Journal of Supramolecular Structure 10: 433–441 (1979)

Phosphoprotein Intermediate in the Ca^{2+}-Dependent ATPase Reaction of Macrophage Plasma Membrane

C. Schneider, C. Mottola, and D. Romeo

Istituto di Chimica Biologica, Università di Trieste, 34127 Trieste, Italy

ATPase activity and phosphorylation by [γ-^{32}P] ATP of isolated plasma membrane of alveolar macorphages are stimulated in a parallel fashion by physiologic concentrations of Ca^{2+}, with half-maximal activating effect of this ion at $(3-7) \times 10^{-7}$ M. For various membrane preparations, a direct proportionality exists between Ca^{2+}-dependent ATPase activity and amount of ^{32}P incorporated. Labeling of membrane attains the steady-state level by 10 sec at 0°C, and is rapidly reversed by adenosine diphosphate (ADP). K^{+} decreases the amount of membrane-bound ^{32}P, mainly by enhancing the rate of dephosphorylation of the ^{32}P-intermediate. Hydroxylamine causes a release of about 90% of ^{32}P bound to the membrane, thus indicating that the ^{32}P-intermediate contains an acyl-phosphate bond. When the labeled plasma membrane is solubilized and electrophoresed on acrylamide gels in the presence of sodium dodecyl sulphate, the radioactivity appears to be largely associated with a single protein fraction of 132,500 ± 2,000 apparent molecular weight. These features of the macrophage Ca^{2+}-ATPase suggest that the enzyme activity might be part of a surface-localized Ca^{2+}-extrusion system, participating in the regulation of Ca^{2+}-dependent activities of the macrophage.

Key words: macrophage (alveolar), plasma membrane, Ca^{2+}-ATPase reaction, membrane phosphorylation, Ca^{2+} buffering

The alveolar macrophage is recognized as the major cellular defense system in the lower respiratory tract [1]. Phagocytosable particles and surface-reactive stimuli are known to activate macrophage functions (chemotaxis, secretion of hydrolitic enzymes and other factors, generation of oxidative antimicrobial compounds) [2–7], which are instrumental to the defense mechanisms of this cell. The coupling between the stimulus and the activation of these functions in macrophages as well as in other phagocytic cells is thought to be provided by a transient elevation of cytosolic Ca^{2+} (reviewed in Romeo et al [8]). This very likely results from mobilization of Ca^{2+} from intracellular stores and/or change in activity of intracellular Ca^{2+} buffering systems [8]. By virtue of its proximity to the cell environment, an adenosine triphosphate-dependent (ATP-dependent) calcium extrusion system localized at the cell surface might be a likely target for regulation by extracellular factors.

Received February 1, 1979; accepted April 26, 1979.

0091-7419/79/1004-0433$02.00

We have recently described a Ca^{2+}-ATPase, distinct from myosin and (Na^+, K^+) Mg^{2+}-ATPase, in the plasma membrane of rabbit alveolar macrophages [9]. A clue toward the understanding of the function of this enzyme might be provided by the study of the mechanism of the ATPase reaction. The Ca^{2+}-dependent ATPase of erythrocyte ghosts, brain microsomes, cardiac sarcolemma and sarcoplasmic reticulum, which is associated with Ca^{2+} transport [10–18], as well as the sodium pump (Na^+, K^+)-ATPase [19, 20], undergo phosphorylation by ATP as a step in their reaction sequence. The experiments described here show that concentrations of Ca^{2+} that activate the ATPase activity also stimulate the phosphorylation by ATP of a protein of macrophage plasma membrane with an apparent molecular weight of 132,500, that this phosphorylation is very rapid and is reversed by adenosine diphosphate (ADP), and that it is sensitive to hydroxylamine, as are the acyl phosphate intermediates of transport ATPases [10, 11, 15, 17–19].

METHODS

Plasma Membrane Isolation

Macrophages were harvested from the lungs of rabbits three weeks after their vaccination with Bacillus Calmette-Guérin [9] and disrupted by lysis in ice-chilled 1 mM $NaHCO_3$ for 60–90 min. The particulate material separated from the cell lysate by centrifugation at 1,000g for 20 min was fractionated by centrifugation through a discontinuous sucrose gradient containing 5 mM $MgCl_2$ as reported elsewhere [9, 21] and the plasma membrane fraction was collected from the 30/40% sucrose interface.

