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Ultrastructural localization of adenosine triphosphatase activity in lymphocytes activated *in vitro* by phytohaemagglutinin

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Summary

The ultrastructural localization of Ca^{2+}, Mg^{2+}-activated ATPase was studied in phytohaemagglutinin activated lymphocytes and in normal unstimulated lymphocytes. Cells, fixed in paraformaldehyde–glutaraldehyde, were incubated in a medium containing 3 mM ATP, 5 mM $CaCl_2$ and 2.4 mM $Pb(NO_3)_2$ in 0.1 M tris buffer at pH 8.5, the optimum pH for histochemical demonstration of this enzyme. Reaction product was localized in the endoplasmic reticulum, nuclear membrane, Golgi apparatus and mitochondria and on the membrane surrounding large electron-dense bodies. Cytoplasmic vesicles and the plasma membrane were negative. Activity in unstimulated lymphocytes showed a similar localization but the amount of endoplasmic reticulum was much less than in activated lymphocytes.

The pH of the medium was critical for the localization of the enzyme. At pH 7.5, the cytoplasmic reaction was almost completely inhibited but a dense precipitate was present on the outer surface of the plasma membrane. The reaction was stimulated by either Ca^{2+} or Mg^{2+} and was greatly decreased in the absence of these cations or in the presence of *p*-chloromercuribenzoate or *N*-ethylmaleimide. Oligomycin inhibited selectively the reaction in mitochondria but not the reaction at other sites. While the reaction in mitochondria showed complete substrate specificity, a mild reaction was obtained at the other sites with uridine diphosphate or sodium β-glycophosphate as substrate. ATP was, however, the preferential substate.

Introduction

It has recently been reported (Cuschieri, 1982) that a very high activity of ATPase can be demonstrated histochemically in lymphocytes activated *in vitro*. The activity has a localized, granular distribution confined mainly to a perinuclear area of cytoplasm, is stimulated by Ca^{2+} and Mg^{2+} and has a pH optimum between 8.5 and 9.0 but it is not inhibited by oligomycin. This last feature does not support a mitochondrial localization of the enzyme and, at the light microscope level, precise localization of the activity is not

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possible. In this paper, we report the cytochemical localization of this enzyme at the electron microscope level.

The ultrastructural localization of ATPase presents many problems. Apart from the *p*-nitrophenyl phosphate method for K^+-stimulated membrane ATPase (Ernst, 1972), there does not appear to be a well-established method of proven reliability for other types of ATPase, although modifications of the Wachstein & Meisel (1957) and of the Padykula & Herman (1955) techniques have been used with varying degrees of success (Firth, 1978). The lead methods in particular have been severely criticized on the grounds that lead can inhibit certain types of ATPases, can form a complex with ATP and can cause non-enzymatic hydrolysis of ATP (Moses *et al.*, 1966; Tice, 1968; Jacobsen & Jorgensen, 1969). While the Wachstein & Meisel method has indeed proved to be quite unsatisfactory for the localization of Na^+, K^+-membrane ATPase, some histochemists tend to dismiss the lead methods as unreliable for any ATPase. It has been pointed out, however, that for the mitochondrial and other divalent cation-dependent ATPases the lead methods might still provide reliable results if appropriate conditions and controls were used (Novikoff, 1967, 1970; Firth, 1978). Moreover, several biochemically distinct types of ATPase are known to exist in different tissues. It cannot be assumed that a technique which is unsatisfactory for demonstrating one type of ATPase is necessarily unsatisfactory for other ATPases.

In a previous study (Cuschieri, 1982), it was shown that the ATPase in activated lymphocytes was not inhibited by 3 mM lead nitrate, and that the pH of the incubation medium was critical for the demonstration of the enzyme. The lead nitrate technique at pH 7.2 gave a negative reaction in the cytoplasm but at pH 8.5 to 9.0 gave a reaction identical to that obtained with the Padykula & Herman calcium–cobalt technique. Based on these findings, we developed a lead nitrate technique which gave sharp ultrastructural localization of the enzyme activity and we used appropriate controls to validate the results.

Materials and methods

Lymphocytes were isolated on Lymphoprep from heparinized blood of normal, healthy donors as previously described (Cuschieri, 1982). The cell suspensions, which contained over 95% lymphocytes, were washed three times in Hank's balanced salt solution. Cultures were set up in RPMI medium 1640, containing 15% foetal calf serum and phytohaemagglutinin M (Gibco; 0.15 ml/10 ml medium). Samples of uncultured lymphocytes and of activated lymphocytes from 72 h cultures were processed for ATPase cytochemistry as described below. Experiments and controls were performed on 72 h cultured lymphocytes.

Fixation

Following centrifugation, the cell pellets were resuspended in 0.5 ml Hank's balanced salt solution, and 8 ml fixative was added with gentle agitation. The following fixatives in 0.1 M cacodylate buffer, pH 7.4, were used at room temperature for 15 min: (a) 2% paraformaldehyde; (b) 2.5% glutaraldehyde; (c) 2% paraformaldehyde plus 0.5% glutaraldehyde. After fixation, the cells were centrifuged and washed three times in 0.1 M cacodylate buffer, pH 7.4, for a total of 45

min. Two per cent paraformaldehyde plus 0.5% glutaraldehyde gave the best results and was used for all the following experiments. At each step in the subsequent procedures, the cells were kept in suspension and centrifuged for changing the medium.

Incubation procedure

Cells in suspension were incubated for 1 h at 37° C in a medium containing 0.1 M Tris HCl buffer, pH 8.5, 3 mM ATP, 2.4 mM lead nitrate and 5 mM calcium chloride. The incubation medium was filtered through a Millipore filter before use. Control incubations were performed, in which the above medium was modified as follows:

(1) The pH of the medium was varied to 7.2, 7.5, 8.0 and 9.0;
(2) $CaCl_2$ was replaced by 5 mM $MgCl_2$;
(3) $CaCl_2$ was omitted;
(4) ATP was omitted;
(5) ATP was replaced by uridine diphosphate or adenosine-5-monophosphate or sodium β-glycerophosphate, all at a concentration of 3 mM;
(6) 1 mM oligomycin was added to the medium;
(7) 2.5 mM *p*-chloromercuribenzoate was added to the medium;
(8) 1 mM ethylmaleimide was added to the medium;
(9) 1 mM L-tetramisole was added to the medium.

