

Effects of Quercetin on Magnesium-Dependent Adenosine Triphosphatase and the Metabolism of Human Polymorphonuclear Leukocytes

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The bioflavonoid quercetin was found to exert at least three separate effects on human polymorphonuclear leukocytes. (1) Concentrations of approximately 100 μM inhibited the membrane-associated magnesium adenosine triphosphatase by 60%–80% in either broken cell preparations or intact cells. Lineweaver-Burk plots showed the inhibition to be uncompetitive in nature. (2) Similar concentrations of quercetin inhibited respiratory burst activity of the cells as measured by oxygen consumption,

glucose oxidation, or iodination of protein. All inhibitions were dose-dependent and were observed with either opsonized zymosan or phorbol myristate acetate as stimulus. (3) Quercetin likewise inhibited the transport of the nonmetabolizable hexose, ^3H -2-deoxyglucose. These observations are most consistent with the hypothesis that quercetin exerts a generalized effect at the level of the cell membrane of the neutrophil.

WE HAD previously described a magnesium-dependent adenosine triphosphatase (Mg^{2+} -ATPase) in the plasma membrane of the human polymorphonuclear leukocyte (PMNL).¹ This appeared to be an ecto-enzyme with its active site facing the exterior of the cell, such that intact cells exhibited as much activity as broken cell preparations under conditions where the substrate was impermeable to the plasma membrane. These observations were independently confirmed by Smolen and Weissman.²

The function of this enzyme is obscure. It appears to be distinct from alkaline phosphatase, which has likewise been suggested to reside in the plasma membrane of the human neutrophil.³ Smolen and Weissman² reported that 100 μM suramin inhibited the ATPase by 65%, without appreciably affecting chemotaxis, superoxide anion generation, or phagocytosis, and concluded that the enzyme was not likely to be implicated in those events.

The present communication further investigates the role of Mg^{2+} -ATPase in the function of the human PMNL, employing another inhibitor, quercetin, as a molecular probe.

MATERIALS AND METHODS

Isolation of Leukocytes

Leukocytes were isolated from 60 to 120 ml of heparinized venous blood obtained from healthy volunteers and from 2 patients with chronic granulomatous disease (CGD) of childhood. Informed consent was obtained, and research was carried out according to the Declaration of Helsinki. The blood was mixed with Plasmagel (HTI Corp., Buffalo, N.Y.) in a 3:1 (v/v) ratio and the erythrocytes allowed to sediment at room temperature for 45–60 min. The supernate was centrifuged at 225 g for 8 min to sediment the leukocytes and the cell pellet washed once with Dulbecco's phosphate-buffered saline (PBS). Contaminating erythrocytes were removed by brief hypotonic lysis in deionized water as previously described.⁴ The cell pellet was resuspended to approximately 10^8 cells/ml in PBS. Total cell counts were performed in a hemocytometer, and differential counts were obtained on Wright-stained slides

prepared in a cytocentrifuge. The differential counts typically yielded greater than 80% neutrophils and less than 20% mononuclear cells in both normal and patient samples. Viability, as judged by exclusion of trypan blue dye, was always greater than 95%. Cells to be used in assays employing whole cell preparations were kept on ice suspended in PBS and were used immediately following isolation. When cell homogenates were employed, the cells were resuspended in 0.34 M sucrose to a concentration of 10^8 cells/ml and homogenized with a tight-fitting Potter-Elvehjem homogenizer. Homogenization was performed on ice for a total time of 5 min with the motor driven at full speed (10,000 rpm). Such homogenates could be stored frozen at -4°C for at least 2 wk with less than 10% loss in enzyme activity.

