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An adenosine triphosphate-dependent calcium uptake pump in human neutrophil lysosomes.

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Research Article

Regulation of the cytosolic free calcium concentration is important to neutrophil function. In these studies, an ATP-dependent calcium uptake pump has been identified in human neutrophil lysosomes. This energy-dependent Ca++ uptake pump has a high affinity for Ca++ (Michaelis constant [Km] Ca++ = 107 nM) and a maximum velocity (Vmax) of 5.3 pmol/mg of protein per min. ATP was the only nucleotide that supported Ca++ uptake by lysosomes. The Km for ATP was 177 microM. ATP-dependent Ca++ uptake by neutrophil lysosomes was temperature- and pH-sensitive with optimal Ca++ pump activity at 37 degrees C and pH 7.0-7.5. Mg++ was also essential for ATP-dependent Ca++ uptake by lysosomes. Azide and antimycin A had no effect on the energy-dependent uptake of Ca++ by neutrophil lysosomes. The chemotactic peptide formyl-methionyl-leucyl-phenylalanine inhibited ATP-dependent Ca++ accumulation by isolated lysosomes. Butoxycarbonyl-phenylalanine-leucine-phenylalanine-leucine-phenylalanine , a competitive antagonist of the chemotactic peptide, blocked this inhibitory effect. These studies demonstrate the presence of an ATP-dependent Ca++ uptake pump in human neutrophil lysosomes that functions at physiologic intracellular concentrations of Ca++, ATP, and H+ and may be important to regulating neutrophil function by modulating cytosolic Ca++.

An Adenosine Triphosphate-dependent Calcium Uptake Pump in Human Neutrophil Lysosomes

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Abstract

Regulation of the cytosolic free calcium concentration is important to neutrophil function. In these studies, an ATP-dependent calcium uptake pump has been identified in human neutrophil lysosomes. This energy-dependent Ca^{++} uptake pump has a high affinity for Ca^{++} (Michaelis constant [K_m] Ca^{++} = 107 nM) and a maximum velocity (V_{max}) of 5.3 pmol/mg of protein per min. ATP was the only nucleotide that supported Ca^{++} uptake by lysosomes. The K_m for ATP was 177 μM. ATP-dependent Ca^{++} uptake by neutrophil lysosomes was temperature- and pH-sensitive with optimal Ca^{++} pump activity at 37°C and pH 7.0–7.5. Mg^{++} was also essential for ATP-dependent Ca^{++} uptake by lysosomes. Azide and antimycin A had no effect on the energy-dependent uptake of Ca^{++} by neutrophil lysosomes. The chemotactic peptide formyl-methionyl-leucyl-phenylalanine inhibited ATP-dependent Ca^{++} accumulation by isolated lysosomes. Butoxycarbonyl-phenylalanine-leucine-phenylalanine-leucine-phenylalanine, a competitive antagonist of the chemotactic peptide, blocked this inhibitory effect. These studies demonstrate the presence of an ATP-dependent Ca^{++} uptake pump in human neutrophil lysosomes that functions at physiologic intracellular concentrations of Ca^{++}, ATP, and H^+ and may be important to regulating neutrophil function by modulating cytosolic Ca^{++}.

Introduction

The intracellular free Ca^{++} concentration is widely recognized to influence stimulus–response coupling in numerous eukaryotic and prokaryotic cells. For polymorphonuclear leukocytes (PMN),[1] changes in cytoplasmic free Ca^{++} concentration follow stimulation with ionophores and chemotactic peptides and have been implicated in control of PMN migration, phagocytosis, degranulation, and superoxide generation (1–5). Ca^{++} is highly suited to regulate such functions because the concentration of the free ion in the cytosol is maintained at an exceedingly low level (10^{-7} M, [3–6]) compared to other cations in the cytosol or compared to the Ca^{++} concentration in extracellular fluid ($\sim 10^{-3}$ M). Recent attention has focused on the presence of Ca^{++} pumps in the plasma membranes of PMN and other cells that serve to maintain the low cytoplasmic free Ca^{++} concentration by extruding Ca^{++} (7–11). In many other cells, a major contribution to maintenance of the low cytosolic free Ca^{++} concentration also comes from Ca^{++} pumps that compartmentalize Ca^{++} within intracellular organelles such as mitochondria and microsomes (12–14). PMN have few mitochondria and sparse endoplasmic reticulum and the major intracellular organelles within PMN are lysosomes. Therefore, studies were performed to determine whether these organelles might contribute to intracellular Ca^{++} homeostasis by containing an energy-dependent Ca^{++} pump that would serve to compartmentalize Ca^{++} within lysosomes. This report demonstrates and characterizes, for the first time, an ATP-dependent Ca^{++} uptake pump in lysosomes of human PMN.

Methods

Chemicals. ATP, ADP, ITP, GTP, UTP, CTP, hexokinase, antimycin A, 4-methylumbelliferylsulfate, and 4-methylumbelliferone, formyl-methionyl-leucyl-phenylalanine (FMLP), and ionophore A23187 were obtained from Sigma Chemical Co., St. Louis, MO; ^{45}Ca (as $CaCl_2$) from New England Nuclear, Boston, MA; Hydrofluor from National Diagnostics Inc., Somerville, NJ; sodium azide from Mallinckrodt Chemical Co., St. Louis, MO; butoxycarbonyl(BOC)-phenylalanine-leucine-phenylalanine-leucine-phenylalanine (BOC-Phe-Leu-Phe-Leu-Phe) from Peninsula Laboratories, Inc., Belmont, CA; silicone oil (type SF1250) from General Electric Co., Waterford, NY; and Percoll from Pharmacia Fine Chemicals, Piscataway, NJ.