For comparison, cells were incubated for 1 h at 37° C in the medium of Padykula & Hermann (1955) containing 2.8 mM ATP, 18 mM $CaCl_2$, and 20 mM sodium barbitone at pH 9.4, followed by treatment in 2% lead nitrate for 5 min before subsequent processing.

Post-incubation procedure

The cells were washed in the same buffer as used in the incubation medium, washed again in 0.1 M cacodylate buffer, pH 7.4, post-fixed for 30 min in 1% osmium tetroxide in 0.1 M cacodylate buffer of pH 7.4, dehydrated in graded ethanol and embedded in Araldite. Ultra-thin sections were cut on a LKB ultratome III or V using diamond knives. Sections were examined without counterstaining in a Jeol 100 CX electron microscope.

Results

Fixation in 2% paraformaldehyde alone gave poor cellular preservation and disruption of organelles, resulting in poor localization with considerable diffusion of the enzyme reaction product. 2.5% glutaraldehyde gave excellent cellular preservation but the reaction was largely inhibited. A mixture of 2% paraformaldehyde plus 0.5% glutaraldehyde for 15 min was found to be the best fixative giving good preservation of cellular detail and a dense, sharply localized reaction product with no diffusion.

In activated lymphocytes from 72 h cultures, a dense reaction product was uniformly distributed throughout the lumen of the endoplasmic reticulum and nuclear membrane (Fig. 1). In places, continuity between the endoplasmic reticulum and the nuclear membrane was clearly observed. Nuclear pores were also distinctly outlined by the reaction product (Fig. 3). The mitochondria (Fig. 3) contained reaction product which was distributed throughout the matrix as well as on the cristae. No reaction was present on the outer mitochondrial membrane. Activity in mitochondria was somewhat variable

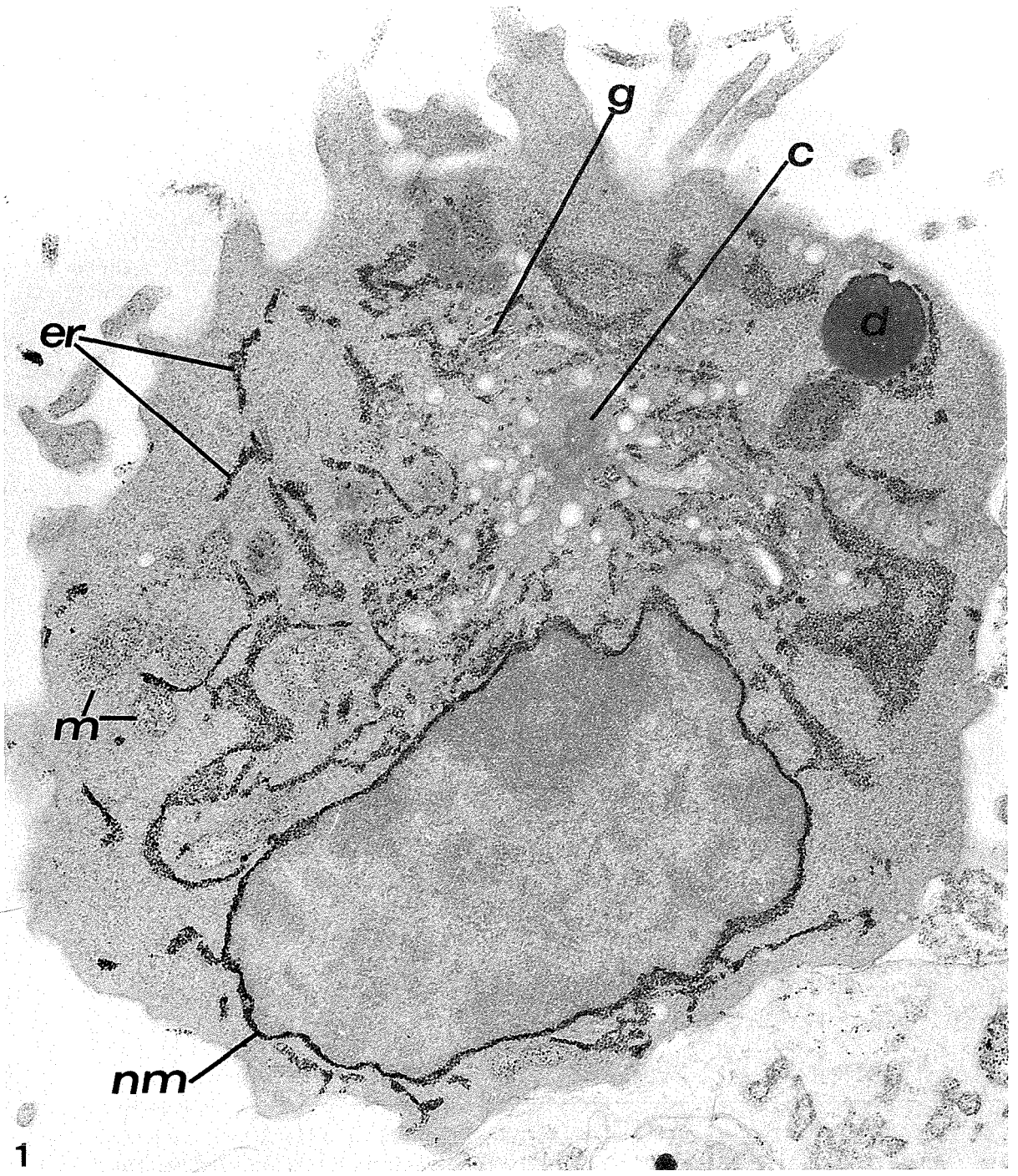


Fig. 1. Activated lymphocyte from a 72 h PHA-activated culture showing ATPase activity in the nuclear membrane (nm), the extensive endoplasmic reticulum (er), the mitochondria (m), the Golgi apparatus (g) and the membrane surrounding a large electron dense body (d). The central area of the cytoplasm contains a centriole (c) and numerous clear vesicles which are all free of reaction product. The plasma membrane shows no activity. Incubated in ATP medium containing 5 mM $CaCl_2$ at pH 8.5. × 28 000.

