Adenosine Triphosphate-dependent Calcium Pump in the Plasma Membrane of Guinea Pig and Human Neutrophils

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Abstract. Changes in cytosolic free Ca may function as a second messenger in neutrophils. Since the plasma membrane seems to be a major regulator of intracellular Ca in many cells, we characterized an energy-dependent Ca transport system in plasma membrane-enriched fractions ("podosomes") from phorbol myristate acetate-stimulated guinea pig and human neutrophils. The active Ca transport system in guinea pig podosomes exhibited a high affinity for Ca (Michaelis constant $[K_m]$Ca 280 ± 120 nM) and a maximum velocity of 0.83 nmol Ca/mg protein per min. Uptake showed an absolute requirement for Mg ATP (K_m ATP 67 μM), whereas other trinucleotides were inactive. Ca uptake was optimal at pH 7, was azide insensitive and temperature dependent. Vanadium, an inhibitor of the Ca/Mg ATPase of heart sarcolemma, inhibited Ca pump activity by 50% at 1 μM. Ca transport was not affected in a NaCl-containing medium, an observation arguing against the presence of a Na/Ca exchange system. Calmodulin at 0.5–10 μg/ml stimulated the Ca pumping activity of EGTA-washed podosomes. Calmodulin depletion decreased the affinity of the Ca pump for Ca (K_m Ca 2.07 μM) and its readdition restored it (K_m Ca 0.55 μM).

ATP-dependent Ca transport by podosomes and

phagocytic vesicles was inactivated by exposure to trypsin or to the nonpenetrating sulfhydryl reagent *p*-chloromercuribenzene sulfonate. Human podosomes had a Ca uptake system with properties similar to those of the guinea pig.

These findings demonstrate the presence of a Ca pump in the neutrophil plasma membrane, which is active at physiological concentrations of free cytosolic Ca. By changing Ca concentrations at the cell periphery, this pump could control various motile functions of the neutrophil, such as locomotion or degranulation.

Introduction

Polymorphonuclear leukocytes (PMN), the first line of defense against invading microorganisms, are highly motile cells, which also produce toxic oxygen metabolites such as superoxide and hydrogen peroxide and release granular enzymes that kill bacteria, fungi, and parasites (1–3). The intracellular mechanisms modulating and synchronizing these various PMN functions are largely unknown.

There is increasing evidence that the intracellular concentration of ionized Ca may play a crucial role in modulating the activity of PMN, for example, the stimulation of the cell by Ca^{2+} ionophores (4–7) and the inhibition of cell function by Ca^{2+} antagonists (8–11). Intracellular Ca-binding proteins such as gelsolin and calmodulin seem to be implicated in the regulation of PMN movement and metabolism. Therefore, it becomes important to understand how phagocytes are capable of controlling their levels of intracellular Ca. In particular, Ca movements across the plasma membrane could be of great physiologic importance, since membrane receptor activation immediately generates a series of events, including changes in cytosolic free Ca concentration (14–16).

By using a plasma membrane-enriched fraction that contains a subpopulation of inside-out vesicles, we demonstrated that PMN have a calcium transport system that pumps Ca from the cytoplasm to the external surface of the membrane. A detailed examination of this energy-dependent Ca pump forms the basis of this report.

Methods

Chemicals. Ruthenium red and *N*-ethylmaleimide (NEM)[1] were obtained from Aldrich-Europe, Beerse, Belgium; Ionophore A23187 from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA. ADP, cytosine triphosphate (CTP), diisopropylfluorophosphate (DFP), EGTA, guanosine triphosphate (GTP), inosine triphosphate (ITP), catalase, soybean trypsin inhibitor (SBTI), phorbol myristate acetate (PMA), *p*-chloromercuribenzene sulfonic acid (pCMBS), uridine triphosphate (UTP), trypsin type IX, and ouabain were obtained from Sigma Chemical Co., St. Louis, MO. Sodium caseinate and lipopolysaccharide B were purchased from Difco Laboratories, Detroit, MI. Triton X-100 and phenylmethylsulfonylfluoride (PMSF) were purchased from Merck, AG, Darmstadt, Federal Republic of Germany; sodium orthovanadate from Fisher Scientific Co., Pittsburgh, PA. Aquasol and ^{45}Ca (as CaCl$_2$) were obtained from New England Nuclear, Boston, MA. Calmodulin was prepared from bovine brain as described previously (17).

Guinea pig neutrophils. 20 ml of a 12% solution of sodium caseinate in saline was injected intraperitoneally into guinea pigs (18). 14–16 h later, the animals were killed and the peritoneal cavity was opened and washed with isotonic saline. The lavage fluid containing the harvested cells was filtered through a sterile gauze. These cells were washed three times by centrifugation at 4°C (250 *g*, 10 min) and finally resuspended in phosphate-buffered saline (PBS) (130 mM NaCl, 4 mM KCl, 10 mM sodium-phosphate buffer, pH 7.4) supplemented with 5 mM glucose, and kept at 0°C until used. Yields ranged from 7×10^8 to 1.5×10^9 PMN cells/guinea pig, representing >90% of the harvested cells. Cell viability was assessed by trypan blue exclusion.