Isolation of lysosomes. Human PMN were isolated from healthy, adult volunteers by Ficoll-Hypaque gradient centrifugation and dextran sedimentation according to the method of Boyüm (15). Two methods were used to isolate lysosomes. In the first method, lysosomes were isolated from PMN after cell disruption by nitrogen cavitation and differential centrifugation as described in detail elsewhere (16). Briefly, PMN suspended in modified Hanks' balanced salt solution (2×10^7/ml, pH 7.4) containing 2.5 mM $MgCl_2$ but without added Ca^{++} were equilibrated at 350 psi of N_2 for 20 min at 4°C in a cell disruption bomb (Artisan Industries, Waltham, MA). After release from the cavitation bomb, the suspension was collected into an EDTA (trisodium salt) solution so that the final concentration of EDTA was 2.5 mM. After an initial centrifugation (1.0×10^4 *g*/min, 4°C) to sediment undisrupted cells, nuclei, and mitochondria, the supernatant was centrifuged (4.5×10^5 *g*/min, 4°C) and the lysosome-containing pellet isolated. The undisturbed pellet was washed three times in ice-cold transport buffer composed of KCl 100 mM, $MgCl_2$ 5 mM, Hepes 20 mM, and NaCl 20 mM, pH 7.2, and finally resuspended in this buffer at a protein concentration of 100 μg/ml. Protein was determined by the method of Bradford (17) with bovine serum albumin as standard. In some experiments, $MgCl_2$ was omitted from the transport buffer.

In separate experiments, PMN lysosomes were isolated and frac-

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1. *Abbreviations used in this paper:* BOC, butoxycarbonyl; ER, endoplasmic reticulum; FMLP, formyl-methionyl-leucyl-phenylalanine; PMN, polymorphonuclear leukocyte(s).

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tionated according to the method of Borregaard et al. (18), modified as follows. PMN, suspended in ice-cold "relaxation buffer" containing KCl 100 mM, NaCl 3 mM, ATP 1 mM, $MgCl_2$ 3.5 mM, piperazine-*N*-*N*'-bis 2-ethane-sulfonic acid 10 mM, pH 7.4, were disrupted by N_2 cavitation (350 psi, 20 min 4°C) and the cavitate was collected into EGTA (final concentration, 1.25 mM). After removal of nuclei and unbroken cells by centrifugation at 500 *g* for 10 min at 4°C, the supernatant was decanted and centrifuged at 15,000 *g* for 20 min at 4°C to sediment the lysosome-containing pellet. This fraction was resuspended in "relaxation buffer" and loaded onto discontinuous Percoll gradients prepared as described in reference 18. Two distinct bands (α and β, in order of decreasing density) were identified after centrifugation of the loaded discontinuous Percoll gradients at 48,000 *g* for 15 min at 4°C. No other visible bands were observed in these gradients. Material forming the α-band, previously identified as the azurophil granules (18), and β-band (specific granules contaminated by ~10% of the azurophil granules) were isolated by aspiration. In some preparations, Percoll was removed from these fractions by centrifugation at 170,000 *g* for 15 min, and the lysosomes, which layered on a hard-packed pellet of Percoll, were aspirated and suspended in transport buffer at a final protein concentration of 100 μg/ml. Sulfatase C activity, a marker for endoplasmic reticulum, was measured in the cavitate, the resuspended lysosome suspension, and the α- and β-bands from the Percoll gradient according to the method of Canonico et al. (19). The assay of sulfatase C activity depends on the fluorometric measurement of the conversion of 4-methylumbelliferylsulfate to 4-methylumbelliferone under conditions which inhibit sulfatase A and sulfatase B activity (19). A sham Percoll gradient was run in parallel with the lysosome-containing gradient, and sulfatase C activity was measured in Percoll fractions of comparable density to the α- and β-bands.

Calcium uptake studies. Calcium uptake by lysosomes was measured in the transport medium supplemented with varying concentrations of ATP and $CaCl_2$ as follows. Lysosomes suspended in medium at 100 μg of protein/ml were prewarmed to 37°C for 5–10 min and agitated, and the reaction was started by the addition of prewarmed $CaCl_2$ (usually 100 μM containing 1 μCi/ml of ^{45}Ca) and prewarmed ATP solution (final concentration usually 1 mM). The pH of all solutions was preadjusted to 7.2 except where otherwise noted. The suspension was agitated and incubated at 37°C. At intervals, 1.0-ml aliquots were removed and layered over 0.5 ml of silicone oil (type SF1250) in microcentrifuge tubes (Beckman Instruments, Inc., Fullerton, CA). Lysosomes were rapidly sedimented from the medium by centrifugation at 12,000 *g* for 3 min at room temperature as previously described (20). A 10-μl aliquot of the supernatant was removed from each sample, placed in 10 ml of scintillation fluid (Hydrofluor), and counted for ^{45}Ca radioactivity in a liquid scintillation counter (Beckman Instruments, Inc.). The remainder of the supernatant and the top 0.3 ml of silicone oil were aspirated and discarded, and the entire lysosome pellet was harvested by cutting off the pellet containing portion of the microcentrifuge tube. The lysosome pellet was placed in 10 ml of liquid scintillation fluid and counted for ^{45}Ca radioactivity. In that initial experiments demonstrated that Ca^{++} uptake by lysosomes did not occur in the absence of ATP (see Fig. 1), Ca^{++} uptake rates are expressed as nanomoles of Ca^{++} per milligram of protein per minute after subtracting the ^{45}Ca radioactivity in the lysosome pellet in the absence of ATP.