Mg^{2+} -ATPase Assay

Enzyme activity was assayed by the method of Harlan et al.,¹ scaled down to allow use of lower concentrations of substrates and cells. Activity was measured at pH 7.4 in a reaction mixture that was 100 mM in Tris-HCl, 0.10 mM in MgCl_2 , 0.10 mM in ATP, and containing sufficient γ -[^{32}P] ATP to give 100,000 cpm per assay. The total reaction volume was 2.0 ml. The reaction was started by the addition of 0.05 mg of homogenate protein and was generally incubated for 60 min at 37°C . When whole cells were employed, 10^6 cells suspended in 100 mM Tris-HCl, pH 7.4, were added per assay. In some experiments, enzyme activity was measured as a function of pH. Tris-HCl was used as the buffer from pH 7.5 to 9.0, and 2-amino-2-methyl-1-propanol · HCl buffer was used from pH 9.0 to 10.5. The reaction was stopped by the addition of 1.0 ml of cold 10% trichloroacetic acid (TCA). The inorganic phosphate formed in the course of the reaction was complexed with ammonium molybdate, extracted into isobutanol, and quantitated in a Beckman LS-100C scintillation counter as previously described.¹

Some Mg^{2+} -ATPase assays contained quercetin, a highly colored

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compound, which was extracted into the organic phase in the separation procedure described; the bright color caused quenching and prevented accurate determination of ^{32}P , in the samples. ATPase assays containing quercetin were stopped with 1.0 ml of 10% (w/v) acid-washed charcoal in 10% (w/v) TCA as described by Smolen and Weissman.² The charcoal was removed by filtration through a 0.45- μ Millipore filter, and the ^{32}P in the supernate was determined by liquid scintillation counting. The activated charcoal adsorbed both unreacted nucleotides and quercetin, resulting in a colorless supernate. Comparative studies demonstrated that the two methods yielded similar results in the absence of quercetin.

Protein determinations were performed by the method of Lowry et al.⁵

Hexose Monophosphate Shunt Assay

Glucose metabolism via the hexose monophosphate shunt (HMS) was determined by the oxidation of [1- ^{14}C] glucose to $^{14}\text{CO}_2$ as previously described.⁶ Each flask contained unlabeled glucose (0.10 mg/ml) as well as 0.50 μCi [1- ^{14}C] glucose. In some experiments, phagocytosis was initiated by the addition of 0.50 ml of opsonized zymosan (8 mg); in others, cells were stimulated by the addition of phorbol myristate acetate (PMA; Consolidated Midland Corp., Brewster, N.Y.) to a final concentration of 1 $\mu\text{g}/\text{ml}$. In all cases, the reaction was terminated by the addition of 1.0 ml of 5% TCA, and the $^{14}\text{CO}_2$ released in the course of the reaction was trapped in hyamine hydroxide and the radioactivity quantitated in a liquid scintillation spectrometer.

Uptake of Deoxyglucose

The uptake of the glucose analog 2-deoxy-D-glucose by neutrophils was measured by a modification of the method of Bibi et al.⁷ The assay mixture consisted of 5×10^6 cells suspended in PBS, 1.0 μCi of ^3H -2-deoxy-D-glucose, and PBS to a final volume of 3.0 ml. The reaction was initiated by the addition of cells and incubated at 37°C for varying periods of time. The reaction was stopped by immersing the incubation tubes in ice water and immediately centrifuging in the cold at 225 g for 10 min. The cell pellet was washed once with 5.0 ml of cold PBS and the cells collected by centrifugation. The resulting cell pellet was resuspended in 3.0 ml of distilled water and disrupted by sonication for 2 min in a sonicating bath (Heat Systems Ultrasonics Inc., Plainview, N.Y.). The uptake of ^3H -2-deoxy-D-glucose was quantitated by counting 1.0-ml aliquots of each sample in 10-ml Aquasol in a scintillation counter.

