Calcium Ion-Dependent Adenosine Triphosphatase Activity and Plasma-Membrane Phosphorylation in the Human Neutrophil

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A plasma-membrane fraction was isolated from a post-nuclear extract of human neutrophils by centrifugation through a linear sucrose density gradient. This fraction exhibited a Ca^{2+} -dependent adenosine triphosphatase (ATPase) activity that could be differentiated from mitochondrial or myosin ATPase and from plasma-membrane Mg^{2+} -dependent ATPase. When assayed in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the Ca^{2+} -dependent ATPase reaction resulted in the formation of an acid-resistant hydroxylamine-sensitive bond between the $\gamma\text{-}^{32}\text{P}$ phosphate group and a membrane protein subunit with an apparent mol.wt. of 135000. Half-maximal activating effect of Ca^{2+} was found at 82 nM and 0.18 μM for the ATPase and the formation of the ^{32}P -membrane complex respectively. Generation of the phosphorylated product attained the steady state at 0°C by about 30 s, and was rapidly reversed by ADP. These results suggest that the Ca^{2+} -activated ATPase reaction occurs through the formation of a phosphoprotein intermediate, similar to that described for some Ca^{2+} -dependent ATPase enzymes associated with Ca^{2+} transport. The possibility thus exists that the neutrophil Ca^{2+} -dependent ATPase catalyses a process of Ca^{2+} extrusion from the cell, thereby participating in the regulation of several Ca^{2+} -dependent neutrophil functions.

The control of the steady-state concentration of cytosolic Ca^{2+} is crucial for the regulation of properties and functions of eukaryotic cells. Outward-directed Ca^{2+} pumps, localized at the cell surface, provide one possible mechanism of cytosol Ca^{2+} buffering (for review see Lew, 1974; Baker, 1976; Bygrave, 1978; Schatzmann & Bürgin, 1978). In particular, an ATP-driven Ca^{2+} -extrusion mechanism has been described in erythrocyte 'ghosts' (Schatzmann & Vincenzi, 1969; Schatzmann & Rossi, 1971) and in cardiac sarcolemma (St. Louis & Sulakhe, 1978). Evidence has also been furnished that a similar mechanism might operate in nerve and brain plasma membrane (Baker, 1976; Di Polo, 1977; Robinson, 1978).

This transport system is manifested in the isolated plasma membrane as a Ca^{2+} -activated ATPase activity, for which a simplified reaction scheme may be written as follows (Knauf *et al.*, 1974; Katz & Blostein, 1975; Rega & Garrahan, 1975; Schatzmann & Bürgin, 1978; Robinson, 1978; St. Louis & Sulakhe, 1978):

The transport of Ca^{2+} across the plasma membrane of intact neutrophils and the effect of chemotactic

factors on this transport have been studied by several investigators (Gallin & Rosenthal, 1974; Boucek & Snyderman, 1976; Naccache *et al.*, 1977; Petroski *et al.*, 1979). These studies have suggested the presence of a Ca^{2+} pump at the neutrophil surface (Boucek & Snyderman, 1976; Naccache *et al.*, 1977; Petroski *et al.*, 1979). The experiments reported in the present paper were thus carried out with the aim of identifying a neutrophil membrane Ca^{2+} -dependent ATPase that might be the enzymic manifestation of a Ca^{2+} pump.

Materials and Methods

Purification of plasma membrane

Neutrophils were isolated from freshly drawn venous blood, generously supplied by the Blood Bank of two Hospitals, as described previously (Zabucchi & Romeo, 1976). Isolated leucocyte suspensions containing more than 93% neutrophils were washed once and suspended in 0.24 M-sucrose/1 mM- NaHCO_3 . Post-nuclear supernatants (200 g for 10 min at 0–4°C) derived from homogenized neutrophils (85–90% disruption) were layered on linear 30–50% (w/w) sucrose gradients. The equivalent of approx. 6×10^8 neutrophils was layered on three gradients. These were centrifuged in a Beckman SW 25.1 rotor for 10 min at 0–4°C and 400 g (r_{av} . 9.1 cm), accelerated to 40700 g and centrifuged for an

additional 50 min. The uppermost visible band was collected with a syringe, diluted 10-fold with 1 mM- NaHCO_3 and pelleted (30 min at 0–4°C) at 81000g (r_{av} , 5.9 cm) in the Beckman rotor no. 40.

The sedimented plasma-membrane fraction (1–2 mg of protein) was suspended in 0.24 M-sucrose at a concentration of 1.5–2.5 mg of protein/ml, and stored at –20°C.