Because endoplasmic reticulum (ER) and mitochondria both contain ATP-dependent Ca^{++} uptake pumps (12–14) and contaminate the lysosome suspension before Percoll gradient centrifugation (16), additional studies were done to determine whether ATP-dependent Ca^{++} uptake by the unfractionated lysosomes could be attributed to these organelles. Sedimentation of ER through the silicone oil layer was monitored by measurement of NADH-oxidoreductase activity (21) in the lysosome suspension and in the supernatant (above the oil layer) and pellet of a lysosome suspension centrifuged through silicone oil. In parallel, ATP-dependent Ca^{++} uptake was measured in the lysosome suspension and in the lysosome pellet obtained after sedimentation through silicone oil. Because Percoll interfered with the measurement of NADH-oxidoreductase activity, in experiments that utilized Percoll gradient-fractionated lysosomes, sulfatase C activity was measured in the various fractions as described above. The effects of sodium azide (5 mM) and antimycin A (5×10^{-7} M), which completely inhibit the mitochondrial electron transport chain and Ca^{++} uptake pump activity (12, 22), on ATP-dependent Ca^{++} uptake by the lysosome suspension were also determined.

The free Ca^{++} concentration of solutions in the presence of ATP and EGTA was calculated from the contaminating Ca^{++}, measured with a Ca^{++} sensitive electrode (model F2112; Radiometer, Inc., Copenhagen, Denmark), plus the amount of Ca^{++} added, using the computer program of Perrin and Sayce (23) with the following logarithmic association constants for metals and H^+ to EGTA: H^+ to $EGTA^{4-}$, 9.46; H^+ to $H\ EGTA^{3-}$, 8.85; H^+ to H_2EGTA^{2-}, 2.68; H^+ to H_3EGTA^{1-}, 2.0; Ca^{++} to $EGTA^{4-}$, 11.0; Ca^{++} to $H\ EGTA^{3-}$, 5.33; Mg^{++} to $EGTA^{4-}$, 5.21; Mg^{++} to $H\ EGTA^{3-}$, 3.37. Kinetic parameters (Michaelis constant [K_m] and maximum velocity [V_{max}]) were determined from double reciprocal plots drawn by computer-assisted least squares analysis. In addition to characterizing the kinetic parameters of Ca^{++} uptake by PMN lysosomes as to [Ca^{++}] and [ATP], the effects of varying temperature, pH, [Mg^{++}], and nucleotides were also examined.

Effect of FMLP on ATP-dependent Ca^{++} accumulation by lysosomes. Lysosomes suspended in transport medium at 100 μg of protein/ml were warmed to 37°C to 5–10 min in the presence and absence of varying concentrations of FMLP. Calcium uptake was initiated by the addition of prewarmed $CaCl_2$ (containing ^{45}Ca) and ATP as described for the standard Ca^{++} uptake studies. The effect of the chemotactic peptide antagonist BOC-Phe-Leu-Phe-Leu-Phe, alone or in combination with FMLP, was also assessed. Lysosomes were warmed to 37°C for 5 min in the presence and absence of BOC-Phe-Leu-Phe-Leu-Phe (10^{-5}) followed by the addition of prewarmed transport medium or FMLP (10^{-10}). After 5 min at 37°C, Ca^{++} uptake was initiated by adding prewarmed $CaCl_2$ and ATP.

To determine whether Ca^{++} leakage from the lysosomes was affected by FMLP treatment, lysosomes were allowed to accumulate $CaCl_2$ (containing ^{45}Ca) for 10 min at 37°C in the presence of ATP (standard uptake assay). At the plateau of Ca^{++} uptake, prewarmed EGTA (2.5 mM, pH 7.2) with or without FMLP (10^{-10} M) was added to the lysosome suspension and lysosome-associated $^{45}Ca^{++}$ was determined at intervals.

Additional experiments were performed to determine whether FMLP accelerated the efflux of Ca^{++} from the lysosomes against a concentration gradient. At the plateau of active Ca^{++} uptake by the lysosomes, ATP was effectively removed from the medium by the addition of glucose (5 mM) and hexokinase (20 U/ml) (24). At intervals, the amount of ^{45}Ca retained by the lysosomes in the presence and absence of FMLP (10^{-10} M) was determined.

