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Ca^{2+}-DEPENDENT ATPase ACTIVITY OF ALVEOLAR MACROPHAGE PLASMA MEMBRANE

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Summary

A plasma membrane fraction was isolated from lysates of Bacillus Calmette-Guérin-induced alveolar macrophages of rabbit. On the basis of morphological and biochemical criteria this fraction appeared to be minimally contaminated by other subcellular organelles. Concentrations of Ca^{2+}, but not of Mg^{2+}, from $6 \cdot 10^{-8}$ to $1 \cdot 10^{-5}$ M markedly stimulated the basal ATPase (EC 3.6.1.3) activity of the plasma membrane, with an apparent K_m (Ca^{2+}) of $1 \cdot 10^{-6}$ M. The specific activity of the Ca^{2+}-ATPase assayed at pCa = 5.5 was enriched about 8-fold in the plasma membrane fraction over the macrophage lysate. In contrast, the specific activity of the K^+, EDTA-activated ATPase, associated to macrophage myosin, increased only 1.3-fold. Oligomycin and -SH group reagents exerted no influence on the Ca^{2+}-ATPase activity, which was on the contrary inhibited by detergents such as Triton X-100 and deoxycholate. The activity of the Ca^{2+}-ATPase was maximal at pH 7, and was decreased by 50 mM Na^+ and 5 mM K^+. On the contrary, the activity of Mg^{2+}-ATPase, also present in the plasma membrane fraction, had a peak at about pH 7.8, and was stimulated by Na^+ plus K^+. On account of its properties, it is suggested that the Ca^{2+}-ATPase is a component of the plasma membrane of the alveolar macrophage, and that its function may be that of participating in the maintenance of low free Ca^{2+} concentrations in the macrophage cytosol.

Introduction

The alveolar macrophages exhibit a variety of physiological activities such as motility, endocytosis and secretion, which tender them uniquely suitable to play a central role in the lower airway defense mechanism against noxious living and non-living particulate inhalants, and in general in inflammatory and

immunological phenomena [1—4]. The mechanisms by which the lung macrophages or other phagocytic cells regulate such diverse biological functions have only partially been clarified. Since these functions are expression of a cell surface activity, investigations on the enzyme composition, receptor specificity and turnover of the plasma membrane should help understand the physiological properties of macrophages at the molecular level. In this view, an analysis of plasma membrane-bound enzyme systems presumably related to active ion transport is of particular interest. In fact, the possibility exists that changes in the steady-state levels of cations occurring in the cytoplasm of macrophages, as well as of other phagocytic cells, may be involved in the initiation or control of cell activity [5—10].

In this paper we present results of our studies on a Ca^{2+}-dependent ATPase (EC 3.6.2.1) activity of plasma membrane of rabbit alveolar macrophages, and compare the properties of this enzyme activity with those of the Mg^{2+}-dependent ATPase, already described as associated to the surface membrane of these cells [11].

Materials and Methods

Cells. 10 mg viable *Bacillus Calmette-Guérin* (kindly donated by Istituto Vaccinogeno Antitubercolare, Milan), suspended in 1 ml sterile saline containing 1% (v/v) Tween 80, were injected into the marginal ear vein of outbred rabbits (2—2.5 kg). 2 weeks later, the animals were killed by air embolism and macrophages were removed from the lungs by means of tracheobronchial lavages [12]. Cells, collected by centrifugation at $200 \times g$ for 10 min, were washed once in 0.9% NaCl. Contaminating erythrocytes were lysed for 30—60 s with cold 0.2% NaCl, the suspension was made isotonic with cold 1.2% NaCl and the white cells were sedimented at $200 \times g$ for 10 min. Differential counts carried out on May-Grünwald-Giemsa stained smears showed that macrophages were more than 85% of the total white cells.

Plasma membrane isolation. Cells were suspended in 1 mM $NaHCO_3$ at a concentration of $5 \cdot 10^6$ cells/ml and, after 10 min at room temperature, maintained in ice for further 50—80 min, their lysis being monitored by phase-contrast microscopy.