Enzyme assays

ATPase (EC 3.6.1.3) was assayed in the presence of a Ca^{2+} -EGTA buffer as described by Gennaro *et al.* (1979). Cell homogenate or plasma membrane (50–120 μg or 7–20 μg of protein equivalent respectively) was used for this assay, and the time of incubation at 37°C was 30 min. The activity of (K^+ +EDTA)-activated myosin ATPase was measured at 37°C in the medium described by Stossel & Pollard (1973). P_i released from ATP was determined after complexation with Malachite Green (Penney, 1976). Alkaline phosphatase (EC 3.1.3.1), β -glucuronidase (EC 3.2.1.31), peroxidase (EC 1.11.1.7), lysozyme (EC 3.2.1.17) and succinate-cytochrome *c* reductase (EC 1.3.99.1) were assayed as reported elsewhere (Sottocasa *et al.*, 1967; Gennaro *et al.*, 1978).

Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

^{32}P Labelling of membranes

The plasma membrane (40 μg of protein) was incubated at 0°C in a reaction mixture (150 μl) consisting of 4.5 μmol of imidazole, brought to pH 7 with HCl, 30 μmol of sucrose, 0.15 μmol of EGTA and the appropriate amount of CaCl_2 and/or MgCl_2 to give the desired free-ion concentration (Portzehl *et al.*, 1964). Blanks contained an equivalent amount of bovine serum albumin, replacing the plasma membrane. The reaction was started with 0.16 nmol of [γ - ^{32}P]ATP (sp. radioactivity > 14 Ci/mmol) and stopped at the time indicated (usually 30 s) by the simultaneous addition of 50 μl of bovine serum albumin (40 mg/ml) and 1 ml of a solution containing 100 g of trichloroacetic acid/litre, 10 mM- KH_2PO_4 and 1 mM-ATP (acid solution). After 10 min in ice, the suspension was centrifuged in an Eppendorf 3200 centrifuge at the maximum speed for 2 min and the pellet was washed three times by the same procedure. The denatured protein was solubilized in 0.5 ml of formic acid (900 ml/litre), added to 10 ml of Aqualuma and counted for radioactivity in a Beckman 230 liquid-scintillation counter. The radioactivity counts per min were converted into pmol by simultaneously counting a portion of the [γ - ^{32}P]ATP stock solution under the same conditions.

To study the effect of hydroxylamine, the phosphorylated membrane (40 μg of protein) was

suspended in 1 ml of 80 mM-sodium acetate, pH 5.3, containing 0.8 M-hydroxylamine hydrochloride (or 0.8 M-NaCl as a control). After 20 min at 20°C, the material was sedimented, washed 3 times, solubilized and counted for radioactivity as above.

Polyacrylamide-gel electrophoresis

This was performed in sodium dodecyl sulphate/sodium phosphate buffer at pH 2.4 exactly as described by Fairbanks & Avruch (1972). The surface of the precipitate of acid-denatured phosphorylated membrane (150–200 μg of protein; bovine serum albumin omitted from the acid solution) was rinsed with 1 ml of 0.5 M-sucrose and drained thoroughly. The membrane was then suspended in 100 μl of 20 mM-sodium phosphate buffer, pH 2.4, containing 0.25 M-sucrose, 20 mg of sodium dodecyl sulphate/ml and 50 mM-dithiothreitol. After incubation at 37°C for 3 min, the membrane solution was clarified by centrifugation in the Eppendorf centrifuge for 2 min. After addition of fuchsin (20 μg /ml), the solubilized membrane (containing about 80% of the original radioactivity) was electrophoresed on 5.6% polyacrylamide-gel columns (8 cm $\times$ 0.5 cm) in 20 mM-phosphate buffer, pH 2.4, containing 10 mg of sodium dodecyl sulphate/ml. At the end of the electrophoretic separation (4 mA/gel; running time for 7.5 cm tracking dye migration of about 2.5 h), the gels were left in ice-cold trichloroacetic acid (50 g/litre) for 30 min and then sliced in 2 mm pieces. These were incubated at 110°C with 0.5 ml of 30% H_2O_2 in glass vials until dry, and counted for radioactivity thereafter in 10 ml of Instagel.

The gels were calibrated with rabbit skeletal-muscle myosin (a gift from Dr. Dalla Libera, University of Padova, Padova, Italy), *Escherichia coli* β -galactosidase, bovine serum albumin, rabbit-muscle aldolase and hen egg-white lysozyme, as described by Weber & Osborn (1969).