Results

The uptake of Ca^{++} by isolated human PMN lysosomes in the presence and absence of ATP (1 mM) is shown in Fig. 1. In the absence of ATP, 0.4±0.1 nmol of Ca^{++}/mg of protein was associated with the lysosome pellet during the first 2–5 min of incubation. There was no further association of Ca^{++} with the lysosome pellet during the 30-min incubation. In the presence of ATP and $MgCl_2$ (5 mM), there was a rapid uptake of Ca^{++} by the lysosomes which was almost linear over the first 5 min. Ca^{++} uptake continued at a slower rate over the next 5 min. As shown in Fig. 2, ATP-dependent Ca^{++} uptake by lysosomes required Mg^{++}. In the absence of Mg^{++}, Ca^{++} uptake by the lysosomes was similar to Ca^{++} uptake in the absence of ATP. That the uptake of Ca^{++} by lysosomes in the presence of ATP

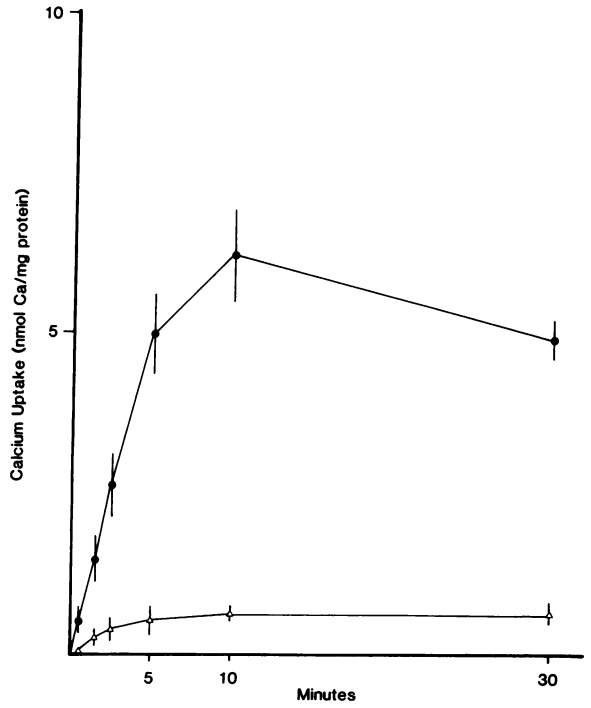

Figure 1. Ca uptake by human neutrophil lysosomes. Lysosomes were suspended in transport buffer (KCl 100 mM, NaCl 20 mM, $MgCl_2$ 5 mM, Hepes 20 mM, pH 7.2) containing 100 μM $CaCl_2$ with 1 μCi/ml of ^{45}Ca. Ca uptake was measured at 37°C in the presence (●) and absence (△) of ATP (1 mM). Results are the mean±SEM of seven experiments.

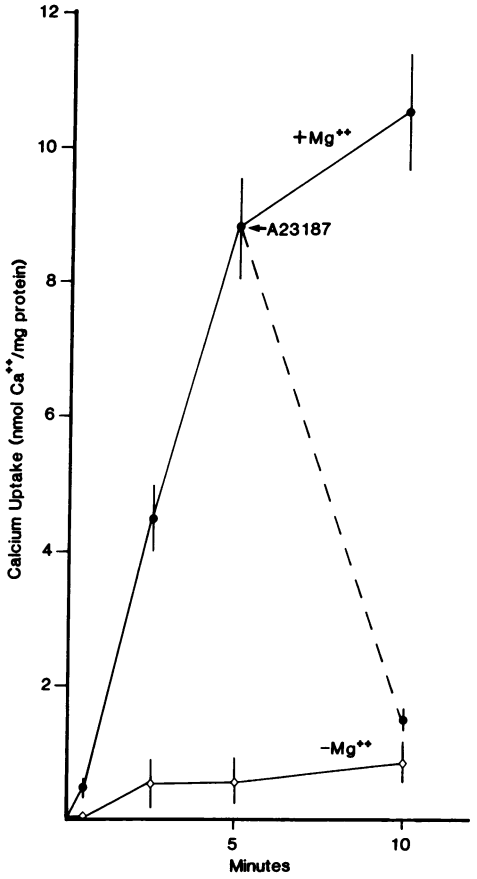

Figure 2. Mg^{++} requirement and effect of A23187 on Ca uptake by lysosomes. ATP (1 mM)-dependent Ca uptake by lysosomes was measured in the same transport medium as Fig. 1 with (——) and without (◇) added $MgCl_2$ (5 mM). At 5 min, A23187 (5 μM) was added to the complete incubation mixture (-----). Results are the mean±SEM of four experiments.

and $MgCl_2$ was against an electrochemical gradient is also shown in Fig. 2. Addition of the Ca^{++} ionophore A23187 (5 μM) after 5 min of Ca^{++} uptake by the lysosomes caused a rapid release of Ca^{++} to near basal levels.

To examine further whether the ATP-dependent uptake of Ca^{++} could be attributed to the lysosomes and not to the small amount of contaminating ER, the ER marker enzyme NADH-oxidoreductase was measured in the organelle suspension before and after passage over silicone oil. Almost all of the ER was retained above the silicone oil layer. In four separate experiments, the specific activity of NADH-oxidoreductase (expressed as micromoles of NADH oxidized per milligram of protein per minute) was 2.4±0.1, 11.3±1.7, and 1.1±0.2 in the lysosome suspension, layer above the silicone oil, and lysosome pellet below the silicone oil layer, respectively. Of the total protein in the lysosome suspension, 10.6±2.5% was retained above the silicone oil layer (n = 4). In contrast, depletion of contaminating ER from the lysosome suspension by centrifugation through silicone oil had no significant effect on ATP-dependent Ca^{++} uptake activity (1.20±0.15 nmol of Ca^{++}/mg of protein per min in the lysosome suspension vs. 1.19±0.19 nmol of Ca^{++}/mg of protein per min in lysosomes obtained after centrifugation through silicone oil; n = 2).