Assays

ATPase activity was measured by the release of inorganic phosphate from ATP (Sigma) [9]. Inorganic phosphate was determined after complexation with malachite green [22]. Protein was determined by the method of Lowry et al [23], with bovine serum albumin (BSA) as standard. All the other enzyme assays were performed as previously reported [9].

Concentrations of Ca^{2+} and Mg^{2+} given in this paper refer to the ions in equilibrium with their EGTA (ethyleneglycol bis-(β-aminoethylether)-N,N′-tetraacetate) complex [24]. The stability constant of the Ca^{2+}-EGTA complex, used for the calculations, was $10^{10.65}$ [25] for the ATPase assay, and 10^{11} [24] for the membrane phosphorylation assay, to account for the different temperatures at which the two assays were carried out.

Labeling of the membrane was performed by two similar procedures. In method A, the plasma membrane (150 μg of protein) was incubated at O°C in a reaction mixture (150 μl) consisting of 4.5 μmoles imidazole, brought to pH 7 with HCl, 30 μmoles sucrose, 0.15 μmoles EGTA and the appropriate amount of $CaCl_2$ and/or $MgCl_2$ to give the desired free ion concentration (see above). Blanks with an equivalent amount of BSA replacing the plasma membrane were run in parallel. The reaction was started with 0.16 nmoles of [γ-^{32}P] ATP (> 14 Ci/mmole, The Radiochemical Centre) and stopped at the time indicated (usually 30 sec) with 4 ml of freshly prepared, cold 5% (w/v) trichloroacetic acid(TCA), containing 10 mM KH_2PO_4 and 1 mM ATP. After standing in ice for 10 min, the suspension was centrifuged at 35,000 rpm for 30 min (rotor No. 40, Beckman centrifuge) at 0–4°C. The precipitate was carefully suspended in 1 ml of 5% TCA containing 10 mM KH_2PO_4, transferred to a tube of an Eppendorf 3,200 centrifuge, and spun at maximal speed for 10 min (0–4°C). This washing procedure was repeated twice. The pellet was then dissolved by boiling for 15 min in 0.5 ml of 0.1 M NaOH containing 2%

Na_2CO_3. An aliquot of the solution was analyzed for protein and the remaining solution was counted in 10 ml of Instagel (Packard) in a Beckman 230 liquid scintillation counter. The recovery of protein was never less than 70% and the results were expressed relative to the recovered protein.

In method B, 40 μg of membrane protein was used, and the reaction was stopped by the simultaneous addition of 50 μl of BSA (40 mg/ml) and 1 ml of 10% (w/v) TCA, containing 10 mM KH_2PO_4 and 1 mM ATP. After 10 min in ice, the suspension was centrifuged in the Eppendorf centrifuge for 2 min and washed three times with 1 ml of 10% TCA, containing 10 mM KH_2PO_4 and 1 mM ATP. The denatured protein was solubilized in 0.5 ml of 90% formic acid, added to 10 ml of Aqualuma (Lumac System), and counted as above.

Since the two procedures provided identical results, the latter, which is less tedious and permits the use of a lower amount of membrane, was selected for most of the experiments.

To study the effect of hydroxylamine, the phosphorylated membrane (0.3 mg of protein) was suspended in 0.5 ml of 80 mM sodium acetate, pH 5.3, with 0.8 M $NH_2OH \cdot HCl$ (or with 0.8 M NaCl, as control). After 20 min at 20°C, 0.5 ml of 10% TCA containing 10 mM KH_2PO_4 was added and the precipitated protein was washed three times with the TCA solution before solubilization and counting.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) was performed in phosphate buffer at pH 2.4 as described by Fairbanks and Avruch [26]. The surface of TCA-denatured membrane (150–200 μg of protein), phosphorylated by method A, 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, 2% SDS, and 50 mM dithiothreitol. After incubation at 37°C for 3 min, the solubilized membrane (containing about 80% of the original radioactivity) was separated from the insoluble residue by centrifugation in the Eppendorf centrifuge for 2 min. After addition of fuchsin (20 μg/ml), the solubilized membrane was electrophoresed on 5.6% polyacrylamide gel columns (8 × 0.5 cm) in 20 mM phosphate buffer (pH 2.4) containing 1% SDS. At the end of the electrophoretic separation (4 mA per gel; running time for 7.5-cm tracking dye migration, about 2.5 h), the gels were left in ice-cold 5% TCA for 30 min and then sliced into 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 thereafter in 10 ml of Instagel.