An aliquot of the cell lysate was saved for analysis and the remainder was used for purification of the plasma membrane fraction by the method of Wang et al. [13]. Briefly, the particulate material separated from the cell lysate by centrifugation at $1500 \times g$ for 20 min (0—4°C), after one wash in cold 1 mM $NaHCO_3$ was suspended in 55% (w/w) sucrose containing 5 mM $MgCl_2$ (8 ml per pellet derived from $2 \cdot 10^8$ cells). This suspension was placed at the bottom of a tube of the SW 25.1 rotor (Beckman Instruments), overlaid with 8 ml of 45% sucrose followed by 8 ml of 40% sucrose and finally by 6 ml of 30% sucrose (all containing 5 mM $MgCl_2$), and centrifuged at 25 000 rev./min for 2 h (0—4°C). The material at the 30/40% and 40/45% interfaces was withdrawn with a pasteur pipette, pooled, and diluted with 1 mM $NaHCO_3$ to a density of 1.04 g/ml. This material, designated the plasma membrane fraction, was collected by centrifugation at $6000 \times g$ for 20 min (0—4°C) and resuspended with 0.24 M sucrose (brought to pH 7 with $NaHCO_3$) at a concentration of

0.5—0.8 mg protein/ml. After performing the assays of the marker enzymes, the plasma membrane preparations were divided in 1 ml portions and stored frozen at —20°C.

Enzyme assays. Alkaline phosphodiesterase I activity was assayed at 37°C by following the kinetics of hydrolysis of the substrate thymidine-5′-monophospho-*p*-nitrophenyl ester (0.5 mM) (Boehringer) at 410 nm [14] in 0.05 M diethanolamine-HCl buffer (pH 9.7) containing 0.025% (v/v) Triton X-100. Succinate cytochrome *c* reductase and rotenone-insensitive NADPH cytochrome *c* reductase activities were determined according to Sottocasa et al. [15]. The assay medium of the latter enzyme also contained 15 μg/ml superoxide dismutase (Truett Laboratories), to prevent cytochrome *c* reduction by O_2^- possibly generated by macrophage NADPH oxidase [16,17]. β-Glucuronidase activity was assayed as reported by Gennaro et al. [18] using 4-methyl umbelliferyl-β-D-glucuronide (Sigma) as substrate. Protein was determined by the method of Lowry et al. [19], with bovine serum albumin as standard.

The basic medium for the assay of ATPase activity (0.5 ml final volume) contained 30 mM imidazole-HCl, pH 7.0, 0.2 M sucrose, and 1 mM EGTA (ethyleneglycol bis-(β-aminoethylether)-*N,N′*-tetraacetate). The amounts of 10 mM $CaCl_2$ or $MgCl_2$ solutions, which were added to the assay medium to reach the desired concentrations of the metals in ionized form, were calculated from the stability constant of the Ca^{2+}-EGTA or Mg^{2+}-EGTA complexes [20]. The dissociation constants of EGTA and the stability constant of Mg^{2+}-EGTA given by Portzehl et al. [20] were not corrected for temperature or ionic strength, whereas the stability constant of Ca^{2+}-EGTA was changed for 37°C to $10^{10.65}$ [21]. When Mg^{2+} and Ca^{2+} were both present in the medium a small correction was applied for any intended Ca^{2+} concentration [20]. All the concentrations of Ca^{2+} and Mg^{2+} given in this paper refer to the ions in equilibrium with their EGTA complexes and no distinction is made between the portion of ions bound to ATP and that actually present in free form.

When the assay of ATPase activity was run at different pH values, various buffers were used as reported in the legend to Fig. 2. In this case, the amounts of $CaCl_2$ and $MgCl_2$ to be added to the assay medium were calculated for any given pH, since the apparent stability constant of the Ca^{2+}-EGTA and Mg^{2+}-EGTA complexes are dependent on the H^+ concentration.

3 to 6 μg or 1—2 μg of protein equivalent of cell lysate or plasma membrane fraction, respectively, were used for the determination of Mg^{2+}-ATPase activity. These amounts of protein were increased to 12—24 μg or 3 -8 μg, respectively, for the determination of Ca^{2+}-ATPase activity. The enzyme assays were initiated by the addition of either Tris-ATP or Na-ATP (both from Sigma) at 0.5 mM final concentration. With cell lysates the hydrolysis of the substrate was determined by measuring the amount of P_i released between the 2nd and the 17th min of incubation at 37°C. With the plasma membrane fractions, the incubations were carried out for 15 min and blanks were run by omitting the plasma membrane. Under the assay conditions employed the amount of P_i produced never exceeded 10—15% of the ATP originally present in the medium, thus warranting high ATP/ADP ratios throughout the assay.