The effects of two inhibitors of mitochondrial respiration and ion transport on the energy-dependent uptake of Ca^{++} by the lysosomes were also examined. Sodium azide (5 mM) had no effect on ATP-dependent Ca^{++} uptake by human PMN lysosomes (0.89±0.17 nmol of Ca^{++}/mg of protein per min without azide vs. 0.81±0.21 nmol of Ca^{++}/mg of protein per min with azide; n = 2). Antimycin A (5×10^{-7} M), which inhibits mitochondrial electron transfer at a different site than sodium azide, also had no effect on ATP-dependent uptake of Ca^{++} by the lysosomes (0.82±0.22 nmol of Ca^{++}/mg of protein per min without antimycin A vs. 0.82±0.16 nmol of Ca^{++}/mg of protein per min with antimycin A).

A second method was also used to isolate PMN lysosomes which permits fractionation of the lysosomes into azurophil (α-band) and specific (β-band) granules, removes any contaminating mitochondrial marker enzyme (18), and, as shown below, removes contaminating ER. As previously reported for the mitochondrial marker cytochrome c oxidase (18), no monamine oxidase activity was detected in the Percoll gradient fractions (n = 3, data not shown). While the activity of the ER marker enzyme, sulfatase C, was low in comparison to cells that synthesize protein (19), it was detected in the cavitate of the PMN suspension and in the resuspended, unfractionated lysosome suspension. No latency for the sulfatase C activity could be detected in either the cavitate or the lysosome suspension. The whole cell cavitate from 1×10^9 cells converted 75.2±3.6 nmol of 4-methylumbelliferylsulfate to 4-methylum-

belliferone after 20 min of incubation. The unfractionated lysosome suspension contained 29.9% of the whole cell cavitate sulfatase C activity (22.5 nmol of 4-methylumbelliferone generated after 20 min of incubation). By using glucose-6-phosphatase activity as another ER marker enzyme, we previously found that the lysosome suspension contained 20.0% of the cell cavitate activity (16). In contrast, both the azurophil (α-band) and specific (β-band) lysosomes recovered from the discontinuous Percoll gradients contained no detectable sulfatase C activity (n = 3). Sulfatase C activity was not inhibited by the presence of Percoll and the fluorescent signal from 4-methylumbelliferone was actually increased from 10 to 16% in its presence. Because the total sulfatase C activity in the cell cavitate and lysosome suspension was low and no other bands were visible in the Percoll gradients (n = 3) (18), the locale of the ER could not be determined with certainty and ER could not be recovered from the gradients.

Unlike the complete removal of sulfatase C activity from the alpha and beta Percoll gradient fractions, ATP-dependent Ca^{++} uptake was clearly present in both lysosome fractions. In a representative experiment, the ATP-dependent Ca^{++} uptake activity in the unfractionated lysosome suspension was 0.72 nmol of Ca^{++}/mg of protein per min, 1.67 nmol of Ca^{++}/mg of protein per min in the azurophil granules, and 1.16 nmol of Ca^{++}/mg of protein per min in the specific granules. While there was invariably energy-dependent Ca^{++} uptake by both azurophil and specific granules (n = 4), the specific activity for Ca^{++} uptake was variable largely owing to incomplete removal of Percoll from the gradient-fractionated lysosomes. Incubation of unfractionated lysosomes with Percoll had no direct effect on Ca^{++} uptake activity (data not shown), however, residual Percoll in the gradient-fractionated lysosomes interfered with the complete recovery of lysosomes after centrifugation through silicone oil.

The kinetic parameters for Ca^{++} uptake by unfractionated lysosomes at various free Ca^{++} concentrations are shown in Fig. 3. The K_m for Ca^{++} was 107 nM and the V_{max} was 5.3 pmol of Ca^{++}/mg of protein per min.

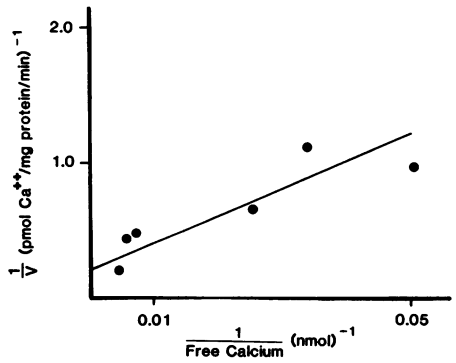

Figure 3. Lineweaver–Burk plot of Ca uptake by lysosomes at various free Ca concentrations. Free Ca concentrations (20–400 nM) in transport buffer (see Fig. 1) were adjusted by the addition of various concentrations of EGTA according to the measurements and calculations described in the text. Results are the mean of four experiments. K_m = 107 nM; V_{max} = 5.3 pM.

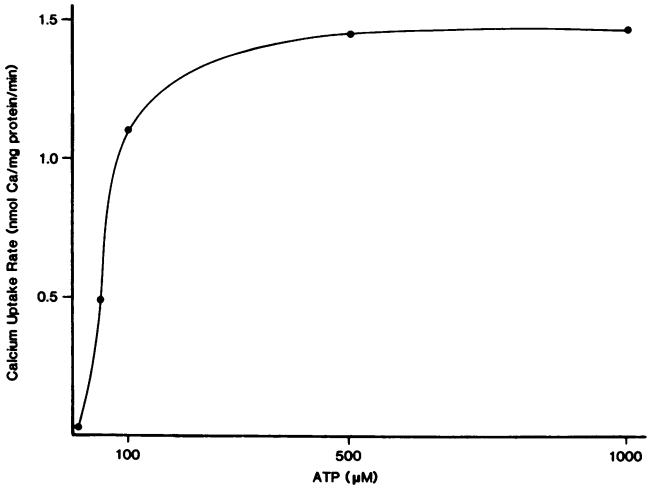

Figure 4. Ca uptake rate by lysosomes at various ATP concentrations. Uptake of Ca^{++} was measured in transport buffer (Fig. 1) supplemented with ATP: 25, 50, 100, 500, and 1000 μM. Results are the mean of three experiments.