Materials

All analytical reagent-grade chemicals were obtained from: BDH Chemicals, Poole, Dorset, U.K.; Sigma Chemical Co., St. Louis, MO, U.S.A.; C. Erba, Milano, Italy; or Merck, Darmstadt, Germany. Sodium dodecyl sulphate was purchased from BDH Chemicals, and acrylamide from Bio-Rad Laboratories, Richmond, CA, U.S.A. [γ - ^{32}P]ATP (triethylammonium salt) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The scintillation 'cocktails' Aqualuma and Instagel were purchased from Lumac Systems A.G., Basel, Switzerland, and Packard Instrument S.p.A., Milano, Italy, respectively.

The solutions of ATP, CaCl_2 and MgCl_2 were standardized as previously described (Gennaro *et al.*, 1979).

Results

Purification of neutrophil plasma membrane

The isolated plasma membrane fraction contains 40–50% of the activity of alkaline phosphatase, a marker enzyme of human neutrophil plasma membrane (Olsson, 1969; Bretz & Baggiolini, 1974; Spitznagel *et al.*, 1974) and 2–4% of protein of the original cell homogenate. Table 1 shows that this fraction is significantly purified according to biochemical criteria. In fact, the specific activities of β -glucuronidase, peroxidase and lysozyme, marker enzymes for human neutrophil granules (Olsson, 1969; Bretz & Baggiolini, 1974; Spitznagel *et al.*, 1974), and that of mitochondrial succinate-cytochrome *c* reductase, decrease significantly in the plasma-membrane fraction when compared with the original cell homogenate. In contrast, the specific activity of alkaline phosphatase markedly increases in this fraction over the cell homogenate. An identical increase in specific activity is exhibited by Ca^{2+} -dependent ATPase, which thus qualifies as a plasma-membrane-associated enzyme. This conclusion is also supported by the observation that the Ca^{2+} -dependent ATPase activity is insensitive to oligomycin (from 0.3 to 3 μg of antibiotic/mg of membrane protein), a known inhibitor of mitochondrial and microsomal ATPases (Van Groningen & Slater, 1963). Finally, one can exclude the possibility that the Ca^{2+} -dependent ATPase reaction is catalysed by membrane-bound myosin (Shibata *et al.*, 1972), since the specific activity of (K^+ +EDTA)-activated ATPase, characteristic for this protein (Stossel & Pollard, 1973), increases in the plasma membrane to a much lesser extent than the activity of both alkaline phosphatase and Ca^{2+} -dependent ATPase.

Ca^{2+} -dependent ATPase activity and membrane phosphorylation

Fig. 1 shows that stimulation by Ca^{2+} of ATPase and membrane labelling by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ follows a

Fig. 1. ATPase activity and labelling by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of neutrophil plasma membrane as a function of free Ca^{2+} or Mg^{2+} concentration

The ATPase activity ($\circ$ and $\triangle$) of 7–20 μg of protein equivalent of plasma membrane was assayed in a medium (0.5 ml) consisting of 15 μmol of imidazole, brought to pH 7 with HCl, 100 μmol of sucrose, 0.25 μmol of ATP (sodium salt), 0.5 μmol of EGTA and various amounts of CaCl_2 or MgCl_2 to reach the desired concentration of Ca^{2+} ($\circ$) or Mg^{2+} ($\triangle$) [Portzehl *et al.*, 1964; the stability constant of Ca^{2+} -EGTA was changed for 37°C to $10^{10.65}$ (Schatzmann & Rossi, 1971)]. The incubation temperature was 37°C and the reaction time 30 min. Plasma-membrane labelling ($\bullet$) was carried out by incubating 40 μg of protein equivalent of membrane at 0°C in a reaction mixture (150 μl) consisting of 4.5 μmol of imidazole, brought to pH 7 with HCl, 30 μmol of sucrose, 0.15 μmol of EGTA and appropriate amounts of CaCl_2 to give the desired free-ion concentration (Portzehl *et al.*, 1964). After addition of 0.16 nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity > 14 Ci/mmol), the reaction was allowed to proceed for 30 s and then stopped with a solution of trichloroacetic acid, KH_2PO_4 and ATP, as described in the Materials and Methods section. The points are means $\pm$ S.E.M. for three experiments carried out with individual membrane preparations.