Iodination Assay

The ability of PMNL to covalently bind ^{125}I in the presence of opsonized zymosan was determined in the presence of varying concentrations of quercetin. Each tube contained 5×10^6 PMNL, 6.6 mg preopsonized zymosan, carrier-free ^{125}I (100,000 cpm), and the indicated concentrations of quercetin in a final incubation volume of 1.0 ml. All solutions were made in Ca^{2+} -free PBS because of the insolubility of calcium iodide. The reaction was initiated by the addition of cells and terminated after 30 min at 37°C by the addition of 2.0 ml of 5% trichloroacetic acid. The resulting precipitate was washed 4 times with 2.0 ml of TCA each time, resuspended in TCA, and the radioactivity determined in a gamma well counter. Iodination in a cell-free system was performed under similar conditions except that 0.01 mM H_2O_2 and 0.02 mg of a crude granule fraction from human neutrophils were employed.

Oxygen Uptake

Measurement of oxygen uptake was performed under standard conditions with a Clark electrode. The assay contained 5×10^6 PMNL in a total volume of 1.80 ml. Cells were equilibrated with varying concentrations of quercetin before stimulation by opsonized zymosan (1.3 mg) or PMA (1.8 μg).

RESULTS

The effect of varying concentrations of quercetin on human neutrophilic Mg^{2+} -ATPase is illustrated in Fig. 1. In this and subsequent experiments, the quercetin was dissolved in dimethyl sulfoxide before addition to the assay mixture. Controls were always incubated with an equal amount of the vehicle alone. In this experiment, maximal inhibition amounted to 80% of

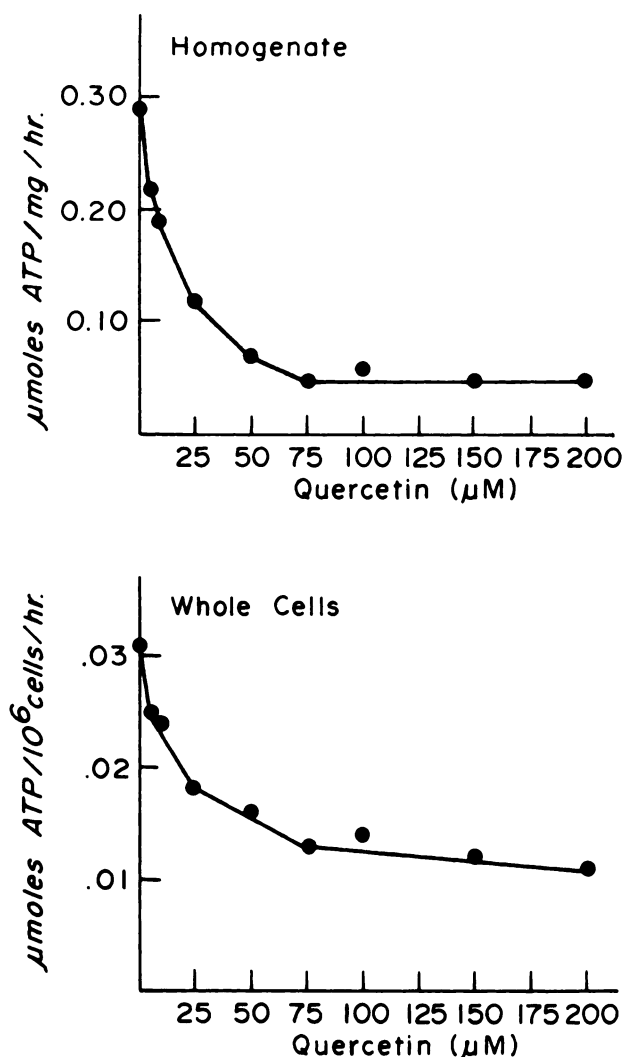


Fig. 1 The effect of varying concentrations of quercetin on human leukocyte Mg^{2+} -ATPase in a homogenate preparation and in whole cells. These data are from a single experiment that is representative of three separate experiments; each point is the mean of duplicate determinations.

the initial activity of the homogenate and 65% of the whole cells. In a number of experiments, maximum inhibition of enzyme activity was observed at approximately 100 μM quercetin and varied between 60% and 85%. There was no consistent difference noted between the inhibition with the homogenate and that with whole cells.