Human neutrophils. Peripheral blood granulocytes were collected from healthy donors in acid-citrate-dextrose and purified by dextran and Ficoll-Hypaque sedimentation as described by Böyum (19). Erythrocytes were removed by hypotonic lysis. The cells, of which >95% were PMN, were washed, suspended in PBS supplemented with glucose, and kept at 4°C until used.

Sera. Guinea pig serum was obtained from freshly clotted blood after cardiac puncture of anesthetized animals. Sera were used on the same day or stored at −70°C until used.

Preparation of neutrophil plasma membrane-enriched vesicles (podosomes). Plasma membrane-enriched vesicles were prepared as described previously (20, 21). The preparation, which is virtually free of organelles, is a mixture of right-side-out and inside-out "blebs." Both human peripheral or guinea pig exudate PMN were used. PMN (2×10^8 in 5 ml) were incubated at 37°C in glass conical centrifuge tubes in the presence of 1 μg/ml of PMA in PBS with glucose for 5 min. After incubation, the suspension was placed in a sonication bath at 37°C for 8–10 s, then placed in an ice bath. The samples were centrifuged at 250 *g* for 10 min to allow the residual cell bodies to sediment. The resulting cloudy supernatant was centrifuged at 10,000 *g* for 10 min. The pellet was washed in a solution containing 250 mM sucrose-30 mM imidazole-HCl, pH 7, resuspended in the same solution, and kept on ice until used.

A Ca transport system can be demonstrated in podosomes prepared in the absence of PMA. Nevertheless, we elected to characterize the Ca

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1. *Abbreviations used in this paper:* CTP, cytosine triphosphate; DFP, diisopropylfluorophosphate; DMSO, dimethyl sulfoxide; GTP, guanosine triphosphate; ITP, inosine triphosphate; NEM, *N*-ethylmaleimide; pCMBS, *p*-chloromercuribenzene sulfonic acid; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonylfluoride; SBTI, soybean trypsin inhibitor; UTP, uridine triphosphate.

pump of podosomes prepared in the presence of this phorbol ester, because PMA activates the NADPH-dependent superoxide generating system (22). This would allow the simultaneous study of both the Ca transport and superoxide generating systems.

The Ca transport activity of human podosomes was more labile than that obtained from guinea pig neutrophils. The activity of human podosomes stored at 0°C decreased rapidly to ∼30% after 2 h in contrast to guinea pig podosomes where the activity was stable 2 h after preparation. When the sonication of human PMN was performed in the presence of PMSF (5 mM) and catalase (10,000 U/ml), loss of activity was decreased.

To obtain an estimate of the proportion of inside-out vesicles in podosomes, we measured the activity of 5′-nucleotidase, an ectoenzyme of guinea pig neutrophils, in this fraction, in the absence and presence of 0.1% Triton X-100. The proportion of inside-out vesicles was calculated as described for erythrocytes (23, 24):

$$\% \text{ Inside-out vesicles} = \left[1 - \frac{\text{normal}}{\text{Triton X-100}} \right] \times 100.$$

Preparation of phagocytic vesicles. This technique, described in detail elsewhere (25), is outlined briefly below. Diisododecylphthalate (1 ml) (practical grade; Matheson, Coleman and Bell, E. Rutherford, NJ) was added to 3 ml of medium containing lipopolysaccharide B from *Escherichia coli* 026: B6 at a final concentration of 10 mg/ml, and the suspension was sonicated for 90 s to form oil droplets coated with lipopolysaccharide.

Lipopolysaccharide particles were incubated with fresh or freshly thawed guinea pig serum for 20 min at 37°C, during which time an opsonic fragment of the third component of complement interacts with them (opsonized particles) (26). Cells were suspended in Krebs-Ringer's phosphate medium (130 mM NaCl, 4 mM KCl, 1.3 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM sodium phosphate buffer, pH 7.4) and warmed to 37°C. Suspensions of opsonized particles (20%, vol/vol) and cells (5%, vol/vol) were mixed and agitated gently in a shaking bath at 37°C for 5 min.

The cells were incubated with 5 mM DFP for 5 min at 0°C to inhibit serine proteases (27). The cells which had ingested opsonized particles were washed once in cold 0.15 M NaCl, suspended in deionized ice-cold water, and immediately centrifuged at 4°C. Exposure to water caused the cells to swell but not to rupture, and rendered subsequent homogenization easier to perform.

The cell pellets were suspended in an equal volume of ice-cold 0.34 M sucrose containing 5 mM EGTA, 5 mM dithiothreitol, 30 mM imidazole-HCl buffer, pH 7.4, and several protease inhibitors, including 1 mM PMSF, 0.25 mg/ml of α-1-antitrypsin, and 0.25 mg/ml of SBTI. The cells were homogenized in this medium in a 5-ml Dounce homogenizer with a tight-fitting pestle. Progressive cell rupture was assessed by determining the percentage of intact cells by phase-contrast microscopy. About 25 strokes were required to break 90% of the cells. Ca (CaCl$_2$) was then added to a final concentration of 5 mM. Thereafter, the homogenate was transferred to a 30-ml Sorvall centrifuge tube and carefully overlaid by means of a peristaltic pump (Pharmacia Fine Chemicals AB, Uppsala, Sweden) with 2 vol of a solution containing 250 mM sucrose, 30 mM imidazole-HCl pH 7, and centrifuged for 60 min at 20,000 *g*. The floating white layer, representing isolated phagocytic vesicles, was then carefully removed and kept at 0°C until it was further diluted for use.