The gels were calibrated with rabbit skeletal muscle myosin (a gift from Dr. Dalla Libera, University of Padova), Escherichia coli β-galactosidase, BSA, rabbit muscle aldolase, and hen egg white lysozyme, as described by Weber and Osborn [27].

TABLE I. Specific Activities of Marker Enzymes in Lysates and Plasma Membrane Fractions of Rabbit Alveolar Macrophages

	Lysate	Plasma membrane
Alkaline phosphodiesterase I	50 ± 5	933 ± 87
Ca^{2+}-ATPase (pCa 5.5)	38 ± 0.5	385 ± 39
Succinate cyt. c reductase	13.6 ± 2.4	4.2 ± 1.7
NADPH cyt. c reductase	6.1 ± 1.3	1.2 ± 0.2
β-Glucuronidase	4.5 ± 0.3	2.0 ± 0.2

Specific activities are given in munits per milligram protein (1 unit corresponds to 1 μmole of substrate converted per minute). The values represent mean values ± SEM for seven experiments.

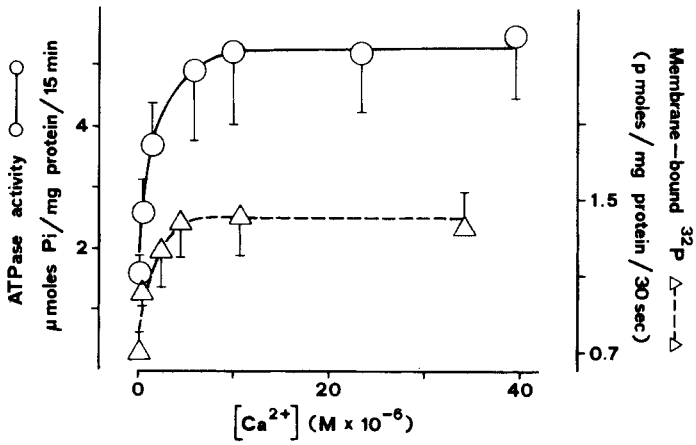

Fig. 1. ATPase activity and ^{32}P labeling of macrophage plasma membrane as a function of free Ca^{2+} concentration. ATPase activity was assayed in a medium (0.5 ml) consisting of 3–8 μg of protein equivalent of membrane, 15 μmoles imidazole brought to pH 7 with HCl, 100 μmoles sucrose, 0.5 μmoles EGTA, and various amounts of $CaCl_2$ to reach the desired concentration of Ca^{2+}. After addition of 0.25 μmoles ATP, the reaction was allowed to proceed at 37°C for 15 min. Membrane labeling was carried out in a reaction mixture of 150 μl with components at the concentrations indicated above, except for protein (40–150 μg) and [γ-^{32}P] ATP (1.1 μM). The incubation temperature was 0°C and the reaction time 30 sec. The values represent mean values ± SEM for three (ATPase activity) or six (membrane labeling) membrane preparations. Basal (EGTA) ATPase activity and membrane phosphorylation were 0.71 ± 0.19 and 0.40 ± 0.10, respectively. The right-hand ordinate has been shifted downward to avoid superimposition of the two curves.

RESULTS

Table I shows that the specific activities of the plasma membrane marker enzyme alkaline phosphodiesterase I [21] and of Ca^{2+}-ATPase increase in the isolated plasma membrane fraction about 20-fold and 10-fold, respectively, over the cell lysate. In contrast, the specific activity of marker enzymes of mitochondria (succinate cytochrome c reductase), endoplasmic reticulum (NADPH cytochrome c reductase) and lysosomes (β-glucuronidase) significantly decreases in this fraction when compared to the original macrophage lysate. This indicates that the preparations of plasma membrane used throughout our experiments meet the classical biochemical criteria of purity.

When assayed for Ca^{2+}-ATPase activity, in the range 0.2–40 μM Ca^{2+}, the macrophage plasma membrane shows a typical saturation curve of activity (Fig. 1). By means of a plot of the data of Fig. 1 according to Lineweaver and Burk, a half-maximal activating effect of Ca^{2+} is found at a concentration of about 0.7 μM (correlation coefficient, r^2, 0.99). An identical pattern of activation by Ca^{2+} is shown by the ATP-dependent phosphorylation of the plasma membrane (Fig. 1), with half-maximal activity of Ca^{2+} at about 0.3 μM (r^2 0.97).

