in this fraction showed a 6-fold decrease in V_{max} in comparison to the homogenate. The different directional change in V_{max} of the two activities after partial purification from alkaline phosphatase suggests that the ADPase and ATPase activities in the 55–75% ammonium sulphate precipitate are due to two separate enzymes.

To investigate the ATPase and ADPase activities further, their inhibition by adenosine 5′-[β,γ-imido]triphosphate (App[NH]p) was studied; this compound is not metabolised by cultured endothelial cells and has been shown to inhibit selectively, the externally facing ADPase of these cells [11]. In a preliminary experiment the effect of 100 μM App[NH]p on the hydrolysis of 50 μM ADP and 500 μM ATP (values approximating to the K_m for these substrates) was assessed. The enzyme containing fraction was incubated with ^{3}H-nucleotide in 50 mM Tris-HCl (pH 7.4), containing 2 mM $MgCl_2$ at 37°C and product formation was monitored by TLC separation [21] with a Berthold linear radioactivity analyser. App[NH]p had no effect on the hydrolysis of 500 μM ATP but reduced the hydrolysis of 50 μM ADP by approx. 60%. Further experiments were performed with a range of nucleotide concentrations. ATP and ADP hydrolysis were inhibited competitively by App[NH]p with apparent K_i values of 400 μM and 100 μM, respectively. In addition, ADP was found to be a poor inhibitor of ATP catabolism with an apparent $K_i > 1$ mM. These results provide further evidence that the ADPase and ATPase activities in the 55–75% ammonium sulphate fraction are due to two separate enzymes.

Discussion

In the present work we have shown that rat lung ADPase is a plasma membrane associated enzyme as indicated by analytical subcellular fractionation studies. This is consistent with the subcellular distribution of the enzyme in cultured vascular smooth muscle cells, endothelial cells [9–11] and rat heart [7]. However, both non-specific alkaline phosphatase and ATPase activities show similar subcellular distributions and similar density shifts when treated with digitonin.

By solubilising the various phosphatases with deoxycholate, after proteolytic digestion of the membranes, we have been able to partially purify the ADPase activity from the other phosphatases present in rat lung. Non-specific alkaline phosphatase and AMPase were precipitated at low ammonium sulphate concentrations (25%) whereas the ATPase and ADPase activities were mainly precipitated by 55–75% ammonium sulphate as found previously for the plasma enzymes [2]. The ATPase and ADPase activities precipitated by 25% salt may be due in part to non-specific alkaline phosphatase but the remainder of the activity appears to be due to a specific ADPase with a K_m of 50 μM at pH 7.5. The detergent-solubilised enzyme thus displays a similar K_m to the values reported by other workers for the membrane-bound enzyme of cultured cells [9] or rat heart membranes [7]. The low levels of adenylate kinase activity in this fraction suggest that the ADPase activity is not due to the concerted action of adenylate kinase and ATPase as observed in rat liver [13].

The specific ADPase fraction also contains an ATPase activity which is probably due to a separate enzyme being present. We ascribe the ATPase and ADPase activities to separate enzymes for three reasons. Firstly, the V_{max} of the ADPase in the 55–75% fraction was higher than in the original homogenate while the V_{max} of ATPase was lower than in the homogenate. Secondly, the two activities display differing sensitivities to the inhibitor App[NH]p. Thirdly, the K_i for ADP inhibition of ATPase activity was more than 20-fold higher than the K_m for ADPase. While these observations strongly suggest the two activities are due to two separate proteins, the evidence is not unequivocal. Further purification of the ADPase will be required to determine the specificity of this enzyme. However, the present work shows for the first time that in rat lung there is a plasma membrane ADPase distinct from non-specific phosphatase which can be solubilised in sufficient quantities to allow further purification.

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