Calcium uptake. Ca uptake by phagocytic vesicles or peripheral vesicles of PMN was measured in a "standard" medium containing 100

mM KCl, 30 mM imidazole-HCl buffer, 5 mM sodium azide at a pH of 7 and at various concentrations of MgCl$_2$, ATP (pH adjusted to 7), and CaCl$_2$ in a total volume of 250 or 500 μl. Human podosomes were tested in the presence of 2 mM PMSF. The test was carried out as follows: Membrane vesicles at concentrations of ~0.2–0.9 mg of protein/ml of medium, were warmed at 37°C in the above medium for 5 min. When it was used, 5 mM ammonium oxalate was added 2 min after the membranes. The suspension was mixed and the reaction was started by the addition of CaCl$_2$ (usually 25 μM containing 1–10 μCi/ml of ^{45}Ca), followed by quick agitation. Samples (75 μl) were taken from the mixture at defined intervals and added to 2 ml of a solution containing 100 mM KCl, 30 mM imidazole-HCl, pH 7. Vesicles were trapped by vacuum filtration with a manifold (Millipore Corp., Bedford, MA) on 0.45-μm pore filters (Millipore Corp.) which were washed with 0.25 M KCl (2 ml), followed by water (11 ml) before addition of test samples. The filters containing vesicles were washed once with 2 ml of the same KCl/imidazole-HCl buffer and were dissolved in Aquasol and counted for ^{45}Ca radioactivity in a scintillation counter (Beckman Instruments Inc., Palo Alto, CA). Ca uptake rates were expressed as nanomoles Ca per milligram total protein per minute after the values bound in the absence of ATP were subtracted. The calcium ionophore A23187 was dissolved with dimethyl sulfoxide (DMSO) and then diluted with the incubation buffer to a final concentration of 5 μM. Control experiments were performed to ensure that DMSO at similar concentrations did not alter calcium uptake by the podosomes.

Calculation and measure of free calcium concentration and kinetic parameters. The free Ca concentration of solutions in the presence of ATP and EGTA was calculated initially by means of the computer program of Perrin and Sayce (28) as described previously (17). The Ca content of all buffers, various reactants, and that of the vesicles was determined by atomic absorption and taken into account when calculating free Ca concentrations. For experiments in which affinity constants for Ca were determined, final free Ca concentrations were measured with a Ca-sensitive electrode. Mini-Ca electrodes were prepared using Ca-selective semiliquid membranes according to Simon et al. (29). The composition of the membrane corresponded to electrode No. 16 shown in Table I of reference 29. A good correlation between calculated and measured free Ca was found down to ~0.3 μM Ca, beyond which the electrodes did not display a linear response any more when in the presence of protein.

Kinetic parameters (Michaelis constant [K_m] and maximum velocity [V_{max}]) of the Ca transport system were drawn according to the fitting of the data obtained by computer analysis, assuming that a single Michaelis-Menten component participated in total Ca transport (30).

Special procedures. To detect inhibition of Ca transport by vanadium, pCMBS, NEM, azide, and trypsin, the vesicles were treated as follows. Podosomes were incubated for 10 min at room temperature and for 5 min at 37°C with the appropriate concentrations of vanadate. Ca uptake was started with the addition of 2.5 mM ATP.

Podosomes or whole PMN were incubated at 0°C for 30 min with 50 μM pCMBS and washed before further processing. For NEM inhibition the following procedure was carried out (31): Podosomes were preincubated 1 h at 0°C with 5 mM NEM, before performing the Ca uptake. Ruthenium red was added to a standard medium without sodium azide at the same time as podosomes. The effect of sodium azide was studied by comparing Ca uptake performed in the presence and absence of sodium azide in the standard medium. Trifluoroperazine was added 2 min before starting Ca uptake with Mg ATP. The trypsinization procedure was performed as previously described (17): Prewarmed trypsin stock solution was added to make a final concentration of 1 mg/ml to a
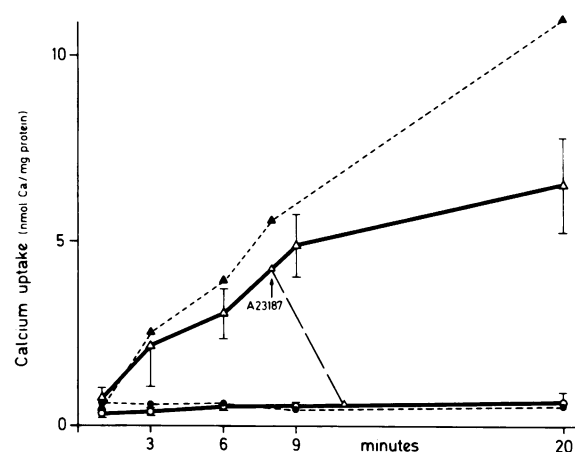
suspension of podosomes, after 5 min at 37°C, SBTI was added to a final concentration of 1 mg/ml.