Figure 2 also suggests that the two measurements, Pi release and membrane phosphorylation, refer to the same enzyme activity. In fact, when the activity of Ca^{2+}-ATPase for various membrane preparations is plotted against the amount of γ-^{32}P of ATP incorporated into the membrane, a direct proportionality is obtained.

The activation of membrane phosphorylation by Ca^{2+} appears to be specific for this cation, Mg^{2+} up to 0.5 mM concentration enhancing the phosphorylation of control

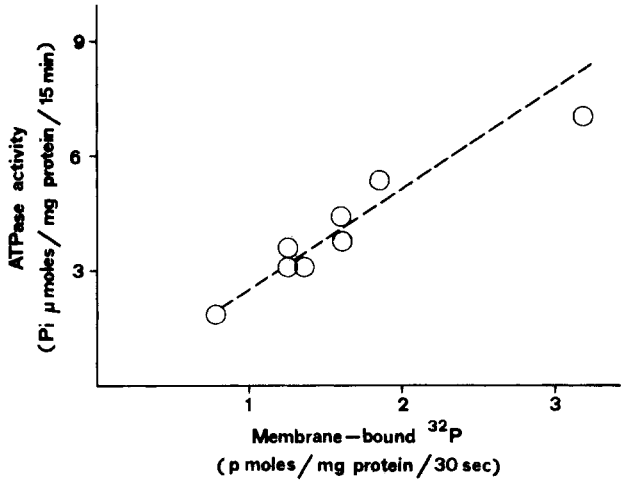

Fig. 2. Relation between Ca^{2+}-dependent ATPase activity and ^{32}P membrane labeling for various preparations of macrophage plasma membrane. ATPase activity and ^{32}P membrane labeling were measured as in Figure 1 at 3 μM Ca^{2+}. ATPase activities are Ca^{2+}-dependent increments above EGTA controls.

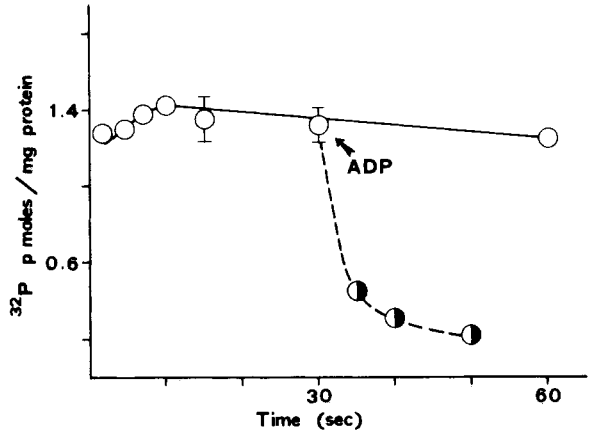

Fig. 3. Time course of Ca^{2+}-dependent ^{32}P membrane labeling and of label chase by ADP. Macrophage plasma membrane was incubated at 0°C for the times indicated in the standard medium, containing 10 μM Ca^{2+} and 1.1 μM [γ-^{32}P] ATP. Data points are mean values (± SEM, where indicated) for two to four experiments. In one experiment, after incubation for 30 sec in the standard medium, ADP was added (final concn. 1 mM), and the residual membrane-bound radioactivity was measured at different times.

(EGTA) membranes by less than 50%. Furthermore, addition of 0.5 mM Mg^{2+} to the assay mixtures does not substantially alter the V_{max} and the affinity for Ca^{2+} of the phosphorylation system.

Labeling of membrane is very rapid at 0°C, attaining the steady-state level by 10 sec (Fig. 3). Addition of excess Ca^{2+} (1 mM) at 30 sec virtually does not modify the pattern of phosphorylation in the following 30 sec. Conversely, addition of excess ADP

