Subcellular Distribution of Ca^{2+} Pumping Sites in Human Neutrophils

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Abstract

The distribution of nonmitochondrial Ca^{2+} pumping sites and the site of action of inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) were studied in subcellular fractions of human neutrophils.

In homogenates, two different Ca^{2+} pools could be observed: a mitochondrial Ca^{2+} pool and a nonmitochondrial, ATP-dependent, Ins 1,4,5-P_3-responsive Ca^{2+} pool.

When the homogenate was separated into microsomes, primary granules, and secondary granules, the nonmitochondrial Ca^{2+} pumping and the Ins 1,4,5-P_3-induced Ca^{2+} release occurred only in the microsomal fraction.

In a gradient developed to separate different microsomal organelles, maximal Ca^{2+} pumping activity occurred in fractions of low densities. Correlations between Ca^{2+} uptake and organelle markers were negative for the endoplasmic reticulum ($r = -0.49$) and positive for plasma membrane ($r = 0.47$), Golgi ($r = 0.62$), and endosomes ($r = 0.96$). Because the Ca^{2+} pumping organelles in these fractions were insensitive to micromolar vanadate and digitonin treatment, they are unlikely to be plasma membrane vesicles.

We conclude first that microsomal fractions of human neutrophils contain organelles that lower the ambient free Ca^{2+} concentration and respond to Ins 1,4,5-P_3. Second, granules are not involved in intracellular Ca^{2+} regulation in neutrophils. Third, nonendoplasmic reticulum organelles, such as endosomes, Golgi elements, or yet undefined specialized structures, play a major role in intracellular Ca^{2+} homeostasis in human neutrophils.

Introduction

A rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)[1] plays a crucial role in neutrophil activation by various physiological stimuli (1–5). $[Ca^{2+}]_i$ of neutrophils is regulated by both intracellular compartments (6) and the plasma membrane (7). To maintain low levels of $[Ca^{2+}]_i$ in resting cells ($\sim$ 100 nM) against the extracellular Ca^{2+} concentration of 2 mM, the intracellular compartments actively take up calcium ions, whereas the plasma membrane Ca^{2+}/Mg^{2+}-ATPase pumps out calcium ions. During cellular activation $[Ca^{2+}]_i$ will rise to micromolar levels, due to release of calcium ions from intracellular Ca^{2+} stores and increased Ca^{2+} permeability of the plasma membrane.

The regulation of $[Ca^{2+}]_i$ by intracellular compartments in neutrophils has been studied using digitonin permeabilized cells (6). Permeabilized neutrophils are capable of maintaining ambient free $[Ca^{2+}]$ (free $[Ca^{2+}]$ in the suspension) at levels that are within the range of resting cytosolic $[Ca^{2+}]$. Two pools appear to participate in this regulation, first, mitochondria that can lower free $[Ca^{2+}]$ to the submicromolar range, and second, ATP-dependent nonmitochondrial pool(s) that lower Ca^{2+} levels further to $\sim$ 200 nM, close to intracellular resting levels in neutrophils.

Inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3), which is produced by membrane phospholipid breakdown in response to a variety of stimuli of neutrophils (8–13), is thought to act as Ca^{2+} mobilizing second messenger. This compound is able to release Ca^{2+} from the nonmitochondrial pool in permeabilized neutrophils.

It is therefore believed that the nonmitochondrial Ca^{2+} pool plays an important physiological role in neutrophils, in both the maintenance of their resting state and their activation.

Studies with various cell types have localized the nonmitochondrial Ca^{2+} pool in the microsomal fraction which contains various components, among them plasma membrane, Golgi, and endoplasmic reticulum (14–23). In some studies positive correlations between Ca^{2+} uptake or Ins 1,4,5-P_3-induced Ca^{2+} release and marker enzymes for the endoplasmic reticulum have been found (24–26). However, a recent study with human neutrophils suggested that granules actively pump Ca^{2+} and thus may be the nonmitochondrial Ca^{2+} pool (27).

The aim of the present study was to perform a detailed examination of the subcellular distribution of the nonmitochondrial Ca^{2+} pumping sites in neutrophils.

Methods

Materials. Analytical grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), Fluka AG (Buchs, Switzerland), E. Merck AG (Darmstadt, FRG), Amersham International (Amersham, UK). Ins 1,4,5-P_3 was a kind gift of Dr. R. Irvine.

Preparation of human neutrophils. Neutrophils were prepared from 500 ml of blood per experiment as described previously (28, 29). Briefly, after dextran sedimentation (30 min), the leukocyte-rich supernatant was centrifuged through a layer of a Ficoll. The remaining erythrocytes were removed by hypotonic lysis. Cells obtained by that method were > 95% neutrophils, and 98% of them excluded trypan blue. The neutrophils were washed twice with NaCl 0.9% containing 1 mM EGTA, to maximally decrease any Ca^{2+} contamination.