Table 1. Specific activities of marker enzymes in human neutrophil homogenates and plasma-membrane vesicles isolated from post-nuclear fractions

Specific activities are given in either m-units (alkaline phosphatase, ATPases, β -glucuronidase and succinate-cytochrome *c* reductase) or units (peroxidase) or μg (lysozyme) per mg of protein (1 enzyme unit corresponds to 1 μmol of substrate converted/min). The values represent means $\pm$ S.E.M. for the numbers of experiments in parentheses.

	Specific activity	
	Homogenate	Plasma-membrane fraction
Alkaline phosphatase (6)	19.4 $\pm$ 1.2	192.3 $\pm$ 17.1
Ca^{2+} -dependent ATPase (p Ca^{2+} 5) (6)	2.3 $\pm$ 0.5	19.5 $\pm$ 2.2
β -Glucuronidase (6)	2.6 $\pm$ 0.4	1.6 $\pm$ 0.3
Peroxidase (5)	6.8 $\pm$ 1.6	4.8 $\pm$ 1.7
Lysozyme (3)	37.3 $\pm$ 4.5	20.5 $\pm$ 4.1
Succinate-cytochrome <i>c</i> reductase (1)	1.6	0.5
(K^+ +EDTA)-dependent ATPase (3)	1.2 $\pm$ 0.8	3.0 $\pm$ 1.8

comparable saturation curve. By subjecting the results of Fig. 1 to Lineweaver-Burk analysis, a half-maximal activating effect of Ca^{2+} is found at a concentration of 82 nM (correlation coefficient r^2 , 0.98) and 0.18 μM (correlation coefficient r^2 , 0.86) for the ATPase and the membrane phosphorylation respectively.

Replacement of Ca^{2+} with Mg^{2+} causes a much smaller stimulation of ATPase activity (Fig. 1) and no significant increase in the basal (EGTA) membrane labelling (results not shown).

Ca^{2+} -dependent membrane phosphorylation is also observed in the presence of 0.5 mM- Mg^{2+} , with a slight enhancement in the steady-state value of ^{32}P labelling. In contrast, Ca^{2+} -dependent stimulation of ATPase activity is not detected in the presence of Mg^{2+} . In fact, if 10 μM - Ca^{2+} is added to an ATPase assay medium containing 0.5 mM- Mg^{2+} , an approx. 20% decrease in enzyme activity is found with respect to the activity measured in the absence of Ca^{2+} . This effect is very likely due to inhibition by Ca^{2+} of Mg^{2+} -dependent ATPase, as reported for other cells (Gennaro *et al.*, 1979).

Fig. 2. Kinetics of Ca^{2+} -dependent membrane labelling by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and of label chase by ADP

Labelling of membrane (inset, 40 μg of protein equivalent) was carried out at 0°C in the presence of 4.4 μM - Ca^{2+} , as described in Fig. 1. At the times indicated, the reaction was stopped with a solution of trichloroacetic acid, KH_2PO_4 and ATP, as described in the Materials and Methods section. In the chase experiment, after incubation of membrane for 30 s with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, non-radioactive ADP was added (final concn. 1 mM), without (○) or with (●) EGTA (neutralized with diethanolamine; final total concn. 3.34 mM), to decrease the free- Ca^{2+} concentration to 83 nM. Results are presented relative to the membrane-bound radioactivity at 30 s.

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of neutrophil plasma membrane labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

Neutrophil plasma membrane (185 μg of protein) was reacted for 30 s with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of EGTA alone (○) or of 4.4 μM - Ca^{2+} (●), as described in the legend to Fig. 1. After solubilization in 100 μl of 10 mg/ml of sodium dodecyl sulphate/50 mM-dithiothreitol/0.25 M-sucrose/20 mM-sodium phosphate buffer, pH 2.4, the samples were applied to sodium dodecyl sulphate/polyacrylamide gels as described in the Materials and Methods section. The distribution of ^{32}P radioactivity was determined after each gel had been cut into 2 mm sections. Background radioactivity was 45 c.p.m. Abbreviation used: T.D., tracking dye. Calibration of gels was carried out by the method of Weber & Osborn (1969), with myosin, β -galactosidase, bovine serum albumin, aldolase and lysozyme as 'standards'.