At the end of the incubations, 0.1—0.5 ml of the assay medium were rapidly transferred to test tubes containing the complement distilled water to 0.5 ml

and 2 ml of 1.6% sodium molybdate · $2H_2O$ in 2 N HCl, followed by an addition of 50 μl of 0.126% malachite green [22]. After 3 min at room temperature, the absorbance of these mixtures was read at 650 nm and compared with standards containing 2—10 nmol of KH_2PO_4. With this method of P_i determination labile phosphates such as ATP suffer insigificant hydrolysis [22], thereby permitting to obtain low blanks. Any reagent added to the assay media of the ATPases was checked for its possible interference with the P_i determination and, when necessary, suitable calibration curves containing the reagent were prepared.

Chemicals. They were analytical grade of BDH or Merck. All solutions were made up in distilled water passed through an ion exchange column.

The concentration of stock solutions of ATP was determined by an enzyme method [23]. The concentration of stock solutions of $CaCl_2$ was controlled by spectrophotometric titration of 0.3 mM murexide in 15 mM Tris-HCl, pH 7.5, at 470 nm [24], by using a calibration curve obtained with standard solutions of $CaCl_2$ (Nyegaard). The concentration of stock solutions of $MgCl_2$ was checked by fluorimetric titration of 2 mM 8-hydroxy-5-quinoline sulfonate (Na) in 50 mM Tris-HCl, pH 6.7 [25], by using standards of Mg in HNO_3.

Results

When analyzed in the electron microscope, the material collected from the 30/40% and 40/45% sucrose interfaces appears to consist of vesicular structures of various sizes, characteristic of plasma membranes of other cells. As shown in Table I, a consistent and large increase in specific activity of the plasma membrane marker enzyme alkaline phosphodiesterase I is seen in this vesicle fraction. In contrast, the specific acitivity of marker enzymes of mitochondria (succinate cytochrome *c* reductase), endoplasmic reticulum (NADPH cytochrome *c* reductase) and lysosomes (β-glucuronidase) significantly decreases in the isolated plasma membrane fraction when compared to the original cell lysate.

The purified plasma membrane fraction was tested for ATPase activity and for specific effect of Ca^{2+} and Mg^{2+} in stimulating the enzymatic splitting of ATP.

With Ca^{2+} in the medium at a concentration as little as $6 \cdot 10^{-8}$ M there is already a 2-fold increase in the basal ATPase activity (Fig. 1). By gradually increasing the Ca^{2+} concentration, the enzyme activity rapidly rises and is about 10-fold higher than the basal value at $1.7 \cdot 10^{-5}$ M Ca^{2+}. From this point up to $1 \cdot 10^{-3}$ M Ca^{2+} the ATPase activity continues to increase, but the rate of increment is very much lower than that observed in the first part of the dose-activity curve. This is more evident when the enzyme activity is plotted vs. $[Ca^{2+}]$ and not vs. *p*Ca (not shown). The ATPase activity stimulated by low concentrations of Ca^{2+}, with an apparent K_m (Ca^{2+}) = $1 \cdot 10^{-6}$ M, is referred to in this paper as Ca^{2+}-ATPase.

In contrast to the effects observed with Ca^{2+}, addition of Mg^{2+} to the assay medium, in a range of concentrations from $1 \cdot 10^{-7}$ M to approximately $1 \cdot 10^{-5}$ M, causes no change in the basal ATPase activity (Fig. 1). A gradual increase in ATPase activity is, however, observed from $1 \cdot 10^{-5}$ M to $1 \cdot 10^{-3}$ M Mg^{2+}, with an apparent K_m (Mg^{2+}) of $5 \cdot 10^{-4}$ M.