Figure 2 is a Lineweaver-Burk plot illustrating the kinetics of the inhibition by quercetin using intact cells. Similar results were obtained with homogenates (data not shown). In this experiment, both unlabeled and radioactive ATP were varied simultaneously to avoid isotope dilution effects. The inhibition by quercetin appeared to be uncompetitive in nature with alterations in both the V_{max} and K_m of the enzyme. In the presence of quercetin, the V_{max} decreased from 0.17 $\mu\text{mole ATP/hr/mg}$ to 0.09 $\mu\text{mole ATP/hr/mg}$, while the K_m decreased from 0.11 mM to 0.07 mM . Substrate inhibition was observed at ATP concentrations above 500 μM for the uninhibited reaction and above 250 μM for the reaction in the presence of 100 μM quercetin.

We next examined the effect of quercetin on a number of functional parameters of human PMNL, especially those associated with activation of the respiratory burst.

The effect of varying concentrations of quercetin on the oxidation of ^{14}C -1-glucose is illustrated in Fig. 3. Quercetin inhibited glucose oxidation in cells that were stimulated by either opsonized zymosan or PMA in a dose-dependent fashion. Maximal inhibition of HMS activity was observed at a concentration of quercetin (approximately 100 μM), which also caused

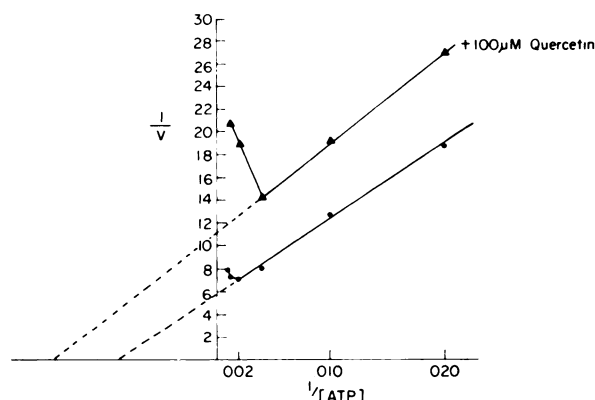


Fig. 2. Lineweaver-Burk plot for human leukocyte Mg^{2+} -ATPase activity in whole cells in the presence and absence of 100 μM quercetin. Velocity is expressed as $\mu\text{mole ATP/hr/mg}$, and the ATP concentration is μM . The data are from a single experiment that is representative of five such experiments (two employing cell homogenates). Each value represents the mean of duplicate determinations.