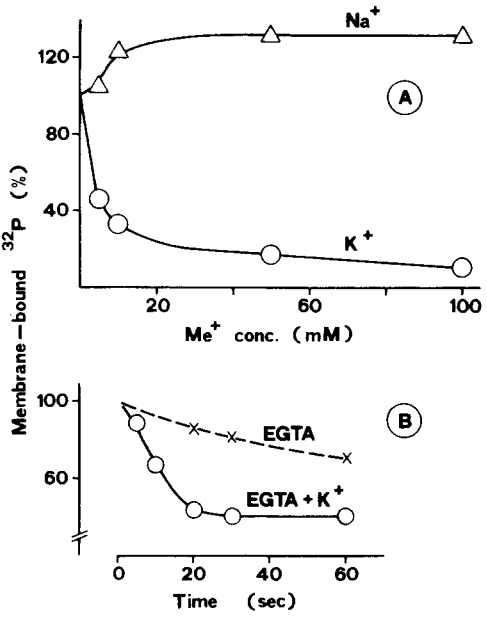

Fig. 4. Effect of alkali ions on the generation and the decay of the ^{32}P-membrane complex. A: Plasma membrane was labeled at 0°C for 30 sec in the standard medium with 4.4 μM Ca^{2+} and 1.1 μM [γ-^{32}P] ATP, in the absence and in the presence of different concentrations of KCl or NaCl. B: Plasma membrane was labeled as in A. At 30 sec EGTA (neutralized with diethanol amine) was added at the final concn. of 3.3 mM (to reduce the free concn. of Ca^{2+} to 0.1 μM), without or with 100 mM KCl (final concn.), and the phosphorylation was stopped with TCA at the times indicated. Data are expressed as percentage of labeling measured in the absence of alkali ions (A) or as percentage of the amount of label at 30 sec (B).

after 30 sec results in a rapid loss of labeling, the amount of membrane-bound ^{32}P falling to 22% within 10 sec (Fig. 3).

When the Ca^{2+}-dependent membrane labeling is carried out in the presence of Na^+, a slight increase in the extent of phosphorylation occurs (Fig. 4A), 0.5 mM ouabain providing some potentiation of this effect. In contrast, K^+ markedly decreases the amount of membrane-bound ^{32}P recovered (Fig. 4A).

To better understand this effect of K^+, the following experiment was devised. After reacting the membrane with [γ-^{32}P] ATP for 30 sec by the usual procedure, EGTA was added to the reaction mixture to reduce the Ca^{2+} concentration to 0.1 μM. Under these conditions the rate of the dephosphorylation reaction should prevail on the rate of the Ca^{2+}-dependent phosphorylation [11]. If K^+ is added simultaneously with EGTA, a rapid and marked acceleration of dephosphorylation is observed (Fig. 4B).

In an effort to understand the chemical nature of the phosphorylated product, various phosphorylated membrane ATPases have been tested for sensitivity to hydroxylamine [10, 11, 15, 17–19]. The release of membrane-bound ^{32}P by this reagent has been interpreted as due to a nucleophilic attack on an acylphosphate bond. Table II shows that acid-denatured ^{32}P-labeled plasma membrane of macrophages are also very sensitive to the hydroxylamine treatment, which releases about 90% of the ^{32}P incorporated in the presence of Ca^{2+}.

TABLE II. Effect of Hydroxylamine Treatment on Ca^{2+}-Dependent Phosphoprotein Intermediate

	^{32}P pmoles/mg protein	
	Ca^{2+}	EGTA
Control	1.39 ± 0.18	0.40 ± 0.15
NH_2OH	0.15 ± 0.15	0.09 ± 0.09
NaCl	1.33 ± 0.14	0.47 ± 0.12

Plasma membrane phosphorylated in the presence of either Ca^{2+}-EGTA buffer (10 μM Ca^{2+}) or EGTA were exposed to either 0.8 M hydroxylamine ·HCl or 0.8 M NaCl in 0.08 M sodium acetate, pH 5.3 for 20 min at 20°C. Control phosphorylated membranes were not incubated at 20°C. The values are means of three experiments ± SEM.

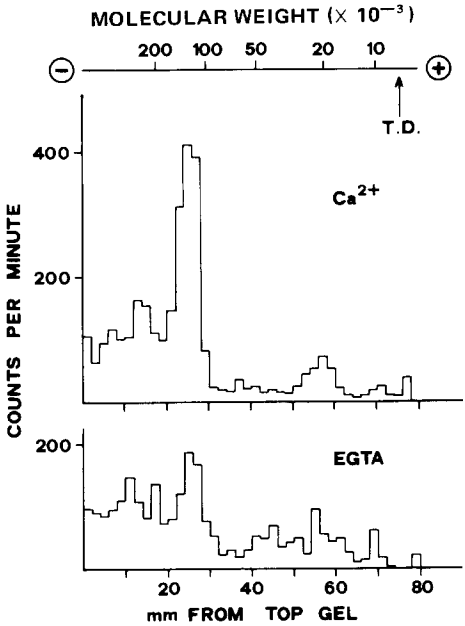

Fig. 5. SDS-polyacrylamide gel electrophoresis of macrophage plasma membrane labeled with [γ-^{32}P] ATP. Membrane was labeled by method A under Methods, solubilized in 1% SDS with 50 mM dithiothreitol and run on pH 2.4 gels (150 μg of protein). TD, tracking dye. Calibration of the gels [27] was carried out with myosin, β-galactosidase, BSA, aldolase, and lysozyme.