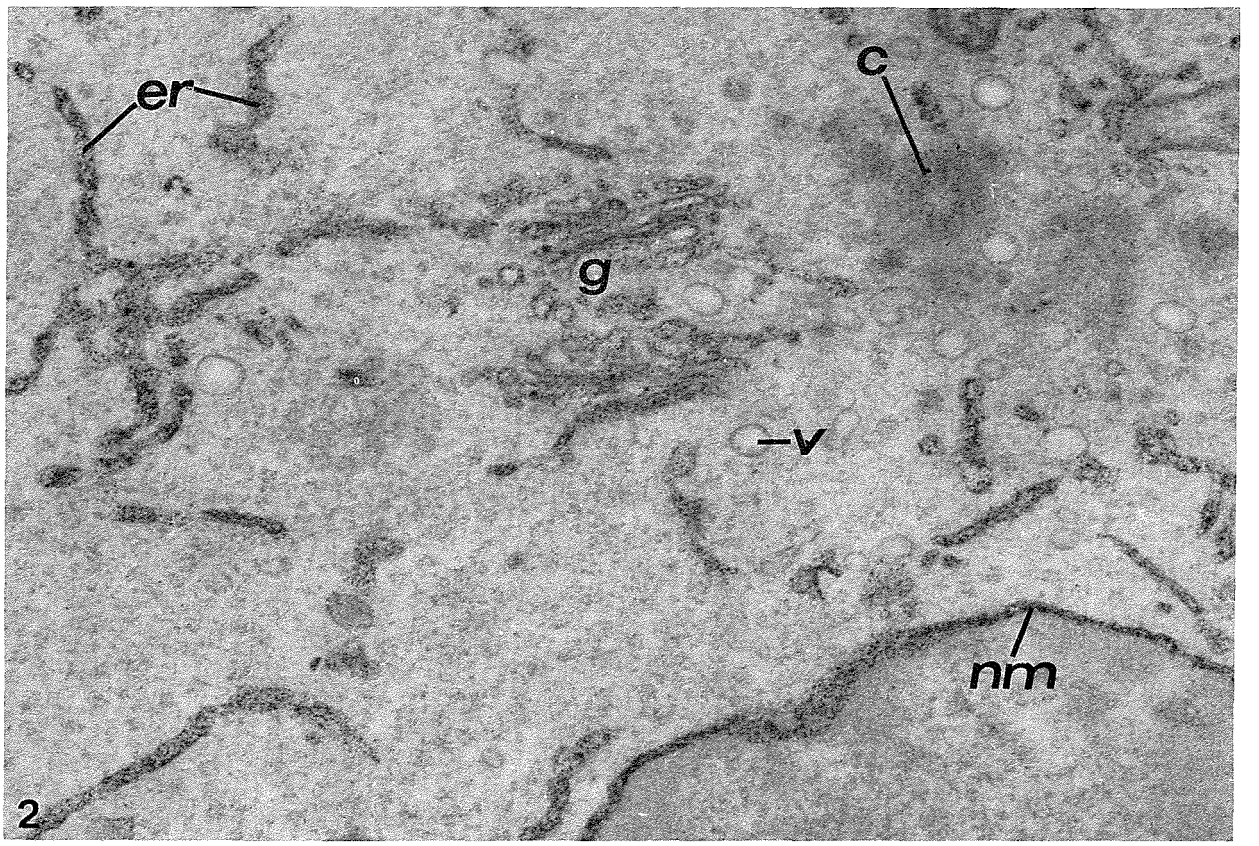


Fig. 2. Ca^{2+}-activated ATPase activity in activated lymphocyte showing localization in the endoplasmic reticulum (er), nuclear membrane (nm) and lamellae and vesicles of the Golgi apparatus (g). The centriole (c) shows minimal activity. Several membrane-bound vesicles (v) show no activity. × 25 000.

in intensity: a few contained sparse reaction product although the surrounding endoplasmic reticulum showed strong activity. The Golgi apparatus (Fig. 2) contained reaction product on the membranes bounding the flattened lamellae, dilated sacs and vesicles. Small membrane-bound vesicles of variable size and with clear contents were frequently observed within the cytoplasm and were invariably negative. Large, round bodies with electron-dense contents (Fig. 1), which occur as a constant feature in activated lymphocytes, were also negative but reaction product was frequently observed on the membrane surrounding them. The centriole sometimes contained a minimal amount of very fine precipitate (Fig. 2). No reaction product was present within the nucleus. The plasma membrane was largely negative except for a slight precipitate in some of the cells (Fig. 3).

Most of the reaction in activated lymphocytes was present in the endoplasmic

reticulum which formed an extensive system of intercommunicating profiles in the abundant cytoplasm to one side of the nucleus. This part of the cytoplasm also contained the well-developed Golgi apparatus, several elongated mitochondria and a small central area in which a centriole and several membrane-bound vesicles were situated. The remaining part of the cytoplasm formed a narrow rim around the nucleus and contained only a few profiles of endoplasmic reticulum and occasional mitochondria (Fig. 1).

In mitotic cells, the nuclear membrane was disrupted and appeared to merge with the endoplasmic reticulum (Fig. 6). Frequently their general cytoplasm was markedly more electron dense than that of non-dividing cells and scanty enzyme reaction product was present in their organelles.