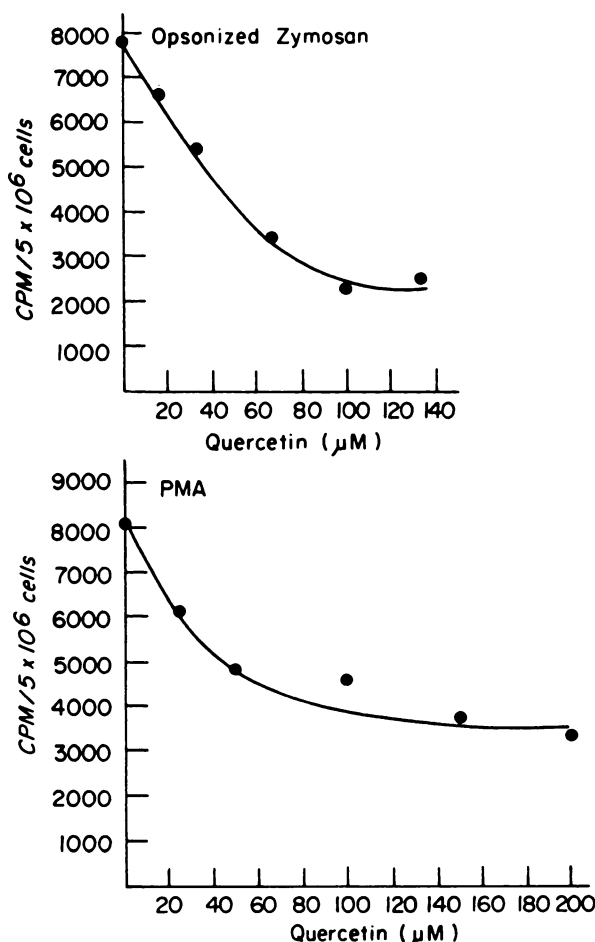


Fig. 3. The effect of varying concentrations of quercetin on the hexose monophosphate shunt activity of human leukocytes. In the upper panel, the cells were stimulated by the addition of opsonized zymosan; in the lower, by the addition of 1 $\mu\text{g/ml}$ of phorbol myristate acetate. Each value represents the mean of triplicate determinations.

maximal inhibition of the Mg^{2+} -ATPase. Further, the extent of inhibition of the two phenomena was similar, ranging from 60% to 85% in different experiments. The inhibition was not observed in the presence of 10% serum, presumably due to serum binding of the quercetin.

Measurement of hexose monophosphate shunt activity requires that extracellular radiolabeled glucose first be transported into the cell for metabolism to occur. Accordingly, we investigated the effects of varying concentrations of quercetin on hexose transport employing the poorly metabolized analog ^3H -2-deoxy-D-glucose. The results in Fig. 4 indicate that quercetin exerts a profound inhibition of hexose transport at concentrations that inhibit both Mg^{2+} -ATPase and glucose oxidation. This experiment employed only radiolabeled deoxy-D-glucose; similar results were obtained in other experiments in which millimolar

quantities of 2-deoxy-D-glucose were incubated with the cells (data not shown).

We next examined the effect of quercetin on oxygen consumption by means of a Clark electrode. Results in Fig. 5 indicate that quercetin inhibits oxygen consumption stimulated by the addition of opsonized zymosan. The inhibition is dose-dependent and appears to be maximal at 100 μM quercetin. Significantly, no inhibition of the basal (resting) level of oxygen consumption was observed even at concentrations of quercetin as high as 200 μM . Similar results were observed when PMA (1 $\mu\text{g}/\text{ml}$) was employed as stimulant.

The effect of quercetin on the iodination of opsonized zymosan is illustrated in Fig. 6. With intact cells, there was a dose-dependent inhibition of iodination similar to that observed for glucose oxidation and oxygen uptake (panel A). This was not due to a direct effect on myeloperoxidase or to its acting as a potent reducing agent since quercetin was completely without effect when iodination was determined in a crude granule fraction of neutrophils (panel B).

The possibility that quercetin was inhibiting transport of ^{125}I into the cell (similar to that observed for ^3H -2-deoxyglucose) was tested. In these experiments, ^{125}I was incubated with neutrophils in the presence or

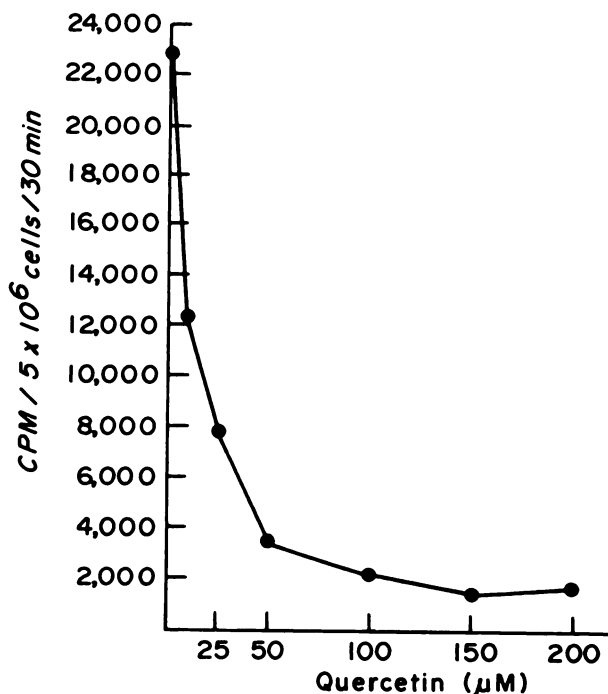


Fig. 4. The effect of increasing concentrations of quercetin on the uptake of ^3H -2-deoxy-D-glucose by human polymorphonuclear leukocytes. This experiment is representative of two separate experiments; each value represents the mean of triplicate determinations.