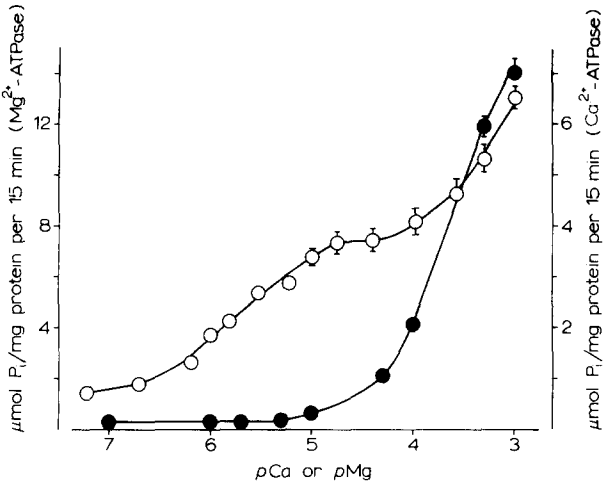

Fig. 1. Ca^{2+}-ATPase (○———○) and Mg^{2+}-ATPase (●———●) activity of macrophage plasma membrane as a function of free Ca^{2+} or Mg^{2+} concentrations. Membranes (1—2 μg or 3—8 μg of protein for Mg^{2+}-ATPase or Ca^{2+}-ATPase, respectively) were incubated at 37°C for 15 min, in 0.5 ml of medium, pH 7.0, containing imidazole-HCl (30 mM), sucrose (0.2 M), EGTA (1 mM), ATP (0.5 mM) and varying concentrations of $CaCl_2$ or $MgCl_2$ (the free concentrations of the cations were calculated from the stability constant of their EGTA complexes). The points are means of activities of 3 individual membrane preparations ±S.E.M. The mean of the enzyme activities (±S.E.M.) measured in the absence of Ca^{2+} and Mg^{2+} was 0.32 ± 0.03.

That the Ca^{2+}-ATPase is a component of the plasma membrane of macrophages is demonstrated by its relative purification in the isolated plasma membrane fraction. As shown in Table I, the specific activity of the Ca^{2+}-ATPase assayed at *p*Ca 5.5 is enriched about 8-fold in this fraction over the cell lysate. The specific activity of Mg^{2+}-ATPase and Na^+, K^+, Mg^{2+}-ATPase also exhibits a several-fold enrichment. In contrast, the specific activity of K^+, EDTA-ATPase,

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES AND OF ATPases IN LYSATES AND PLASMA MEMBRANE FRACTIONS OF RABBIT ALVEOLAR MACROPHAGES

Enzyme activities, assayed at 37°C, are expressed as follows: alkaline phosphodiesterase I = nmol *p*-nitrophenol/mg protein per min; succinate- and NADPH-cytochrome *c* reductase = nmol cytochrome *c* reduced/mg protein per min; β-glucuronidase = nmol 4-methyl umbelliferone/mg protein per 15 min; ATPases = μmol P_i/mg protein per 15 min. The assays of the ATPases were run in the presence of 3 μM Ca^{2+} or 1 mM Mg^{2+} or 1 mM Mg^{2+} plus 50 mM Na^+ and 5 mM K^+; the assays of K^+, EDTA-ATPase were carried out in the medium described by Hartwig and Stossel [26]. The values represent the means of 7 (marker enzymes) or 5 (ATPases) individual experiments ± S.E.M.

	Lysate	Plasma membrane
Alkaline phosphodiesterase I	65.6 ± 2.4	682.2 ± 20.9
Succinate cytochrome *c* reductase	40.8 ± 1.2	25.4 ± 2.0
NADPH cytochrome *c* reductase	10.7 ± 0.4	6.5 ± 0.3
β-Glucuronidase	146.6 ± 8.3	111.0 ± 7.7
Ca^{2+}-ATPase	0.41 ± 0.02	3.10 ± 0.09
Mg^{2+}-ATPase	2.04 ± 0.04	12.60 ± 0.17
Na^+, K^+, Mg^{2+}-ATPase	2.76 ± 0.11	17.32 ± 0.31
K^+, EDTA-ATPase	0.017 ± 0.001	0.022 ± 0.002

associated to macrophage myosin [26], on the average increases only 1.3-fold.

When assayed in buffers at different pH values, the Ca^{2+}-ATPase (pCa 5.5) has a very sharp optimum of activity at pH 7, with a second small peak around pH 9.5 (Fig. 2). On the contrary, the Mg^{2+}-ATPase shows a broader pH-activity curve, with maximal activity at about pH 7.8. An identical profile of activity as a function of pH is also shown by the ATPase in the presence of $1 \cdot 10^{-3}$ M Ca^{2+}. This suggests that, at least in vitro, both bivalent cations at concentrations greater than about $1 \cdot 10^{-5}$ M can activate the Mg^{2+}-ATPase, although to a different extent (see also Fig. 1). This conclusion is consistent with observations reported for ATPases of other cells [27—29].

Addition of 50 mM Na^+ and 5 mM K^+ to the assay medium of Mg^{2+}-ATPase increases the enzyme activity by about 40% (see Table I). This is consistent with the effect of the alkaline cations on the Mg^{2+}-ATPase activity of the plasma membrane of sheep lung macrophages [11]. The addition of the same amounts of Na^+ and K^+ to the assay medium of Ca^{2+}-ATPase (pCa 5.5) leads, on the contrary, to a 35% inhibition of enzyme activity. Parallel assays carried out with either alkali ion shows that this inhibition is essentially due to Na^+.