Properties of the ^{32}P -membrane complex

Ca^{2+} -dependent labelling of membrane is very rapid at 0°C, attaining the steady-state value by about 30 s (Fig. 2). Addition of excess ADP after 30 s results in a rapid loss of radioactivity, the amount of membrane-bound ^{32}P decreasing to 5% in 12 s (Fig. 2). The rate of the ADP-dependent release of ^{32}P from the membrane is under the influence of Ca^{2+} concentration. In fact, if EGTA is added together with ADP to decrease the concentration of Ca^{2+} from 4.4 μM to 83 nM, the rate of decay of the ^{32}P -membrane complex is decreased to about one-half (Fig. 2).

Radioactivity of labelled plasma membrane is readily removed by exposure to 0.8 M-hydroxylamine hydrochloride, but not to 0.8 M-NaCl, suggesting that the terminal $[\text{P}^{32}]\text{phosphate}$ group of ATP is incorporated into an acyl-phosphate bond (Knauf *et al.*, 1974; Katz & Blostein, 1975; Rega & Garrahan, 1975; Schatzmann & Bürgin, 1978; Robinson, 1978).

To further characterize the nature of the phosphorylated product, labelled plasma membrane was solubilized and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels. As shown in Fig. 3, Ca^{2+} markedly enhances the incorporation of ^{32}P into a protein fraction of apparent mol.wt. 135000, with a slight increase of labelling associated with fractions of lower mobility (apparent mol.wt. $>2 \times 10^5$).

Discussion

As judged from the decrease in specific activity of enzymes associated with various subcellular particles and from the increase in specific activity of alkaline phosphatase (Olsson, 1969; Bretz & Baggiolini, 1974; Spitznagel *et al.*, 1974), the fraction we have isolated from post-nuclear supernatants of neutrophil homogenates satisfies the necessary conditions for its being considered a purified plasma-membrane fraction.

The high relative purification of Ca^{2+} -dependent ATPase in the plasma-membrane fraction with respect to the enzymes used as markers of granules, mitochondria and myosin-containing filaments permits us to conclude that the enzyme is a specific component of the plasma membrane. The activation of the plasma-membrane ATPase activity by 60nm – $10\mu\text{M}$ - Ca^{2+} greatly exceeds that provided by similar concentrations of Mg^{2+} . This indicates that the Ca^{2+} -dependent ATPase is an enzyme different from the Mg^{2+} -dependent ATPase associated with the surface of human neutrophils (Harlan *et al.*, 1977).

The Ca^{2+} -dependent hydrolysis of ATP, catalysed by the neutrophil plasma membrane, is accompanied by the formation of an acyl-phosphate bond between the γ -phosphate group of ATP and a membrane protein subunit with apparent mol.wt. 135000. Membrane-bound ^{32}P has a rapid turnover at 0°C , is released on addition of excess non-radioactive ADP and exhibits a dependence of the steady-state value on Ca^{2+} concentration identical with that shown by the Ca^{2+} -dependent ATPase activity. These observations provide strong evidence for the ^{32}P -membrane product being an intermediate in the ATPase reaction.

In the erythrocyte 'ghosts' it has been shown that a similar phosphorylated intermediate of apparent mol.wt. 135000–150000 is formed in the course of the ATPase reaction associated with Ca^{2+} transport (Knauf *et al.*, 1974; Katz & Blostein, 1975; Drickamer, 1975; Schatzmann & Bürgin, 1978). Furthermore, the formation of a phosphoprotein intermediate of apparent mol.wt. about 100000 has been reported to occur in the reaction catalysed by other Ca^{2+} -transport ATPases, such as those present in the sarcoplasmic reticulum (Makinose, 1969; MacLennan *et al.*, 1971), in sarcolemma (St. Louis

& Sulakhe, 1978) and in brain microsomal fraction (Robinson, 1978).

By analogy, we therefore suggest that the Ca^{2+} -dependent ATPase activity of neutrophil plasma membrane is the enzymic manifestation of an ATP-dependent Ca^{2+} -extrusion system. Further investigation is needed to gain insight into the mechanism(s) of regulation of such a pump-ATPase system and its possible relation to the control of the various Ca^{2+} -dependent neutrophil functions such as chemotaxis (Gallin & Rosenthal, 1974; Wilkinson, 1975; Boucek & Snyderman, 1976; Naccache *et al.*, 1977), reduction of O_2 to cytotoxic H_2O_2 and O_2^- (Schell-Frederick, 1974; Romeo *et al.*, 1975; Zabucchi & Romeo, 1976; Root & Metcalf, 1977) and secretion of granule enzymes (Woodin & Wieneke, 1970; Smith & Ignarro, 1975; Zabucchi *et al.*, 1975; Zabucchi & Romeo, 1976).

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