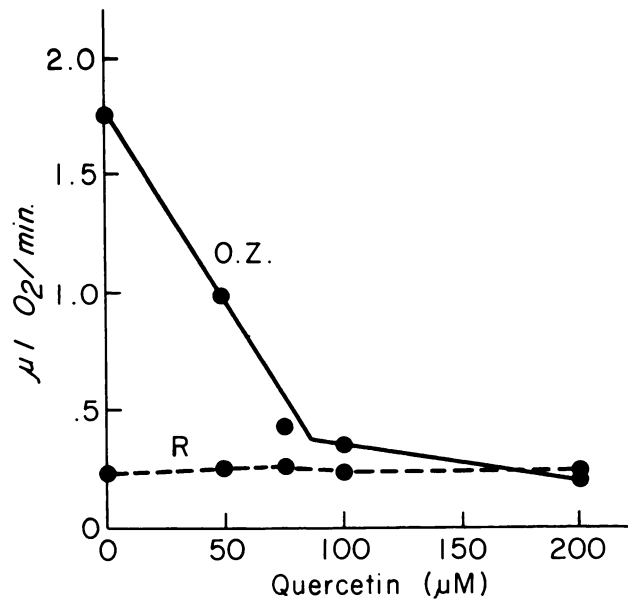


Fig. 5. The effect of varying concentrations of quercetin on oxygen consumption by resting and stimulated human polymorphonuclear leukocytes. Dotted line, resting cells; solid line, cells challenged with opsonized zymosan. The experiment illustrated is representative of two separate experiments; similar results were observed when the cells were stimulated with phorbol myristate acetate in one experiment.

absence of 100 μM quercetin for varying periods of time ranging from 30 sec to 30 min. The uptake was stopped by rapidly centrifuging an aliquot of the cell suspension through 0.50 ml of n-butylphthalate using a Beckman Microfuge. In five separate experiments, the addition of quercetin (100 μM) had no influence on ^{125}I uptake at any time point tested, thus excluding a direct effect on transport.

It seemed unlikely that the effects of quercetin were secondary to an inhibition of phagocytosis, since the compound likewise inhibited PMA-induced oxygen consumption and glucose oxidation. Nevertheless, this was directly tested by measuring the uptake of radio-labeled preopsonized *S. aureus*. In several experiments, a modest inhibition of uptake (30%) was observed in the presence of 200 μM quercetin. This was not sufficient to explain the profound inhibition of oxidative metabolism induced by this compound. Further, lower concentrations (100 μM) did not result in a statistically significant inhibition of phagocytosis.

The similar inhibition of both Mg^{2+} -ATPase and oxidative metabolism by quercetin suggested that the enzyme might be involved in the initiation of the respiratory burst during phagocytosis. Accordingly, we examined ATPase activity in neutrophil homogenates from two patients with well documented CGD whose cells completely fail to exhibit a respiratory burst during phagocytosis.⁸ In these experiments