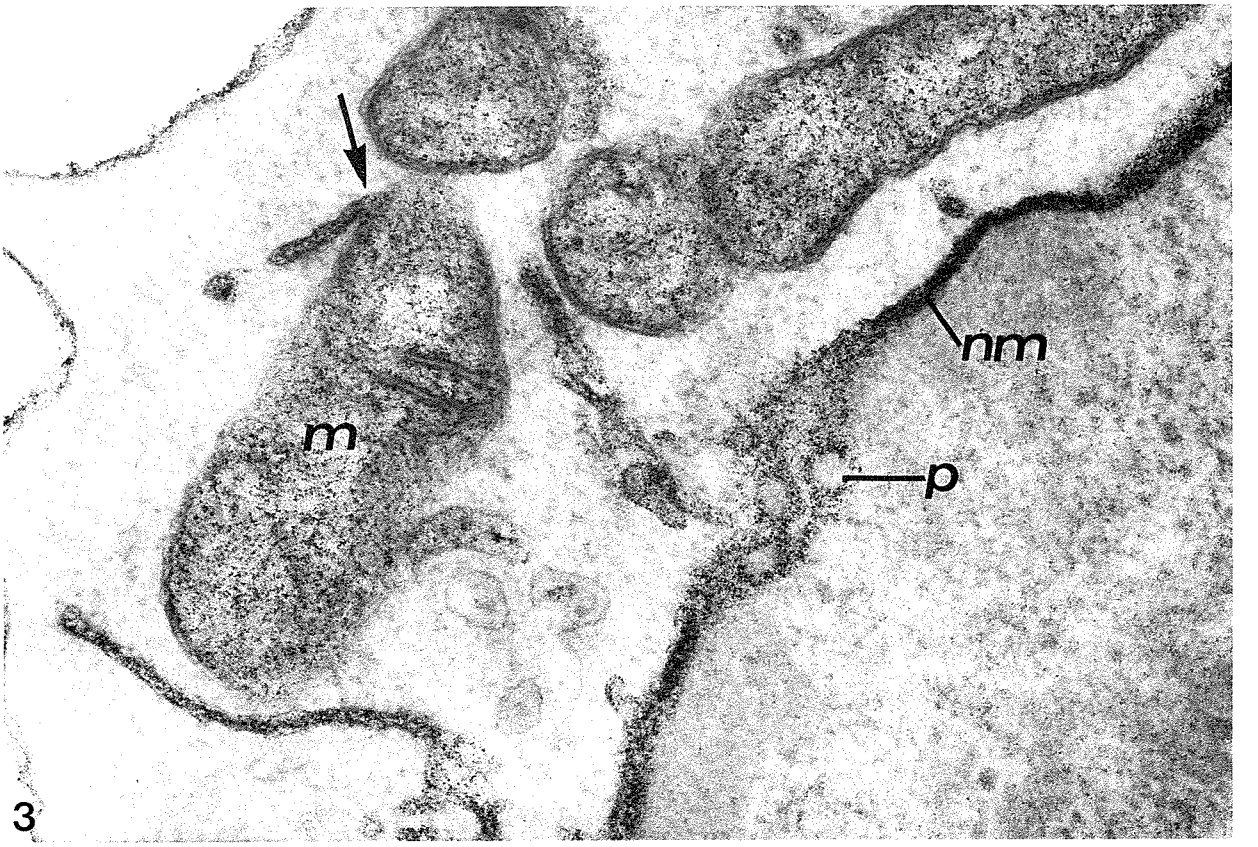
In normal unstimulated lymphocytes, activity was present in the same organelles as in the activated lymphocytes. These cells were smaller than activated lymphocytes, and their cytoplasm contained fewer profiles of endoplasmic reticulum and mitochondria but the Golgi apparatus was usually well developed (Fig. 7).

When the pH of the incubation medium was adjusted to 9.0, a similar localization to that described above was obtained. At pH 7.2, the reaction in the organelles was completely abolished, but a dense precipitate was present on the outer surface of the plasma membrane.

Addition of oligomycin to the incubation medium did not inhibit the reaction in the endoplasmic reticulum, nuclear envelope or Golgi apparatus but caused almost complete inhibition of the reaction in mitochondria (Fig. 4). When calcium chloride was replaced by magnesium chloride, an identical localization of the enzyme reaction product was present (Fig. 5). After elimination of calcium chloride from the medium, the density of the reaction product in all organelles was markedly reduced. Elimination of ATP from the medium gave a completely negative reaction. When ATP was replaced by 5-AMP no reaction was obtained but uridine diphosphate gave a scanty reaction product in the endoplasmic reticulum and nuclear envelope and mitochondria were negative (Fig. 8). With sodium β-glycerophosphate as substrate, a scanty reaction product was present in the endoplasmic reticulum and nuclear envelope. Surprisingly, the mitochondrial membranes were darkly stained but no particulate precipitate was present on the cristae or in the matrix (Fig. 9). 2.5 mM *p*-chloromercuribenzoate and

Fig. 3. Ca^{2+}-activated ATPase activity in activated lymphocytes. The reaction product in the mitochondria (m) is localized on the cristae and scattered throughout the matrix. The endoplasmic reticulum shows continuity with the mitochondrial membrane at one point (arrow). Reaction product is present in the nuclear membrane (nm) in which nuclear pores (p) are clearly outlined. × 66 000.

Fig. 4. Activated lymphocyte incubated in Ca^{2+}-activated ATPase medium containing oligomycin, showing no activity in mitochondria (m). Reaction product is present in the endoplasmic reticulum (er), Golgi apparatus (g) and nuclear membrane (nm). × 45 000.



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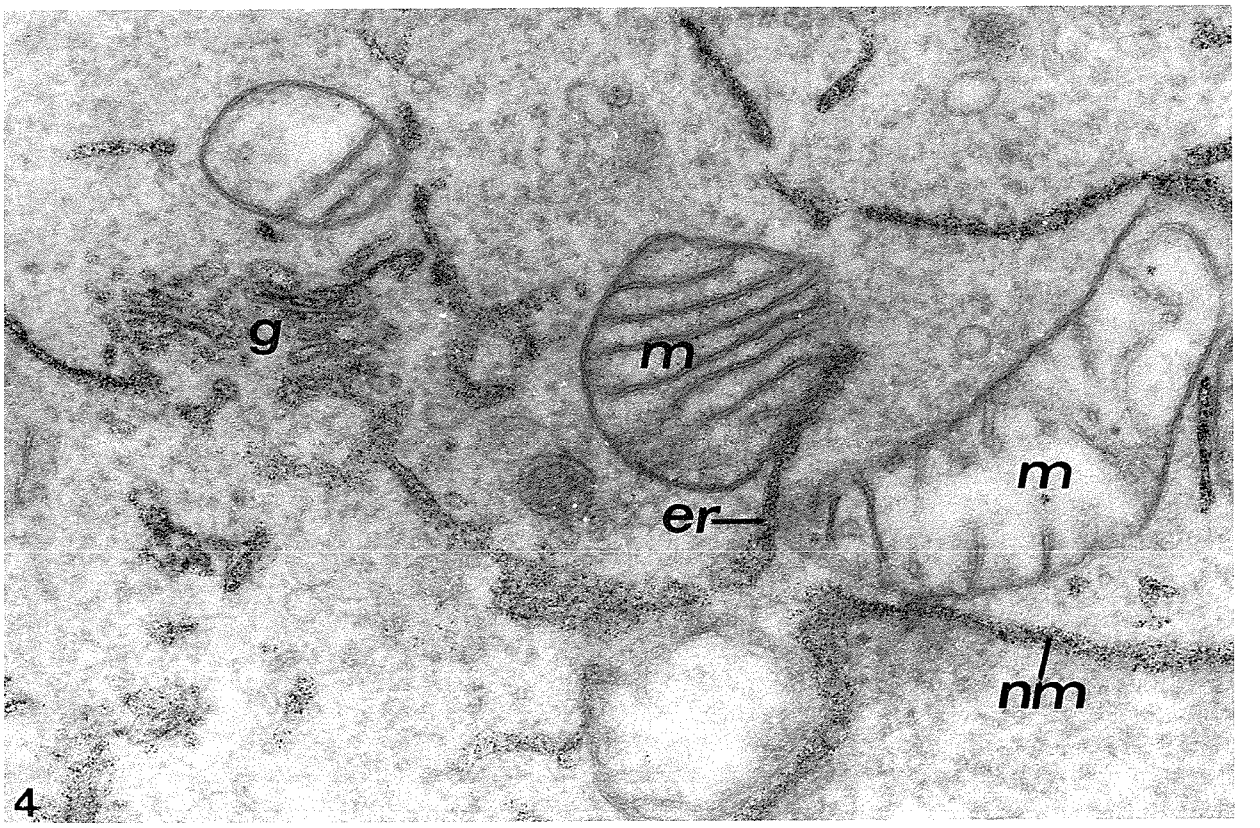