Protein was determined by the method of Lowry by using bovine serum albumin as a standard (32).

Calmodulin activation of calcium uptake into podosomes. These experiments were performed with podosomes suspended in 250 mM sucrose, 30 mM imidazole buffer, pH 7, and incubated for 5 min at 0°C with 1 mM DFP and 2 mM EGTA. The podosomes were then washed in the sucrose-imidazole buffer (10,000 g, 10 min), resuspended in the sucrose-imidazole with 1 mM EGTA but without DFP. They were centrifuged again (10,000 g, 10 min), resuspended in the sucrose-imidazole buffer, and kept at 0°C until used. The aim of these two EGTA washes was to remove calmodulin bound to inside-out vesicles (33, 34). This preparation was sensitive to the extrinsic addition of bovine brain calmodulin (see below). The washed podosome preparation was incubated with various concentrations of bovine brain calmodulin for 5 min at 0°C, with occasional agitation in the standard medium, then warmed for 5 min at 37°C before starting the reaction with Mg ATP.

Results

Uptake of calcium by podosomes. Fig. 1 shows a time course of Ca uptake by podosomes obtained from guinea pig PMN. To understand the following results, it is important to note that in our study only inside-out membranes were active (as demonstrated below), and that calcium was therefore pumped into the vesicles (called "uptake"), as opposed to the situation in intact PMN, where Ca is extruded from the cytoplasm into the extracellular medium. In the absence of ATP or MgCl$_2$, ~0.5 nmol Ca/mg protein became associated with the podosomes in

Figure 1. Calcium uptake by podosomes from guinea pig PMN. Ca uptake was measured in the following medium: 100 mM KCl, 30 mM imidazole-HCl buffer, pH 7, 5 mM sodium azide, 25 μM CaCl$_2$, 10 μCi ^{45}CaCl$_2$/ml in a total volume of 500 μl at 37°C. Standard medium (mean±SD of eight experiments), O; medium with 5 mM MgCl$_2$ and 5 mM ATP (mean±SD of eight experiments), $\triangle$; medium with 5 mM ammonium oxalate, $\bullet$; medium with 5 mM ammonium oxalate, 5 mM MgCl$_2$, and 5 mM ATP, $\blacktriangle$. Ionophore A23187 (5 μM) was added to an incubation medium lacking oxalate at 8 min (- - -).

<1 min and did not increase thereafter. This base-line uptake was very similar whether or not ammonium oxalate was added. In the presence of both Mg and ATP, Ca uptake was continuous for ~20 min, but the Ca uptake rate decreased after 9 min. Addition of the Ca^{+2} ionophore A23187 (5 μM) to the podosomes after 8 min of Ca uptake initiated a rapid release of Ca from the loaded podosomes to basal levels, demonstrating thereby that Ca uptake had occurred against an electrochemical gradient. When ammonium oxalate was added to the incubation medium, Ca uptake continued in a linear fashion for >20 min, and in some experiments up to 40 min (results not shown).

Fig. 2 shows several general characteristics of this Ca transport system. Its activity was proportional to the protein concentration
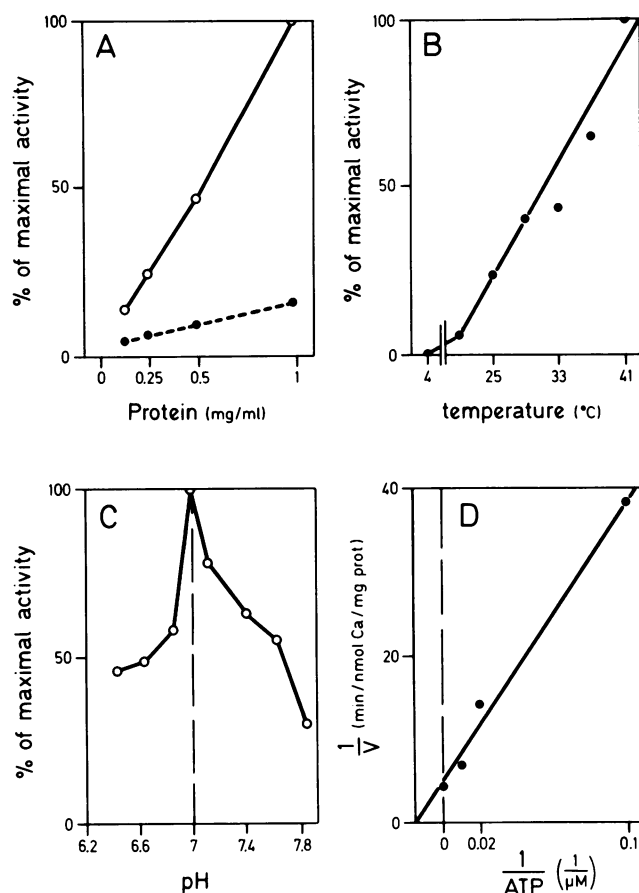