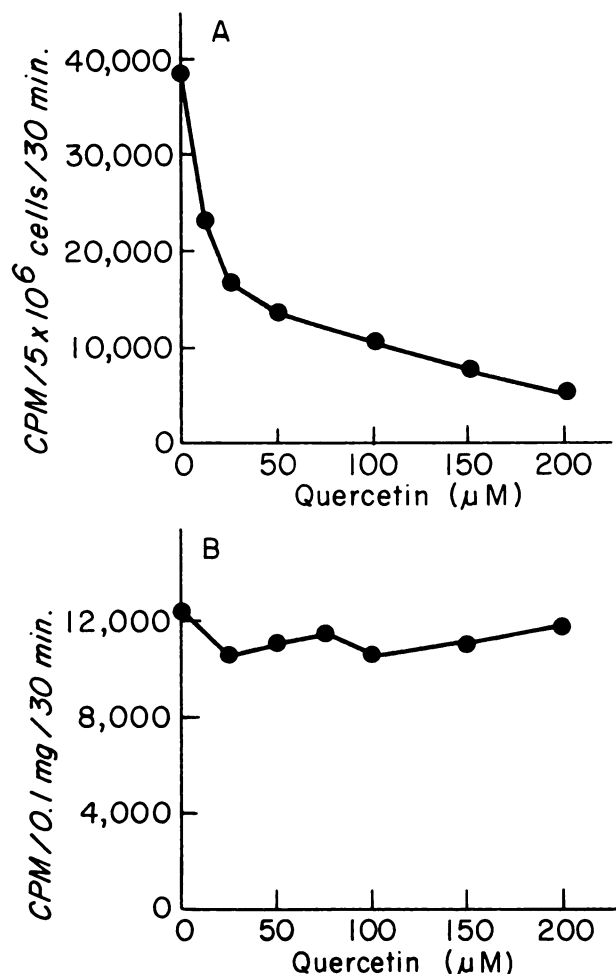


Fig. 6. The effect of quercetin concentration on iodination of preopsonized zymosan. (A) Iodination by intact cells; (B) iodination by a crude granule fraction derived from intact cells. The experiment illustrated is representative of two separate experiments. Each point represents the mean of triplicate determinations.

enzyme activity was measured over a range of pH with patient and control samples prepared and assayed in parallel. Results are shown in Fig. 7. In the case of patient 1, the ATPase activity was somewhat higher than in the control cells, while in the case of patient 2, the two curves were virtually identical. In neither case was there any evidence for a deficiency of this enzyme in CGD.

DISCUSSION

Harlan et al.¹ and Smolen and Weissman have previously described the presence of an ecto Mg²⁺-ATPase on the plasma membrane of the human neutrophil. In an attempt to elucidate the function of this enzyme, we employed quercetin, which has been reported to be a potent inhibitor of ATPase in many systems.^{9,10} At least three different effects were noted.

First, quercetin was repeatedly shown to inhibit human PMNL Mg²⁺-ATPase in either intact cells or a cell homogenate. The inhibition was dose-dependent with a maximal inhibition of 60%–85% achieved at a concentration of approximately 100 μM (Fig. 1). These observations are in contrast to those of Smolen and Weissman who reported in a summary table that concentrations of this compound from 30 to 300 μM were without significant effect on the neutrophilic enzyme.² The reasons for this discrepancy are not clear. It should be noted that Berton et al.¹⁵ observed inhibition of a guinea pig ATPase by quercetin, but the magnitude of inhibition was somewhat less than that observed in the present study. The kinetics of inhibition are illustrated in Fig. 2. The substrate