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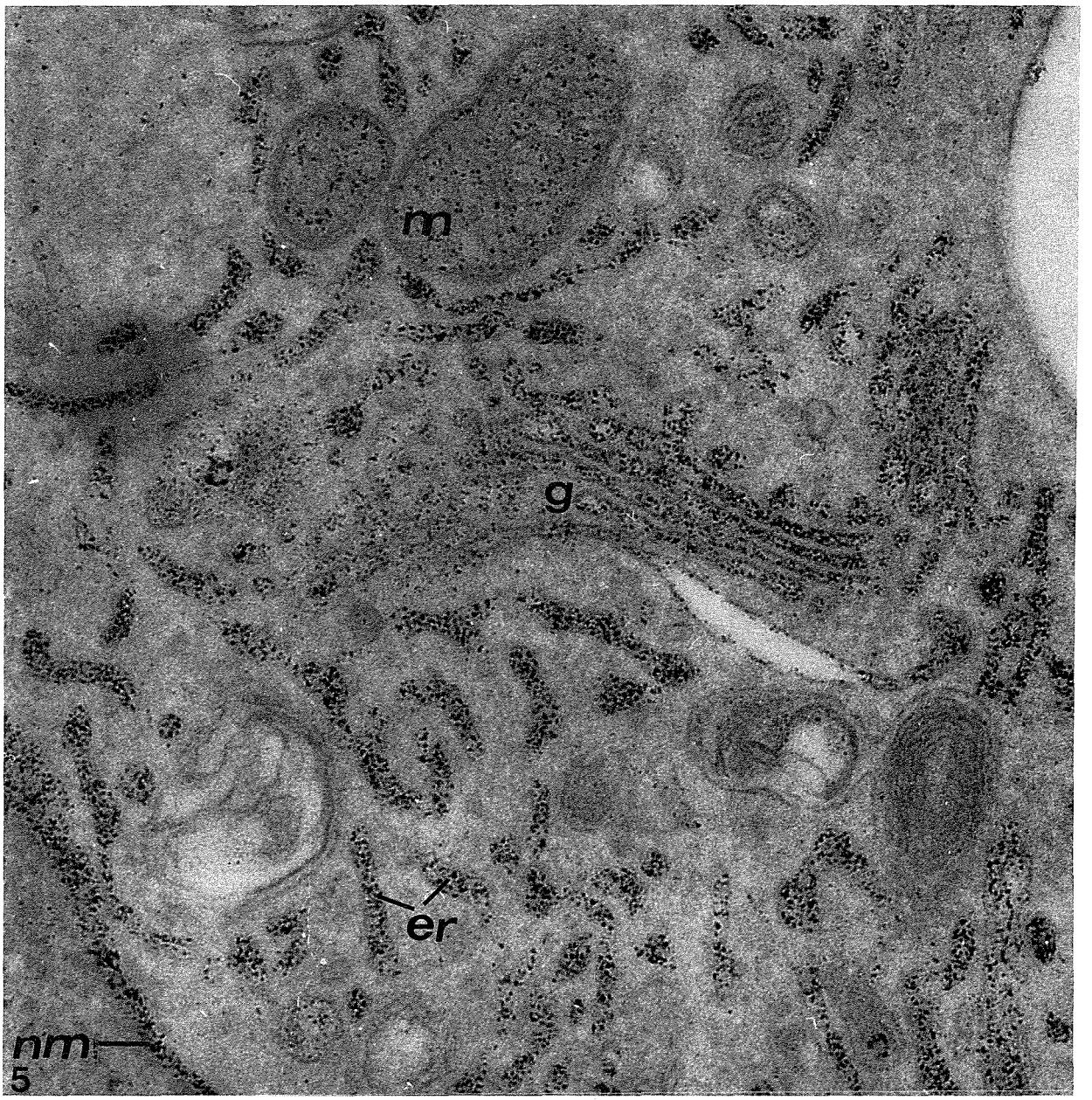


Fig. 5. Activated lymphocyte incubated in ATPase medium containing Mg^{2+} instead of Ca^{2+} showing localization in endoplasmic reticulum (er), nuclear membrane (nm), and Golgi apparatus (g). Reaction product in the mitochondria (m) is mainly on the cristae. × 82 000.

1 mM ethylmaleimide caused a reduction in the density of the reaction product at all sites but the reaction was never completely inhibited. 1 mM tetramisole caused a reduction in the density of the reaction product.

Using the Padykula & Herman incubation medium, followed by treatment in 2% lead nitrate, a localization similar to that described above was obtained but considerable diffusion was present and a fine precipitate was irregularly distributed throughout the whole cell.