within the range 125–1,000 μg protein/ml. There was no detectable Ca uptake at 4°C and increasing the temperature of the incubation medium from 25 to 41°C caused a fourfold increase in Ca uptake. When the temperature was raised to 100°C for 1 min, Ca uptake was totally abolished. Optimal pH of the incubation medium was 7.0, and Ca uptake decreased by 50% at pH 6.6 and 7.6 (Fig. 2 C). The affinity of the Ca uptake activity for ATP was determined, as shown in Fig. 2 D, using a Lineweaver-Burke plot. Plotted from this graph, K_m for ATP was near 67 μM in the presence of 5 mM $MgCl_2$. Nucleotide requirements were rather specific: ADP promoted a Ca uptake which was 13% of that obtained with ATP at the same concentration. The other nucleotides tested, i.e., CTP, GTP, ITP, and UTP, did not promote any detectable Ca^{+2} uptake.

Fig. 3 shows the effect of various free Ca concentration on uptake by podosomes. Calculated K_m for Ca was 280±120 nM (range 110 nM–510 nM) and V_{max} 0.83±0.26 nmol Ca/mg protein/min (mean±SD of six experiments). At high free Ca concentration (~20 μM), Ca uptake rate decreased slightly.

Inhibition of Ca uptake by sodium orthovanadate is shown in Fig. 4. 50% inhibition of Ca uptake was obtained with ~1.5 M vanadate, and 85% (maximal) inhibition with ~15 μM.

As shown in Table I, incubation of podosomes with NEM totally abolished Ca uptake, whereas ouabain, sodium azide, and ruthenium red had no effect. The absence of inhibition by azide and ruthenium red (Table I) excludes a mitochondrial contamination of our preparation. Trifluoperazine (30 μM) resulted in 50% reduction of the Ca uptake. When 100 mM KCl was substituted by 100 mM NaCl in the standard medium, Ca uptake was not modified (Fig. 5). The addition of 80 mM NaCl or 80 mM KCl to a standard reaction medium did not affect significantly the time course of Ca uptake.
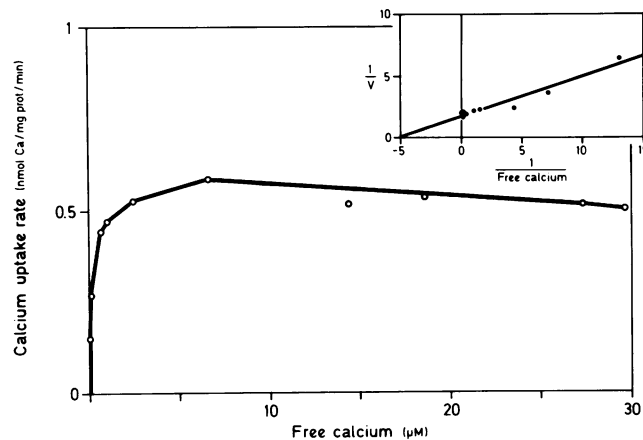
Figure 2. Other characteristics of calcium uptake by podosomes obtained from guinea pig PMN. Ca uptake was performed in the same standard medium as Fig. 1. (*A*) Ca uptake as a function of protein concentration, in the presence of (○) or without (●) 5 mM ATP and 5 mM $MgCl_2$. (*B*) Ca uptake related to temperature of the incubation medium. (*C*) Ca uptake related to pH of incubation medium. The various pHs were measured in the presence of podosomes at 37°C. (*D*) Lineweaver-Burke plot of K_m for ATP in Ca uptake by podosomes. 10 mM $MgCl_2$ and variable concentrations of ATP: 0, 10, 50, 100 and 1,000 μM. The K_m ATP was 67 μM as plotted from this graph.

Figure 3. Rate of calcium uptake by podosomes at various free calcium concentrations. The incubation medium is described in the legend of Fig. 1 except that 100 μM $CaCl_2$ was added to all tubes with various concentrations of EGTA. Free Ca concentrations were calculated and measured as described in the text. K_m for Ca $\cong$ 190 nM and V_{max} $\cong$ 0.58 nmol Ca/mg protein per min in this experiment. *V*, catalytic rate.
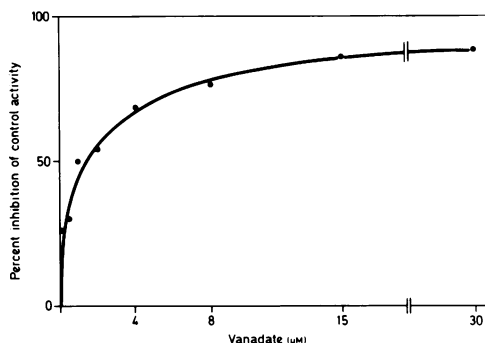
Figure 4. Vanadate inhibition of calcium uptake by podosomes. Ca uptake was initiated by the addition of 2.5 mM ATP. Otherwise the reaction was performed in the same standard medium as Fig. 1. 50% inhibition was obtained with 1.5 μM vanadate.