To further characterize the nature of the ^{32}P-membrane complex, the labeled plasma membrane was solubilized and electrophoresed on acrylamide gels in the presence of SDS. As shown in Figure 5, incorporation of Ca^{2+}-dependent ^{32}P is confined largely to a single fraction, with an apparent molecular weight of 132,500 ± 2,000 (mean of four experiments carried out with different membrane preparations ± SEM).

DISCUSSION

A prerequisite for a Ca^{2+}-ATPase to work as a Ca^{2+}-transport system appears to be the formation of a ^{32}P-enzyme intermediate, similar to that originally described by Skou for the sodium pump (Na^+, K^+) ATPase [19].

$$\text{ATP} + \text{Enz} \rightleftarrows \text{ATP} \cdot \text{Enz} \quad (1)$$
$$\text{ATP} \cdot \text{Enz} \rightleftarrows \text{P-Enz} + \text{ADP} \quad (2)$$
$$\text{P-Enz} \rightarrow \text{Enz} + \text{Pi} \quad (3)$$

Formation of a phosphoprotein intermediate has, in fact, been described for the ATPase associated with Ca^{2+} transport in the sarcoplasmic reticulum [17, 18], in brain microsomes [15], and in the plasma membrane of erythrocytes [10–14] and cardiac cells [16].

The data here reported indicate that the Ca^{2+}-ATPase reaction of macrophage plasma membrane also occurs with the formation of a hydroxylamine-sensitive ^{32}P-intermediate. Evidence in favor of this conclusion is provided by the observation that 1) the dependence on Ca^{2+} of Pi release and of incorporation of ^{32}P into the membrane follows the same pattern; 2) the concentration of Ca^{2+} for half-maximal effect is of the order of 10^{-7} M in both cases; and 3) for various membrane preparations, a direct proportionality exists between the ATPase activity and the amount of ^{32}P incorporated into the membrane.

The formation of the intermediate in the macrophage ATPase reaction shares with other Ca^{2+}-ATPases the property of reaching the steady state in a very short time at 0°C, and of being reversed by ADP. It thus appears that the set of intermediate reactions 1–3 can also be invoked to describe the reaction mechanism of the Ca^{2+}-ATPase of the macrophage plasma membrane.

The dependence of the macrophage Ca^{2+}-ATPase reaction on Ca^{2+} concentrations close to those which are supposed to exist in the cytosol of resting cells [14, 28, 29] strongly suggests that the enzyme is also active in the intact macrophage. The mechanism of its intracellular regulation is still to be clarified. Preliminary experiments have shown that lysis of macrophages in the presence of 2 mM EDTA decreases the Ca^{2+}-ATPase activity of the isolated plasma membrane by about 60%. Whether this is due to an EDTA-promoted release of a Ca^{2+}-dependent cytoplasmic activator of the ATPase, similar to that described for the enzyme of the erythrocyte ghosts [30, 31], remains to be established.

Another important factor for the regulation of the turnover of the ^{32}P-intermediate here described is K^+, which appears to act mainly through a stimulation of the dephosphorylation reaction (reaction 3). The slight enhancement by Na^+ of the formation of the ^{32}P-intermediate is more difficult to explain. One possibility is that Na^+ reduces the rate of turnover of the Ca^{2+}-dependent phosphoprotein intermediate. Alternatively, due to its potentiation by ouabain, a known specific inhibitor of the (Na^+, K^+)-ATPase [20], the Na^+ effect might arise from an activation of a partial reaction of the macrophage (Na^+, K^+)-ATPase [9]. An elucidation of this point will be made possible only by the isolation and purification of the two ATPase activities.