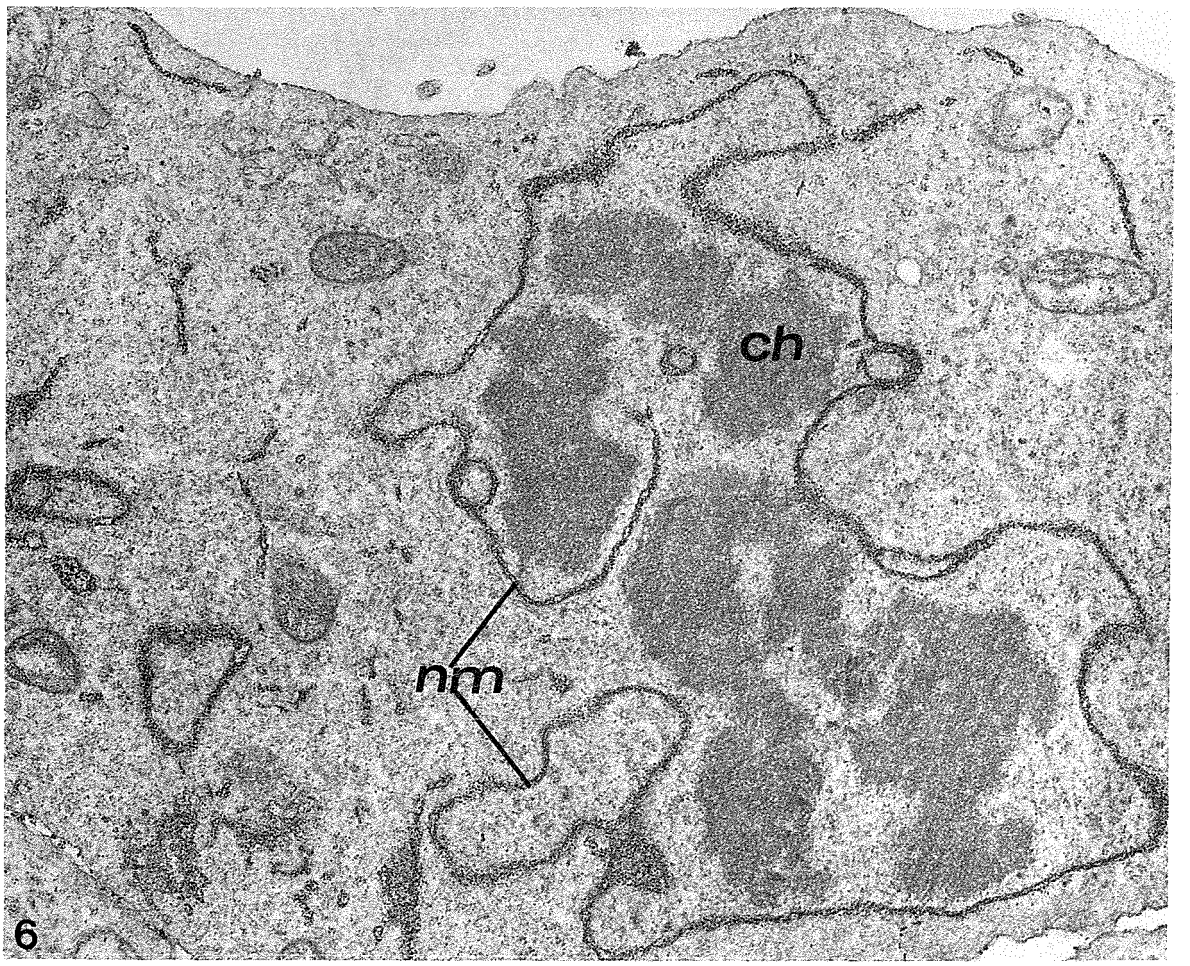


Fig. 6. Mitotic cell from a 72-h lymphocyte culture incubated in Ca^{2+}-ATPase medium. The nuclear membrane (nm), which contains reaction product, is irregular and discontinuous and encloses the chromosomes (ch). × 18 000.

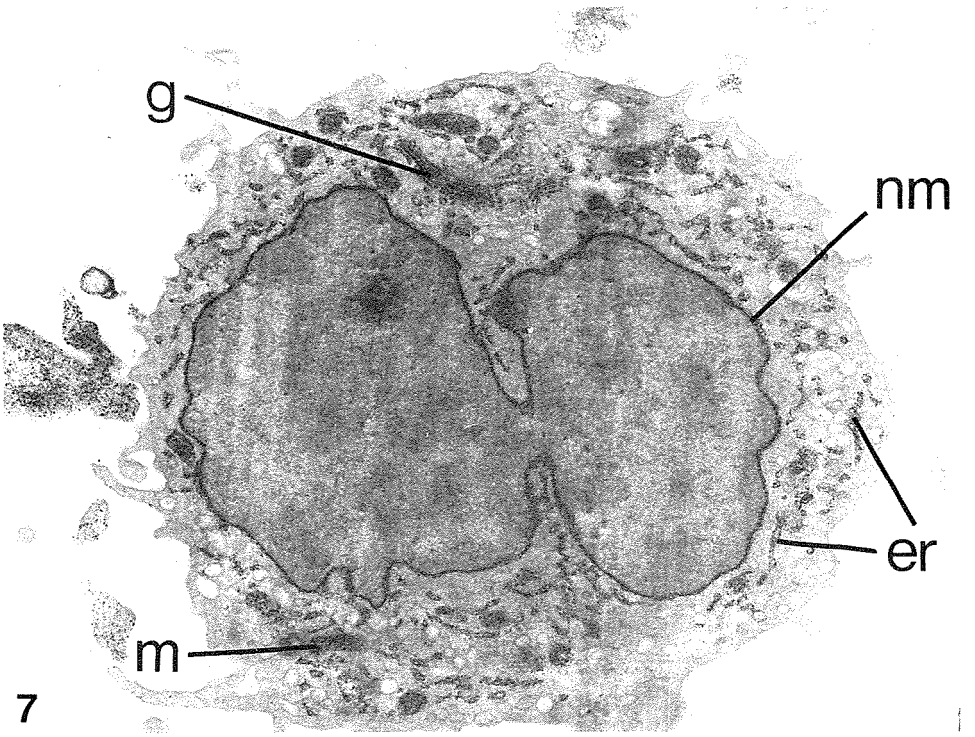
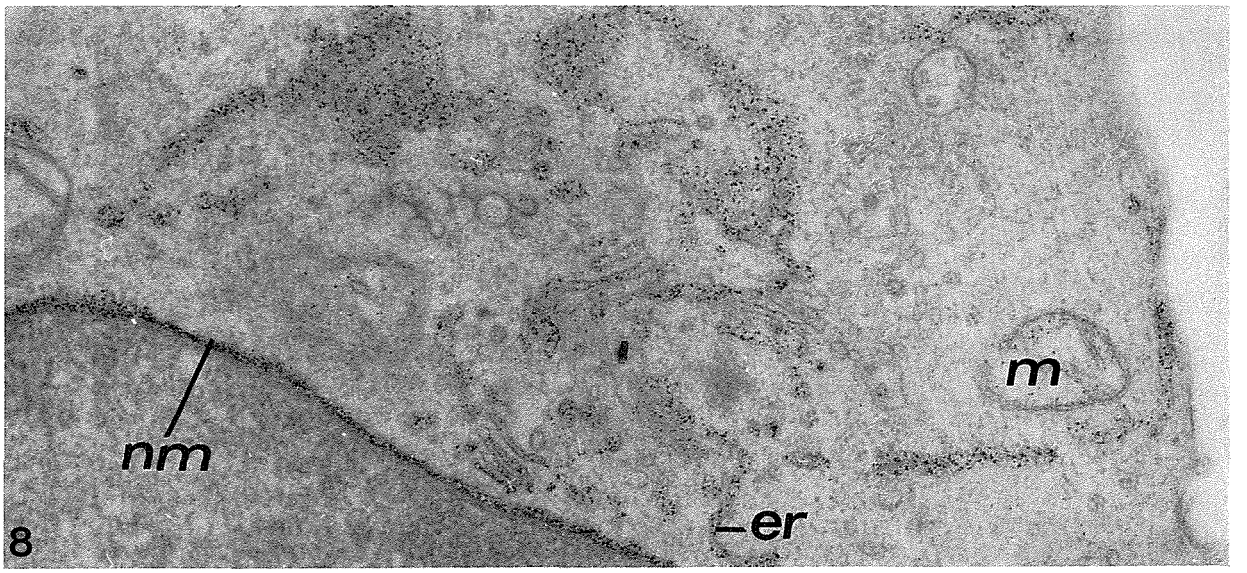