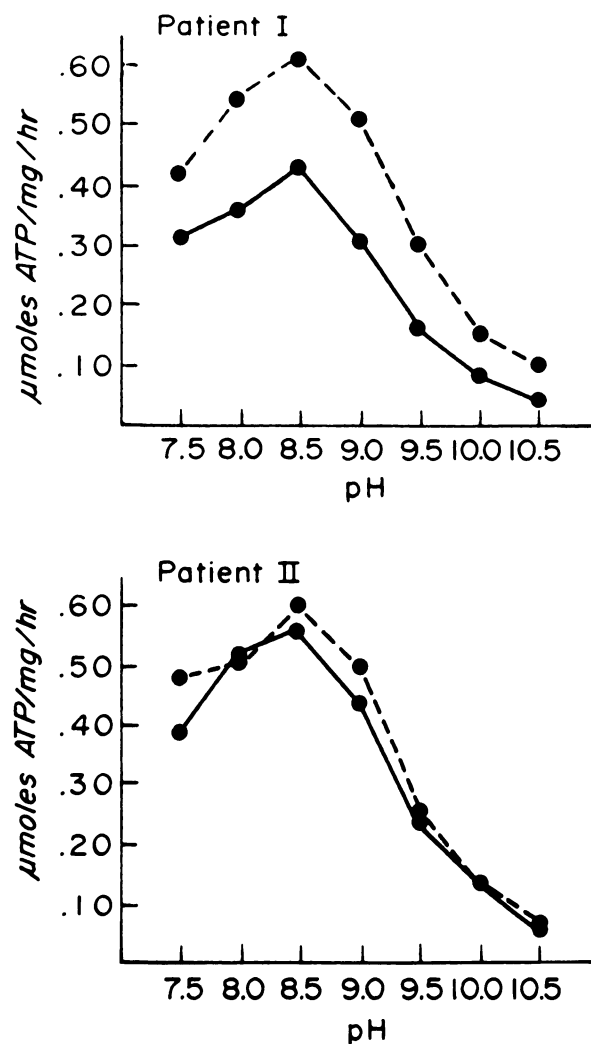


Fig. 7. Leukocyte Mg²⁺-ATPase activity as a function of pH in homogenates from two patients with chronic granulomatous disease and two normal controls. Dotted line, patient samples; solid line, control samples. Each value was determined in duplicate in each experiment.

inhibition observed at high concentrations of ATP is consistent with that observed by Smolen and Weissman.² In the absence of quercetin, the Mg^{2+} -ATPase has an apparent K_m of approximately 0.11 mM and a V_{max} of 0.17 μ mole ATP/hr/mg. This value for the K_m is in reasonable agreement with that reported by Smolen and Weissman² and is substantially lower than that previously reported by us.¹ In five separate experiments, the K_m of the uninhibited reaction varied between 0.052 mM and 0.12 mM with a mean of 0.074 mM. The inhibition by quercetin appears to be uncompetitive with alterations in both V_{max} and K_m . This is similar to the inhibition of Na^+, K^+ -ATPase by quercetin, which was also observed to be uncompetitive.⁹ The data in Fig. 2 reinforce the observation that 100 μ M quercetin is, indeed, an inhibitor of the ATPase of the human PMNL.

Second, quercetin inhibited the stimulated respiratory burst as measured by either glucose oxidation, oxygen consumption, or iodination of opsonized zymosan. This did not appear to be related to an inhibition of phagocytosis since the PMA-induced responses were likewise affected and quercetin had only a minimal effect on the uptake of radiolabeled bacteria. These observations suggest that the compound somehow interferes with the activation of the enzyme responsible for initiation of the respiratory burst, i.e., NADPH oxidase. These observations are in essential agreement with those of Berton et al.¹⁵ who demonstrated that bioflavonoids, including quercetin, inhibited

oxygen consumption by neutrophils in a stimulus-specific manner.

The similar inhibition of Mg^{2+} -ATPase activity and of the respiratory burst by quercetin raised the possibility that the two might be linked. The observation that neutrophils from patients with chronic granulomatous disease have normal levels of Mg^{2+} -ATPase suggests that this may not be the case. This is further substantiated by the experiments of Smolen and Weissman,² who demonstrated that the enzyme activity was unchanged by phagocytosis; these observations were confirmed in our own laboratory (unpublished observations). Further, Smolen and Weissman observed normal superoxide production under conditions where ATPase activity was substantially inhibited by suramin.²

Finally, we demonstrated that quercetin is a potent inhibitor of hexose transport. This is consistent with other reports in which quercetin has been shown to inhibit glucose transport in human erythrocytes,¹² rat lymphocytes,¹³ and cultured human fibroblasts.¹⁴

The diverse effects of quercetin, i.e., inhibition of an ecto-enzyme, of glucose transport, and of the respiratory burst activation, would seem to be most compatible with the hypothesis that quercetin exerts a generalized effect on the cell membrane, as suggested recently by Berton et al.¹⁵

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