Effect of calmodulin addition to calmodulin-depleted podosomes. Ca uptake by podosomes washed twice with EGTA was decreased by a factor of five when compared with unwashed podosomes (Figs. 1 and 6 for comparison). Podosomes washed twice or more in EGTA became sensitive to the addition of calmodulin, whereas unwashed podosomes did not change their Ca uptake after the addition of calmodulin. Increasing the number of washings beyond two, however, progressively and irreversibly inactivated the Ca pump activity.

When exogenous calmodulin was added to the incubation medium, an increase in Ca uptake occurred. At calmodulin concentration up to 10 μg/ml, and at high free Ca concentrations (free Ca = 10 μM) a maximal 50% increase of Ca uptake could be demonstrated (Fig. 6). Higher calmodulin concentration (60 μg/ml) did not produce any further increase of Ca uptake (not shown). Addition of the ionophore A23187 released accumulated Ca and Ca content returned to basal levels in all groups.

The stimulatory effect of calmodulin was more evident in the presence of submicromolar free Ca concentrations (Fig. 7). Fig. 7 shows the effect of various Ca concentrations on Ca uptake by EGTA-washed podosomes in the presence or absence of extrinsic calmodulin. The affinity constants of the Ca pump

Table I. *Effect of Various Substrates on Calcium Uptake*

Addition	Concentration	Activity
None		100±8
Ouabain	2 mM	97±9
Sodium azide	5 mM	95±10
Ruthenium red	2 μM	102±5
Trifluoperazine	30 μM	50±5
NEM	1 mM	0±0

Incubations and Ca uptake were performed as described in Methods. Results are averages of triplicates and are expressed as percentage±SD of control activity.
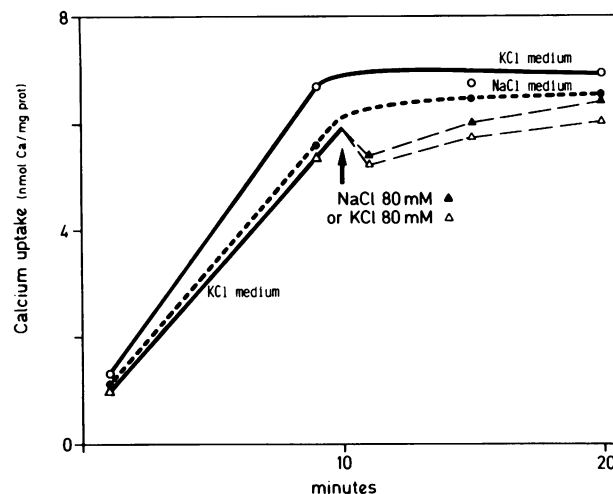

Figure 5. Time course of calcium uptake. Effects of NaCl substitution for KCl in standard medium and of the addition of 80 mM KCl or NaCl to KCl plus imidazole medium. Ca uptake was measured under the same conditions as in Fig. 1, using the same KCl 100 mM plus imidazole 30 mM buffer (O) or NaCl 100 mM plus imidazole 30 mM buffer (●). 80 mM NaCl (▲) or KCl (Δ - - - Δ) was added at 10 min to KCl plus imidazole buffer (Δ —— Δ).

in the absence or presence of calmodulin were as follows: The K_m for Ca was 2.07 μM and the V_{max} 0.3 nmol Ca/mg protein per min in the calmodulin-depleted podosomes. When calmodulin (10 μg/ml) was added, the affinity for Ca increased
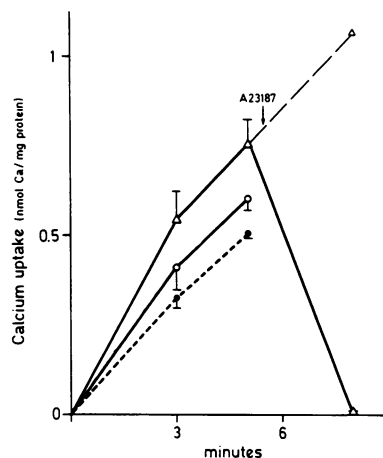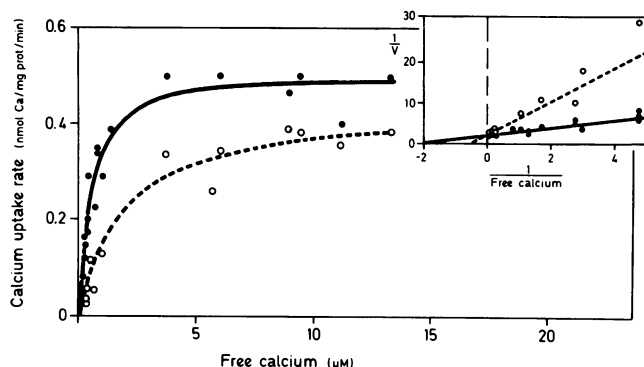

Figure 6. Effect of various doses of calmodulin on the calcium uptake of EGTA-washed podosomes. Podosomes were incubated 5 min at room temperature with calmodulin 5 μg/ml (Δ); 0.5 μg/ml (O); or without calmodulin (●). Results are expressed as means±SD of three experiments using the same membrane preparation. Ionophore A23187 (5 μM) in DMSO was added to each experiment and released Ca to basal levels (shown only for the experiment with 5 μg/ml calmodulin). Addition of the same volume DMSO (- - -) without A23187 did not affect Ca uptake nor induce Ca release from podosomes.