Fig. 7. Unstimulated lymphocyte showing Ca^{2+}-activated ATPase activity in the Golgi apparatus (g), mitochondria (m), endoplasmic reticulum (er) and nuclear membrane (nm). × 18 000.

Discussion

This study has shown that the high ATPase activity demonstrated in activated lymphocytes was localized mainly in the extensive system of rough endoplasmic reticulum, nuclear membrane and Golgi apparatus, and was insensitive to oligomycin. The mitochondrial activity, which was inhibited by oligomycin, was apparently responsible for a relatively small part of the total ATPase activity in the cytoplasm of activated lymphocytes. These results are generally in agreement with previous light microscope observations that the ATPase activity demonstrated histochemically in activated lymphocytes was insensitive to oligomycin (Cuschieri, 1982). Presumably the relatively small amount of inhibition produced by oligomycin in these cells would not be detectable visually in light microscope preparations. The existence of an oligomycin-insensitive ATPase in lymphocytes is also supported by the biochemical studies of Ellegaard & Dimitrov (1972) which indicated that a proportion of the total ATPase activity in normal lymphocytes was insensitive to either oligomycin or ouabain. Although Ellegaard & Dimitrov (1972) demonstrated an increase in ATPase activity following phytohaemagglutinin (PHA) stimulation, their inhibitor studies were



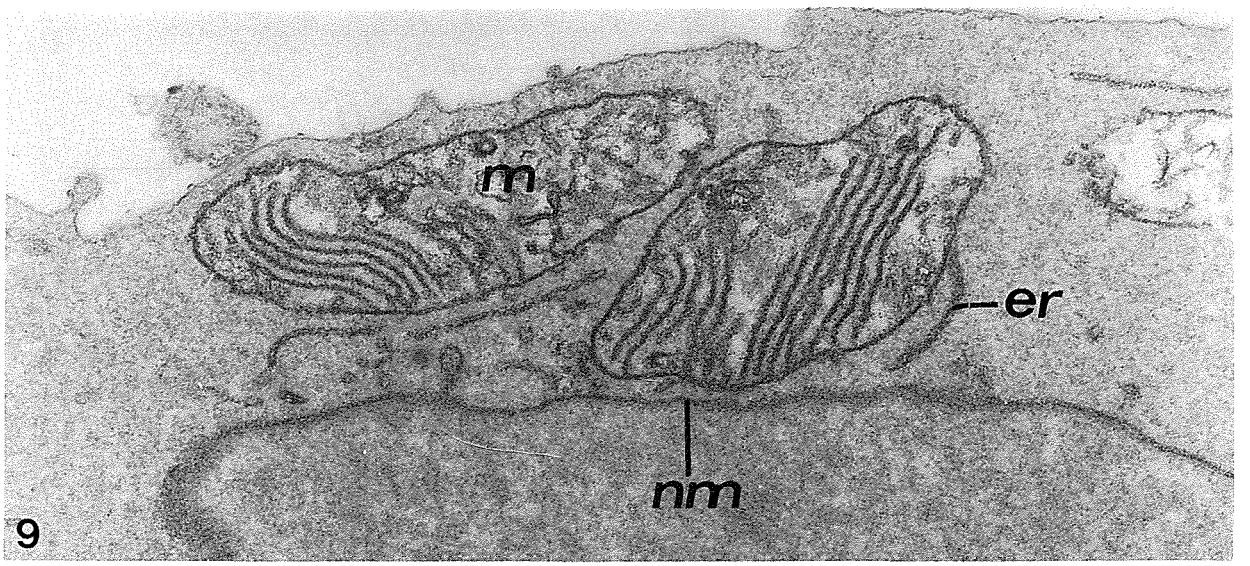


Fig. 8. Incubation in medium containing uridine diphosphate as substrate. Sparse reaction product is present on the endoplasmic reticulum (er) and nuclear membrane (nm). Mitochondria (m) are free of reaction product. × 40 000.

Fig. 9. Incubation in medium containing β-glycerophosphate as substrate. The membranes and cristae of mitochondria (m) are darkly stained. Sparse reaction product is present on the endoplasmic reticulum (er) and nuclear membrane (nm). × 32 000.

performed only on unstimulated lymphocytes. The results of the present study indicated that the increase in ATPase following lymphocyte activation was mainly the result of an increase in the amount of endoplasmic reticulum and the ATPase associated with it.