The Ca^{++} uptake rate by lysosomes at various ATP concentrations is shown in Fig. 4. The double reciprocal plot of these data revealed a K_m for ATP of 177 μM.

As shown in Table I, the nucleotide specificity for support of Ca^{++} uptake by lysosomes was exclusive for ATP. None of the other nucleotides (ADP, UTP, CTP, GTP, or ITP) resulted in Ca^{++} uptake >2% of that supported by ATP.

Temperature and pH had profound effects on the ATP-dependent Ca^{++} uptake by lysosomes. At 4°C, no Ca^{++} uptake was detectable and at 25°C, Ca^{++} uptake was 41% of the uptake observed at 37°C. Fig. 5 shows the effects of pH on Ca^{++} uptake by lysosomes. The optimal pH for Ca^{++} uptake was between 7.0 and 7.5. At very acidic pH (5.5), Ca^{++} pump activity was 29% of maximal and 72% of maximal activity at very alkaline pH (8.0).

FMLP inhibition of ATP-dependent Ca^{++} accumulation by lysosomes. FMLP inhibited ATP-dependent Ca^{++} accumulation

Table I. Nucleotide Specificity for Calcium Uptake by Human Neutrophil Lysosomes

Nucleotide	% activity
ATP	100.0±6.6
ADP	0±0
UTP	1.7±1.2
CTP	1.0±0.4
GTP	0±0
ITP	0±0

Incubation conditions and measurements of Ca^{++} uptake were as described in Methods. All nucleotides were present at a final concentration of 1 mM. Activity is expressed as the percentage (mean±SEM) of activity in the presence of ATP from two separate experiments, each run in duplicate.

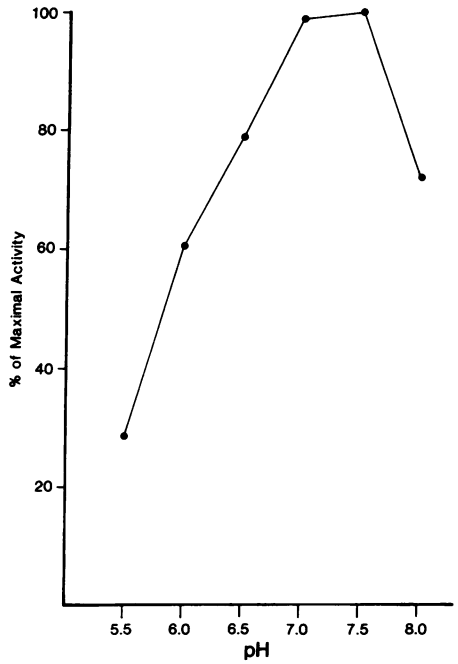

Figure 5. Effect of pH on ATP-dependent Ca uptake by lysosomes. Incubation conditions were as described in Fig. 1, with the pH adjusted at 37°C as indicated. Results are the mean of three experiments.

by lysosomes in a dose-dependent fashion (Fig. 6). Lysosomal accumulation of Ca^{++} was inhibited to a similar degree whether lysosomes were treated with FMLP for 5 or 10 min. There was no effect of FMLP (10^{-6} M) on the amount of $^{45}Ca^{++}$ associated with the lysosome pellet in the absence of ATP

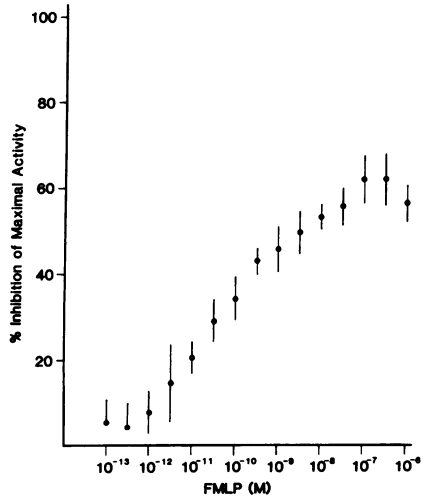

Figure 6. Effect of FMLP on ATP-dependent Ca^{++} accumulation by lysosomes. Lysosomes in transport buffer were incubated for 5–10 min at 37°C in the absence and presence of varying concentrations of FMLP. Uptake of Ca^{++} was then measured as in Fig. 1. Results are the mean±SEM of three to nine separate experiments.

Table II. BOC-Phe-Leu-Phe-Leu-Phe Blocks the Inhibitory Effect of FMLP on ATP-dependent Ca^{++} Uptake by Lysosomes

Addition	Activity
	nmol of Ca^{++} mg of protein per min
None	1.04±0.07
FMLP (10^{-10} M)	0.49±0.10
BOC-Phe-Leu-Phe-Leu-Phe (10^{-5} M)	1.08±0.17
BOC-Phe-Leu-Phe-Leu-Phe (10^{-5} M) + FMLP (10^{-10} M)	1.15±0.14

Incubations and Ca^{++} uptake were performed as described in Methods. Results are the mean±SEM of three experiments, each run in duplicate.