Figure 7. Effect of calmodulin on calcium uptake rate by EGTA-washed podosomes at various free calcium concentrations (Lineweaver-Burke plot in inset). EGTA-washed podosomes were incubated for 5 min at room temperature with (●) or without (○) 10 µg/ml calmodulin. The incubation medium is described in the legend of Fig. 1 except that CaCl₂ 100 µM was added to all tubes with various concentrations of EGTA. Free Ca concentrations were determined as described in the text. The average of duplicates is shown.

(K_m = 0.55 µM) concurrently with the V_{max} (0.5 nmol Ca/mg protein per min), suggesting that the calmodulin effect is mediated by increasing the affinity of the pump for Ca. Under these conditions, calmodulin also increased the V_{max} of the Ca pump.

Calcium uptake by human podosomes. Human podosomes displayed a Ca transport similar to guinea pig podosomes. The uptake of Ca was dependent on the presence of Mg and ATP with a similar affinity constant for Ca but with a lower specific activity (Table II). Ionophore A23187 (5 µM) released Ca from loaded podosomes until basal levels were achieved. The Ca pump activity persisted longer in the presence of ammonium oxalate, ADP promoted ~30% of the activity, measured in the presence of ATP, whereas UTP, CTP, and GTP were inactive. The system was not inhibited by azide or ruthenium red. The

activity was abolished by preincubation of podosomes in NEM or trypsin under similar conditions as described for guinea pig podosomes (Table II).

Arrangement of the calcium pump system in the plasma membrane of neutrophils. To obtain an estimate of the percentage of inside-out vesicles in our preparation of podosomes, 5'-nucleotidase activity, an ectoenzyme of guinea pig granulocytes, was measured in the presence and absence of Triton X-100. The calculated proportion of inside-out vesicles in the podosomes preparation was 40±5% (average±SD of triplicate determinations in three different preparations).

We were also able to demonstrate a Ca pump activity in phagocytic vesicles, i.e., a homogenous population of inside-out plasma membrane vesicles from guinea pig PMN. This activity was Mg- and ATP-dependent and pumped Ca at a rate of 0.7 nmol Ca/mg protein per min in standard medium containing 25 µM Ca. Ca uptake was abolished by pretreating the vesicles with trypsin and pCMBS (Table III). Inactivation of Ca uptake was also observed when podosomes were pretreated with pCMBS and trypsin. By contrast, no effect was seen when whole cells were treated with trypsin or pCMBS before preparation of podosomes. These results are consistent with an asymetrical structure of the Ca pump across the plasma membrane: a proteic moiety and sulfhydric component being exposed at the cytoplasmic surface.

Discussion

The present findings demonstrate the presence of an energy-dependent Ca pump in the plasma membrane of neutrophils, capable of transporting Ca against an electrochemical gradient from the inside of the cell into the extracellular medium. This active transport is an important attribute of plasma membrane ATPases, which have to displace Ca across a 10,000-fold gradient (35). The physiological importance of the Ca pump described is suggested by its optimal activity at free Ca concentrations

Table III. Effect of Trypsin and pCMBS on Ca Pump Activity of Podosomes and Phagocytic Vesicles

Additions	Phagocytic vesicles	Podosomes	Whole PMN
	%	%	%
None (control activity)	100±8	100±6	100±8
Trypsin	0±0	0±0	93±4
Trypsin plus SBTI	not done	110±6	not done
SBTI	not done	106±2	not done
pCMBS	0±0	0±0	100±6

Phagocytic vesicles, podosomes, or whole PMN were incubated either in 0.34 sucrose-30 mM imidazole, pH 7, without any additive or with one of the following compounds: trypsin (1 mg/ml); trypsin (1 mg/ml) plus SBTI (1 mg/ml); SBTI (1 mg/ml); or pCMBS (50 µM). Incubations lasted for 5 min except for pCMBS (30 min). Results are averages of triplicates and are expressed as percentages±SD of control activity.

Table II. Characteristics of Calcium Pump Activity in Podosomes Obtained from Human PMN

Additions	Activity (K_m Ca²⁺, 350 nM)
	nmol Ca²⁺/mg protein per min
None	0.12±0.02
ADP (5 mM)	0.04±0.01
CTP, GTP, UTP (5 mM)	0.00
Azide (5 mM)	0.11±0.03
Ruthenium red (2 µM)	0.12±0.03
NEM (1 mM)	0.00
Trypsin (1 mg/ml)	0.00

* Results of a typical experiment performed on the same day with the same membrane preparation. Average±SD of triplicates.

encountered within the cytoplasm: free Ca concentrations in neutrophils were recently measured, and resting values of 100–200 nM were observed (36). These values increased to micromolar levels upon chemotactic peptide stimulation. Because of its high affinity for Ca, this Ca pump is able to function at physiological free Ca concentrations in resting and stimulated neutrophils.