Addition of Ca^{2+} from $6 \cdot 10^{-8}$ M to $5 \cdot 10^{-4}$ M to the assay medium of Mg^{2+}-ATPase, containing a fixed concentration of 0.5 mM Mg^{2+}, results in a slight stimulation of enzyme activity only at the lowest concentration of Ca^{2+}. Highest Ca^{2+} concentrations, from $1 \cdot 10^{-6}$ M to $5 \cdot 10^{-4}$ M, are progressively inhibitory (from about 15% to about 50%, when referred to the sum of the activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase, as determined in separate experiments). This effect does not appear to be simply due to a change in the bivalent cation/ATP ratio, because, as shown in Fig. 1, the rise of the Mg^{2+} concentration from 0.5 mM to 1 mM enhances the Mg^{2+}-ATPase activity.

When the plasma membrane fraction is exposed to increasing concentrations of the non-ionic detergent Triton X-100 a gradually increasing inhibition of the activity of both Ca^{2+} and Mg^{2+}-ATPases is seen, with an 80—90% inhibition at 0.05% (v/v) detergent concentration. Also the anionic detergent deoxycholate

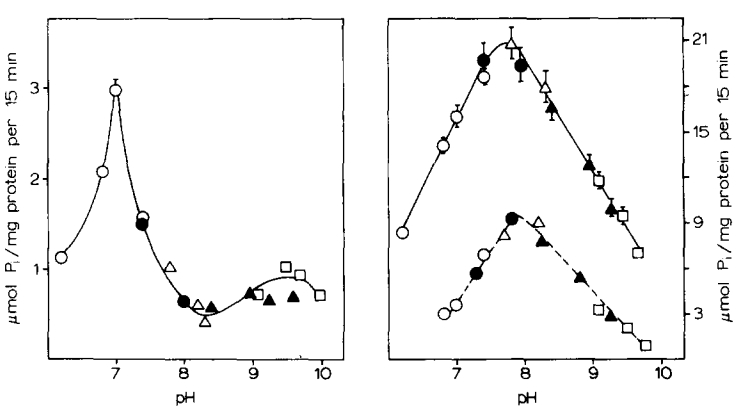

Fig. 2. Activities of macrophages plasma membrane ATPases as a function of pH. Left panel: Ca^{2+}-ATPase (pCa = 5.5); right panel: Mg^{2+}-ATPase (pMg = 3, ———) or Ca^{2+}-ATPase (pCa = 3, - - - - -). Buffers at 30 mM concentration were used: imidazole-HCl (○); triethanolamine-HCl (●); glycylglycine-NaOH (△); diethanolamine-HCl (▲); glycine-NaOH (□) (other conditions as in Fig. 1).

causes an inhibition of the two enzymes, although with a different pattern, and at 0.05% deoxycholate the activity of Ca^{2+}-ATPase (pCa 5.5) and Mg^{2+}-ATPase is decreased by about 20% and 40%, respectively.

Other compounds, known for their inhibitory effect on ATP-splitting enzymes, were also tested. A preincubation (5—30 min at 37°C) of the macrophage plasma membrane with -SH group reagents, such as *N*-ethylmaleimide or *p*-chloromercuribenzene sulphonate, at concentrations from 0.01 mM to 1 mM, does not substantially modify the activity of either Ca^{2+}-ATPase (pCa 5.5) or Mg^{2+}-ATPase. Oligomycin (0.3—30 μg/mg protein), ouabain (1 mM), and the flavonoids quercetin and morin (0.05—0.1 mM) also have no influence on both enzyme activities.

Discussion

The plasma membrane fraction, purified from rabbit alveolar macrophages by the procedure of Wang et al. [13], exhibits a Ca^{2+}-dependent ATPase activity, which has an apparent K_m (Ca^{2+}) of about $1 \cdot 10^{-6}$ M and a pH optimum of 7. The several-fold enrichment in specific activity of the Ca^{2+}-ATPase of the plasma membrane fraction over the original macrophage lysate, paralleled by a decline in specific activity of marker enzymes of mitochondria and endoplasmic reticulum, as well as the insensitivity of the plasma membrane Ca^{2+}-ATPase to oligomycin, an inhibitor of the mitochondrial and microsomal ATPases [30], rule out the possibility that the Ca^{2+}-ATPase activity derives from cytoplasmic organelles contaminating the plasma membrane fraction. Furthermore, the extremely low increase in specific activity of K^+, EDTA-ATPase of myosin [26] in the plasma membrane preparation when compared to the cell lysate, the insensitivitiy of the Ca^{2+}-ATPase to -SH group reagents, inhibitors of myosin ATPase [31], and its inhibition by low concentrations of detergents, particularly by Triton X-100, strongly suggests that the Ca^{2+}-ATPase activity is not associated to subplasmalemmal contractile structures but it is a true component of the plasma membrane itself.