(0.26±0.08 nmol of Ca^{++}/mg of protein vs. 0.23±0.10 nmol of Ca^{++}/mg of protein in the absence and presence of FMLP, respectively, after a 5-min incubation, n = 9). Table II shows the ability of BOC-Phe-Leu-Phe-Leu-Phe to block the inhibitory effect of FMLP on ATP-dependent Ca^{++} accumulation by the lysosomes. The antagonist alone had no significant effect on accumulation of Ca^{++} by lysosomes.

As shown in Fig. 7, the addition of sufficient EGTA to chelate virtually all the extralysosomal Ca^{++} ($[Ca^{++}]_o$ < 1 nM) at the plateau of ATP-dependent Ca^{++} uptake, promoted an efflux of Ca^{++} down its concentration gradient. FMLP (10^{-10} M) did not enhance the efflux of Ca^{++} from the lysosomes, indicating that the effect of FMLP was not to render the lysosomes relatively more permeable to calcium.

Fig. 8 shows the effect of FMLP on Ca^{++} efflux from the

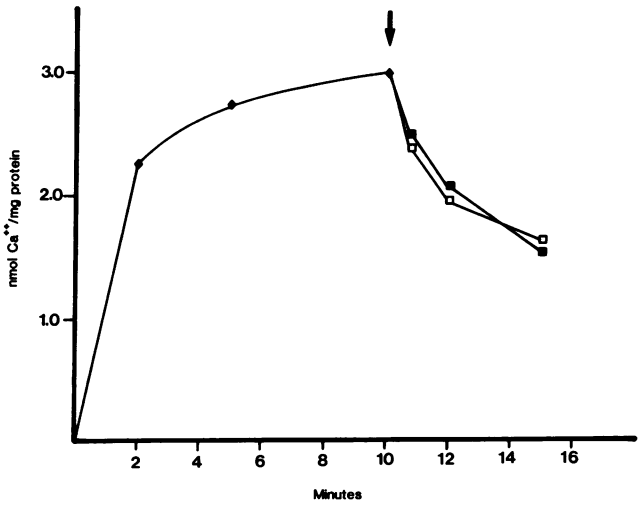

Figure 7. Effect of FMLP on lysosomal permeability to Ca^{++}. At the plateau of Ca^{++} uptake by lysosomes (10 min, *arrow*), EGTA (2.5 mM, pH 7.2) was added with (□) or without (■) FMLP (10^{-10} M). Under these conditions, extralysosomal $[Ca^{++}]$ was reduced to <1.0 nM. At intervals, lysosome-associated $^{45}Ca^{++}$ was measured. Results are the mean of three experiments.

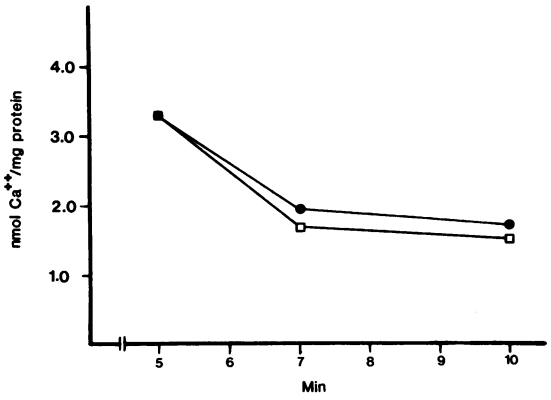

Figure 8. Effect of FMLP on efflux of calcium from lysosomes. After 5 min of active Ca^{++} uptake by lysosomes under standard assay conditions (see Fig. 1), the concentration of ATP was effectively reduced by the addition of glucose (5 mM) and hexokinase (20 U/ml) with (□) or without (●) FMLP (10^{-10} M). Lysosome-associated $^{45}Ca^{++}$ was measured 2 and 5 min after the additions. Results are the mean of three experiments.

lysosomes when the extralysosomal Ca^{++} concentration was not changed. FMLP (10^{-10} M) added at the plateau of Ca^{++} uptake and after lowering the ATP concentration by the addition of glucose and hexokinase did not increase Ca^{++} efflux from the lysosomes, indicating that the inhibition of Ca^{++} accumulation by lysosomes treated with FMLP is the result of inhibited Ca^{++} influx.

Discussion

This report describes the presence of an ATP-dependent Ca^{++} uptake pump in isolated human PMN lysosomes that pumps Ca^{++} against an electrochemical gradient, requires Mg^{++}, and operates at Ca^{++}, ATP, and H^+ concentrations relevant to the cytoplasmic environment. That the Ca^{++} uptake pump activity is attributable to lysosomes and not to contaminating subcellular organelles is supported by several lines of evidence. First is the purity of the lysosome fraction obtained by disrupting PMN by nitrogen cavitation and separating organelles by differential centrifugation. By using ouabain inhibitable Na^+-K^+ ATPase and 5-nucleotidase activities as markers for the plasma membrane, we have previously shown that this preparation of lysosomes is entirely free of contaminating plasma membrane vesicles (16). Moreover, plasma membrane vesicles, which are less dense than ER (16), do not sediment through silicone oil (type SF1250). Mitochondria, which have an active Ca^{++} uptake pump, are sparse in PMN and none are seen in electron micrographs of the lysosome preparation. Almost all of the few mitochondria that are present in the PMN sediment in a denser fraction during the lysosome isolation. Furthermore, concentrations of sodium azide and antimycin A, which completely inhibit mitochondrial electron transport and Ca^{++} pump activity, have no effect on the ATP-dependent uptake of Ca^{++} by lysosomes (13, 22). ER, which has an energy-dependent Ca^{++} uptake pump in several cell types (14, 25), is also quite sparse in PMN. Moreover, centrifugation of the lysosome suspension through silicone oil resulted in depletion of >90% of these low-density microsomes from the lysosome suspension but had no effect on the ATP-dependent Ca^{++} uptake activity of the reisolated lysosomes.