In recent years, high affinity Ca-ATPases as well as ATP and Ca-dependent phosphorylation processes have been shown to be associated with the plasma membrane of neutrophils (37–39). The large number of nonspecific ATPases and kinases present in these cells, however, has precluded the establishment of a correlation of these activities with Ca transport. A Ca pump in phagocytic vesicles of rabbit neutrophils has recently been described (40). The reported K_m for Ca was 2.8 μM, a value which is considerable higher than the K_m values obtained in other nucleated cells (i.e., macrophages: K_m Ca = 480±10 nM; heart sarcolemma: K_m Ca = 300±200 nM) (17, 41–43) and well above the presumed resting cytosolic free Ca levels in rabbit PMN. An analysis of their results indicates, however, a two-component Ca uptake: One of the components was saturable with a low K_m, while the other did not saturate in the range of Ca concentrations used.

In our system, the maximal Ca uptake activity occurred at pH 7, which is the presumed cytosolic pH in resting neutrophils. The pump was active at low ATP concentrations (K_m, 67 μM), indicating that this nucleotide is not a regulator of the pump under physiologic conditions. The nucleotide specificity was similar to that described for the Ca pump of the macrophage plasma membrane, where only ATP was effective, with ADP promoting only partial Ca uptake (17).

Low concentrations of vanadate, such as those used in this study, have proven to be a useful tool for the inhibition of the Ca-ATPase activity of plasma membrane vesicles (44). It has been used in heart tissue to distinguish plasma membrane activity from that of contaminating sarcoplasmic reticulum vesicles (41). Recently, Varecka and Carafoli (45), using 0.5 mM vanadate, have shown a decreased ^{45}Ca efflux from erythrocytes and suggested that this compound led to high levels of intracellular Ca by Ca-ATPase inhibition.

In addition to the Ca transport mediated by the ATPases, several plasma membranes also possess a Na/Ca exchange system. Experiments performed in NaCl-containing medium, or the addition of NaCl to KCl-containing medium after several minutes of Ca uptake have allowed detection of such exchanges in other systems (46). Our negative results militate against such a system in the plasma membrane of guinea pig neutrophils.

By measuring 5'-nucleotidase activity, an ectoenzyme of guinea pig PMN, we were able to estimate the proportion of inside-out vesicles in the podosome preparation. A similar value of ~40% can be calculated from the results obtained by Cohen et al. (47) during Triton X-100 activation of the NADPH-dependent superoxide-generating system, a marker of the inner surface of the plasma membrane in digitonin-activated guinea pig podosomes. Preincubation with trypsin or pCMBS led to an inhibition of Ca uptake by phagocytic vesicles and podosomes, but not in whole cells, suggesting that the Ca pump presents a susceptible protein moiety and a sulfhydryl radical at the cytoplasmic side of the plasma membrane (i.e., at the outer side of the phagocytic vesicle).

An influence of calmodulin on several plasma membrane Ca-ATPases has been shown recently (17, 41, 43, 48). This ubiquitous protein is a modulator of many Ca-dependent processes (49). Its functions can be explored by the use of phenothiazines such as trifluoperazine at appropriately low concentrations (<50 μM), when calmodulin antagonism occurs (49–51). In our hands, trifluoperazine (30 μM) inhibited Ca pump activity, suggesting the involvement of calmodulin in this system. Alternatively, recent studies have shown that trifluoperazine inhibited purified Ca-ATPase that had been incorporated into liposomes, thus indicating a direct effect on the Ca pump (52). In our system, podosomes did not respond to extrinsically added bovine brain calmodulin, unless they had been washed previously with EGTA. On the other hand, depletion of calmodulin from the podosomes was limited by several technical problems, more than two EGTA washings drastically reducing Ca uptake, indicating some kind of membrane damage. After two EGTA washings, however, podosomes retained a satisfactory residual Ca pump activity and the stimulating effect of exogenous calmodulin could be demonstrated. Under these conditions calmodulin shifted the Ca pump to a higher affinity state for Ca, and enhanced the maximal velocity of this Ca transport system. These results are in accord with other observations made with sarcolemma and erythrocyte membranes (41, 53).

Our present findings allow us to speculate that the Ca pump described could play a major role in the control of movement in the neutrophil. There is indeed extensive experimental evidence that movement in ameboid cells depends on a Ca-dependent gel-sol transformation in the peripheral cytoplasm (12, 54). Contact of an opsonized particle with specific surface neutrophil receptors presumably raises the cytosolic Ca, creating solation of the peripheral cytoplasm, which in turn allows free access of granules to the plasma membrane, i.e., degranulation and stimulation of the superoxide generating system (55). Secondary to this Ca rise, Ca pump stimulation by calmodulin binding and other receptor-mediated events, such as pump phosphorylation, may decrease local cytosolic Ca concentrations to levels where gelation occurs, with the formation of a pseudodopod that would progressively surround its prey.

Although the exact sequence of calcium changes in the peripheral cytoplasm during phagocytosis remains to be shown, it is very likely that the calcium pump present in the plasma membrane of neutrophils plays an important role in the regulation of intracellular calcium homeostasis of the resting or stimulated neutrophil.

Acknowledgments

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