The selective inhibition by oligomycin of the activity in mitochondria is to be expected since oligomycin is a specific inhibitor of the mitochondrial ATPase associated with the F_1 particles. It also testifies to the specificity of the reaction for mitochondrial ATPase. However, the localization was found to be diffusely distributed within the mitochondrial matrix and not confined solely to the cristae, where the F_1 particles are situated. It seems that the final reaction product diffuses readily from the actual site of ATPase activity but is strictly confined within the inner mitochondrial membrane. The reason why diffusion should occur at this site is not understood but similar diffusion within the matrix was obtained in most studies on the electron histochemical localization of mitochondrial ATPase (Lazarus & Barden, 1964; Somogyi *et al.*, 1971; Hajos *et al.*, 1974).

The main difference between the method used in the present study and the classical Wachstein & Meisel method was the pH of the medium. As previously reported (Cuschieri, 1982) and confirmed in the present study, the pH of the medium was critical for the demonstration of cytoplasmic ATPase in lymphocytes. Optimal activity was found to be at pH 8.5, and the reaction within the cytoplasm was inhibited at pH 7.2, although a dense precipitate on the surface membrane was present at this pH. Numerous studies have been reported in which the Wachstein & Meisel medium (or minor modifications of it) at or near pH 7.2 was used for the cytochemical demonstration of ATPase in various tissues, including lymphocytes (White & Krivit, 1965). Most of these have reported a reaction product on the surface membrane, and occasionally in mitochondria but, with the exception of skeletal and cardiac muscles, localization in other organelles was not usually found. The fact that the classical Wachstein & Meisel (1957) medium makes use of a 'physiological' pH does not necessarily imply that this is the optimum pH for the cytochemical demonstration of all ATPases. It appears likely that ATPases at different sites and in different tissues differ considerably in their biochemical properties, and might be demonstrable by modifications of the lead nitrate method only if the appropriate conditions, especially as regards pH and ion concentrations, are employed. Benedeczky *et al.* (1972), investigating the cytoplasmic localization of ATPase in adrenal medulla, obtained a negative reaction with the classical Wachstein & Meisel medium, but found localization of the enzyme in the Golgi apparatus when the pH of the medium was adjusted to 6.4, the optimum pH for Ca^{2+}-activated ATPase in adrenal medulla as determined in biochemical experiments.

A dense precipitate on the outer surface of the cell membrane, as found in this study using ATP medium at pH 7.2, has been reported in many tissues investigated by the classical Wachstein & Meisel ATPase technique. Reaction products at this site certainly do not represent the membrane-associated Na^+, K^+-activated ATPase activity (Firth,

1978), and are sometimes interpreted as being artefactual, but the possibility that they are the result of enzymatic activity cannot be excluded. In fact Ohnishi & Yamagachi (1978), using the Wachstein & Meisel procedure at pH 7.2, observed that the reaction product on the outer surface of cultured hepatoma cells was greater at the sites of contact of adjacent cells than on the free borders, and was markedly inhibited by *p*-chloromercuribenzoate but not by ouabain. They interpreted this localization as indicative of an ATPase belonging to a group of 'ecto-enzymes' present on the outer surface of the plasma membrane.

In the present study, it was shown that the ATPase reaction in lymphocytes was stimulated by either Ca^{2+} or Mg^{2+} since in the absence of either of these cations the reaction was considerably less. While the reaction in mitochondria showed complete substrate specificity, the reaction in the endoplasmic reticulum and nuclear membrane was not completely abolished when the ATP substrate was replaced by uridine diphosphate or sodium β-glycerophosphate. The reaction was reduced but not eliminated by the sulphydryl inhibitors *p*-chloromercuribenzoate and ethylmaleinide. Tetramisole also apparently caused some inhibition of the reaction. It seems therefore that the reaction in the endoplasmic reticulum and nuclear envelope is not completely specific for ATPase and that there might be some interference from other nucleotide phosphatases or non-specific alkaline phosphatases which might be present at the same sites. It must be noted, however, that reactions with substrates other than ATP were always mild and that ATP was the preferential substrate, suggesting that ATPase is the enzyme mainly responsible for the observed reaction.

The cytoplasmic localization of ATPase in endoplasmic reticulum has been previously described only in the sarcoplasmic reticulum of skeletal and cardiac muscles (Sommer & Spach, 1964; Tice & Engel, 1966; Somogyi *et al.*, 1972). In muscle, ATPase was also present in the T-system, on myofibrils, in mitochondria and on the sarcolemma and it has been possible to distinguish between different ATPases on the basis of ion-dependence, inhibition by *p*-chloromercuribenzoate and oligomycin, and pH optimum (Khan *et al.*, 1975). ATPase has also been localized in the Golgi apparatus of chromaffin cells in the adrenal medulla (Bendeczky *et al.*, 1972), and in the cisternal spaces and dense tubular system (a derivative of endoplasmic reticulum) of platelets (Cutler *et al.*, 1980).

The role of mitochondrial ATPase in oxidative phosphorylation is widely accepted. However, with the exception of muscle in which the specific roles of various ATPases have been widely investigated, one can only speculate about the possible functions of ATPase in non-mitochondrial sites. Localization of ATPase on the endoplasmic reticulum and Golgi apparatus suggests that the energy released from ATP hydrolysis might be necessary for protein synthesis in the rough endoplasmic reticulum and for other synthetic activities in the Golgi apparatus. Protein synthesis is known to be greatly increased in lymphocytes following activation (Ling & Kay, 1975).

ATPase is thought to play an important role in the active transport of Ca^{2+}, and possibly other divalent cations, across membranes. Such a mechanism is known to

occur in the case of Ca^{2+} uptake into the lumen of sarcoplasmic reticulum which forms an intracellular reservoir of Ca^{2+} (Sandow, 1970). There is growing evidence for the existence of intracellular Ca^{2+} reservoirs in many other cells, although the sites of these reservoirs have not been defined, and the utilization of intracellular Ca^{2+} appears to be an important component in cellular control mechanisms (Berridge, 1975). It is possible that the ATPase located on the endoplasmic reticulum and nuclear membrane, which form a continuous system, might be responsible for uptake of Ca^{2+} into the lumen of this system, thus participating in regulating the level of free Ca^{2+} in the cytoplasm. Biochemical evidence indicates that Ca^{2+} plays a pivotal role in lymphocyte activation (Decker & Marchalonis, 1978). Intracellular calcium mobilization appears to be closely linked to cyclic nucleotide metabolism which, in turn, regulates the phosphorylation of non-histone chromosomal proteins which enter the nucleus to bind specifically to DNA and stimulate RNA synthesis.

Acknowledgements

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