Unlike the alkali ions, addition of Mg^{2+} to the Ca^{2+}-containing assay mixture does not affect the turnover of the ^{32}P-intermediate. This is in contrast with the stimulatory effect of Mg^{2+} on ^{32}P labeling of erythrocyte ghosts [11, 14]. It is important to note, however, that with the latter membranes Mg^{2+} also significantly enhances the Ca^{2+}-

independent phosphorylation, while the Ca^{2+}-specific increment remains essentially unmodified.

In conclusion, we have demonstrated that the Ca^{2+}-dependent ATPase reaction of macrophage plasma membrane occurs through the formation of a high-energy bond between the γ-P of ATP and a membrane protein of apparent molecular weight of 132,500. Since this feature is typical of transport ATPases [10–20], we suggest that the Ca^{2+}-ATPase of macrophages fulfills the function of regulating the concentration of cytosolic Ca^{2+}. Further experiments are required to clarify whether this is the main Ca^{2+} buffering system of the macrophage and by which mechanism(s) extracellular factors could modify its activity, thereby regulating Ca^{2+}-dependent cell functions.

ACKNOWLEDGMENTS

This study was supported by grants from the Consiglio Nazionale delle Ricerche (CT 77.01477.04) and from the University of Trieste.

We acknowledge the excellent assistance of Ms L. Dolzani and Mr. A. Marzullo. C. M. is a recipient of a fellowship from the L. Stock Fund.

REFERENCES

1. Green GM: Ann Rev Med 19:315, 1968.
2. Snyderman R, Mergenhagen SE: In Nelson DS (ed): "Immunobiology of the Macrophage." New York: Academic, 1976, p 323.
3. Ackerman NR, Beebe JB: Nature 247:475, 1974.
4. Schneider C, Gennaro R, de Nicola G, Romeo D: Exp Cell Res 112:249, 1978.
5. Romeo D, Zabucchi G, Marzi T, Rossi F: Exp Cell Res 78:423, 1973.
6. Johnston RB: Fed Proc 37:2759, 1978.
7. Rossi F, Bellavite P, Dobrina A, Dri P, Zabucchi G: In van Furth R (ed): "Functional Aspects of Mononuclear Phagocytes." Oxford: Blackwell Scientific Publishers (In press).
8. Romeo D. Schneider C. Gennaro R, Mottola C: In Escobar ME and Friedman H (eds): "Macrophages and Lymphocytes: Nature, Functions and Interactions." New York: Plenum (In press).
9. Gennaro R, Mottola C, Schneider C, Romeo D: Biochim Biophys Acta 567:238, 1979.
10. Knauf PA, Proverbio F, Hoffman JF: J Gen Physiol 63:324, 1974.
11. Rega AF, Garrahan PJ: J Membrane Biol 22:313, 1975.
12. Katz S, Blostein R: Biochim Biophys Acta 389:314, 1975.
13. Drickamer LK: J Biol Chem 250:1952, 1975.
14. Schatzmann HJ, Bürgin H: Ann NY Acad Sci 307:125, 1978.
15. Robinson JD: FEBS Lett 87:261, 1978.
16. St. Louis PJ, Sulakhe PV: Biochemistry 17:4540, 1978.
17. Martonosi A: J Biol Chem 244:613, 1969.
18. Inesi G, Maring E, Murphy AJ, McFarland BH: Arch Biochem Biophys 138:285, 1970.
19. Skou JC: Biochim Biophys Acta 42:6, 1960.
20. Dahl JL, Hokin LE: Ann Rev Biochem 43:327, 1974.
21. Wang P, Shirley PS, De Chatelet LR, McCall CE, Waite BM: J Reticuloendothel Soc 19:333, 1976.
22. Penney CL: Anal Biochem 75:201, 1976.
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
24. Portzehl H, Caldwell PC, Rüegg JC: Biochim Biophys Acta 79:581, 1964.
25. Schatzmann HJ, Rossi GL: Biochim Biophys Acta 241:379, 1971.
26. Fairbanks G, Avruch J: J Supramol Struct 1:66, 1972.
27. Weber K, Osborn M: J Biol Chem 244:4406, 1969.
28. Brinley FJ: Fed Proc 32:1735, 1973.
29. Baker PF: Fed Proc 35:2589, 1976.
30. Vincenzi FF, Farrance HL: J Supramol Struct 7:301, 1977.
31. Hanahan DJ, Taverna RD, Flynn DD, Ekholm JE: Biochem Biophys Res Commun 84:1009, 1978.