A second recently described method was also used to isolate and fractionate PMN lysosomes into the two major granule classes (18). As previously reported, the mitochondrial marker cytochrome *c* oxidase was not present in the α- or β-bands recovered from the discontinuous Percoll gradient. Although activity of the nonlatent ER marker enzyme, sulfatase C, was present in the whole cell cavitate and the lysosome-enriched fraction, no sulfatase C activity was located in the visible bands from the gradients. In that the density of ER, determined in this buffer system by sedimentation in Percoll gradients, is ~1.06, and the mean density of azurophil and specific granules is 1.13 and 1.09, respectively (26), it would be predicted that the small amount of ER would sediment above the B band. Despite this anticipation, a layer of organelles sedimenting to this density was not observed in these Percoll gradients and could not be detected by spectrophotometric monitoring of the optical density of the gradient fractions in the original report (18). This is not surprising in that ER is very sparse in PMN and in that the total sulfatase C activity in the cavitated PMN was <2% of the activity in cultured macrophages (19) and <1% of the activity in hepatocytes (27). Although ER was removed from the unfractionated lysosome suspension by passage over the Percoll gradients, ATP-dependent Ca^{++} uptake activity was present in both α- and β-granules. These data indicate that both azurophil and specific granules contain an active Ca^{++} uptake pump. Further study will be required to determine whether these pumps differ in their kinetic and functional behavior.

The role of this Ca^{++} pump in PMN function requires considerable further study. However, several recent observations suggest that it may have a major influence on cytoplasmic Ca^{++} homeostasis, which influences each of the functional responses of PMN. We and others have demonstrated that the acute rise in cytosolic free Ca^{++} that follows PMN activation with some soluble stimuli (e.g., the chemotactic peptide, FMLP) results from both extracellular and intracellular stores of Ca^{++} (2, 6, 28). The inhibitory effect of FMLP on ATP-dependent Ca^{++} uptake by lysosomes could serve to liberate Ca^{++} into the cytosol from this intracellular compartment upon PMN activation. Such a role for the lysosomal Ca^{++} pump being directly related to the chemotactic peptide (or other stimulus) induced Ca^{++} transients would require that the stimulus gain access to the cytosol and interact with the lysosomes rapidly. Although the exact kinetics for reaching the maximum cytosolic Ca^{++} concentration upon stimulation of PMN are unclear (2, 3, 6), the rise in cytosolic free Ca^{++} begins almost immediately after addition of FMLP. Although it remains to be determined whether FMLP enters the cytosol, it seems unlikely that the peptide could influence the earliest cytosolic Ca^{++} change through a direct interaction with the lysosomal Ca^{++} pump. Whether the more prolonged rise in cytosolic Ca^{++} that follows stimulation with FMLP could be the result of such a direct interaction or whether FMLP-stimulated generation of cytosolic factors, e.g., phosphoinositides (29, 30), could rapidly regulate

the activity of the lysosomal Ca^{++} pump and thereby influence cytosolic Ca^{++} are currently being studied.

The findings that FMLP inhibits ATP-dependent Ca^{++} uptake by lysosomes and that this inhibition can be blocked by a competitive antagonist of FMLP (BOC-Phe-Leu-Phe-Leu-Phe) is consistent with the finding of FMLP mediating its effect by interacting with a receptor on the lysosome surface. Previous studies have demonstrated a specific FMLP receptor on intact PMN lysosomes (principally on specific granules) (31) and, while binding activity was identified on the cytoplasmic surface, these organelles have been proposed as a possible source for the increased number of FMLP receptors expressed on the cell surface after degranulation (32). Whether or not the inhibition of the lysosomal Ca^{++} uptake pump by FMLP is related to the physiologic effects of FMLP on intracellular Ca^{++} homeostasis, this purified peptide should be useful in better defining, and possibly isolating, the lysosomal Ca^{++} pump.

The lysosomal Ca^{++} pump may also serve to down-regulate or turn off activated PMN. This could result from activation of the Ca^{++} uptake pump of intracellular lysosomes by a rise in cytosolic free Ca^{++}. Alternatively, translocation of lysosomal Ca^{++} pumps to the plasma membrane during degranulation should orient these pumps to extrude cytosolic Ca^{++} and thereby decrease the cytoplasmic Ca^{++} concentration. This hypothesis would be consistent with the recent observation by Lagast et al. (33) that plasma membranes isolated from phorbol myristate acetate-stimulated PMN contain a more active Ca^{++} extrusion pump with an unchanged K_m for Ca^{++} than control plasma membranes (33). Future studies will be directed at examining the regulation of the ATP-dependent Ca^{++} uptake pump of neutrophil lysosomes.

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