A Ca^{2+}-dependent ATPase activity has been demonstrated to be present in the plasma membrane of other cells in addition to a Mg^{2+}-dependent activity [21,27—29,32—37]. Apart from the ATPase of the erythrocyte ghosts, which has a Ca^{2+}-dependence similar to that reported here [21], most of the above enzymes are activated by Ca^{2+} at rather high concentration. This sometimes does not permit to establish whether the same enzyme is activated unspecifically by either Mg^{2+} or Ca^{2+}, although to a different degree, or two enzymes with different cation specificity exist in the membrane. On account of the profile of the pH vs. activity curve, we suggest that at concentrations of bivalent ion greater than about $1 \cdot 10^{-5}$ M the former possibility applies to the macrophage plasma membrane. When the Ca^{2+} concentration is lower than about $1 \cdot 10^{-5}$ M, however, we believe that an enzyme different from Mg^{2+}-ATPase is activated. The following evidence supports this assumption: (a) from $6 \cdot 10^{-8}$ M to $1 \cdot 10^{-5}$ M Ca^{2+}, but not Mg^{2+}, stimulates the enzymatic splitting of ATP; (b) the Ca^{2+}-ATPase (pCa 5.5) has a sharp pH optimum of 7, whereas the Mg^{2+}-ATPase displays maximal activity around pH 7.8; (c) 50 mM Na^+ plus 5 mM K^+ enhance the Mg^{2+}-ATPase activity, but depress the Ca^{2+}-ATPase

activity; (d) the pattern of inhibition of the two enzymes by deoxycholate is different.

Beside activating a specific ATPase, Ca^{2+} also appears to exert an influence on the Mg^{2+}-ATPase. In fact, when the macrophage plasma membrane is incubated with a fixed amount of Mg^{2+} and variable concentrations of Ca^{2+} a progressive decline of ATPase activity is observed. Although this inhibition is not very marked, as a physiological correlation of it one might expect that an increased level of ionized calcium in the cytosol, while activating the plasma membrane Ca^{2+}-ATPase, would interfere with the utilization of ATP by the Mg^{2+}-ATPase, thereby leading to some energy conservation and/or depression of ion transport supported by the activity of Mg^{2+}-ATPase.

It is well known that Ca^{2+} has a number of molecular targets in the cells, such as enzymes, transport systems and cytoskeletal components, whose activity is regulated by the free ion concentration in the cytoplasm. This concentration, which in resting cells is very likely below $1 \cdot 10^{-6}$ M [38,39], is controlled not only by Ca^{2+} leak into the cell and by its binding to, and release from, organelles such as mitochondria, but very likely also by its active extrusion by a specific 'pump' or plasma membrane Ca^{2+}-ATPase [21,34,39,40]. We have no direct evidence whether the Ca^{2+}-ATPase identified in the plasma membrane fraction of the alveolar macrophages is a component of a transport system of this type. The localization of the enzyme at the cell boundaries and its high affinity for Ca^{2+} suggests, however, that it may indeed fulfil this function in the macrophage.

In this assumption, the control of the plasma membrane Ca^{2+}-ATPase activity would have a great relevance in the overall regulation of macrophage activity, and might provide one of the mechanisms by which events such as locomotion, secretion or endocytosis are activated. We have recently shown, for example, that an elevation of Ca^{2+} concentration induced by an ionophore in the alveolar macrophage triggers the secretion of granule enzymes, and that this secretion is several-fold increased by agents which cause a decrease of total ATP concentrations to 5--10% of normal values [10]. More physiological ways of modifying the Ca^{2+}-ATPase activity might ensue from binding of extracellular ligands to the enzyme or to adjacent receptors. Further, since the enzyme is inhibited by Na^{+}, an increase in local Na^{+} concentrations caused by membrane depolarization might depress the enzyme activity, leading to Ca^{2+} accumulation in the cytosol.

Until similar mechanisms are shown to be present in macrophages, these possibilities must remain conjectural. Some speculation may be justified, however, since so little is known of the transduction of external stimuli into a functional macrophage response.

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