Buffers used in this study were abbreviated as follows: KCl buffer, 100 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 25 mM Hepes, 2 mM KH_2PO_4, pH 7. Sucrose buffer, 250 mM sucrose, 5 mM Hepes, pH 7. KCl buffer 10× (tenfold concentrated KCl buffer), 1 M KCl, 250 mM Hepes, 20 mM KH_2PO_4, pH 7. Sucrose buffer 10× (tenfold concentrated sucrose buffer), 2.5 M sucrose, 50 mM Hepes, pH 7.

Treatment of cells with diisopropylfluorophosphate (DFP) (30). Cells (10^9/ml) were suspended in NaCl 0.9% on ice and incubated with DFP (5 mM) at 4°C for 5 min, then washed twice with ice-cold NaCl.

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1. *Abbreviations used in this paper:* CHX, cycloheximide; PHA-P, phytohemagglutinin-P; pHe7, ^{32}P-labeled He7 control probe; SAC, *Staphylococcus aureus* Cowan strain I; UTP, uridine triphosphate.

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Cell disruption. This is a slight modification of a procedure, described before (31). Cells (10^8/ml) were suspended in an ice-cold sucrose buffer containing 1 mM ATP. For the fractionation procedure described as "Percoll procedure number 2" this buffer also contained 1 mM EGTA. This suspension was transferred into a nitrogen cavitation chamber containing a magnetic stirrer. The nitrogen cavitation chamber was kept on ice. Cells were pressurized for 30 min at 24 bars. The cell homogenate was centrifuged at 2,000 rpm (800 *g*, model J 6, Beckman Instruments, Inc., Fullerton, CA, rotor JS-4.2, 4°C) for 5 min to pellet nuclei and unbroken cells. The pellet was washed once with 3 ml of the above mentioned sucrose/Hepes buffer to recover membranes, which stick to nuclei and unbroken cells. 10–20% of total plasma membrane markers can additionally be recovered by this procedure. This postnuclear supernatant was the starting material for all subcellular fractionation experiments. It will be referred to in the text as homogenate.

Differential centrifugation. 0.3–0.4 × 10^9 cells were used per experiment. The homogenate was centrifuged for 10 min at 4°C at 5,000, 10,000, 15,000, and 20,000 rpm (2,000, 7,840, 17,600, and 31,400 *g*, respectively, Beckman J 21 C, rotor JA-20, 4°C). The supernatants of these centrifugations were designated S5, S10, S15, and S20, respectively. Activities of marker enzymes and Ca^{2+} uptake in these supernatants were expressed as percent of the activity found in the homogenate.

Percoll procedure No. 1. The aim of this gradient was to separate primary granules, secondary granules, and microsomes. It was a slight modification of the procedure described in detail by Borregard et al. (32). The whole procedure was performed at 4°C using 0.6–0.8 × 10^9 cells per experiment.

4.5 ml of a low-density Percoll and then 4.5 ml of a high-density Percoll were carefully layered under 4 ml of the homogenate using a narrow piece of Teflon tubing. The low-density Percoll consisted of 14% Percoll, 10% of sucrose buffer 10×, and 76% H_2O; the high-density Percoll consisted of 68% Percoll, 10% of sucrose buffer 10×, and 22% H_2O. The gradient was centrifuged for 10 min at 20,000 rpm (31,400 *g*, Beckman J 21 C, rotor JA-20, 4°C). Three distinct well-separated bands (bottom = fraction 1 = primary granules, middle = fraction 2 = secondary granules, and top = fraction 3 = microsomes) were collected by aspiration from the bottom of the gradient. Percoll was removed by dilution of the fractions with 30 ml of the KCl buffer followed by centrifugation for 15 min at 20,000 rpm (31,400 *g*, Beckman J 21 C, rotor JA-20, 4°C). The bulk of the Percoll stayed in suspension; the biological material was found in the pellet. Fractions 1 and 2 were resuspended in 2 ml and fraction 3 in 0.8 ml of KCl buffer, respectively.

Percoll procedure No. 2. This was a two-step procedure developed to further resolve the subcellular compartments present in the microsomal fraction. The whole procedure was performed at 4°C using 1–1.5 × 10^9 cells per experiment. The first step was a rapid separation of the homogenate into heavy (granular) and light (microsomal) fractions, as described by Record et al. (33). 5 ml of gradient material (61% percoll, 10% KCl buffer 10×, 29% H_2O, pH 7) were layered under 5 ml of homogenate and centrifuged for 10 min at 36,000 rpm (160,000 *g*, Beckman L5-85, rotor SW41, 4°C). Microsomes were then found at the top of the gradient, granules on the bottom.

The advantage of this procedure was a more rapid and slightly better separation between granules and microsomes, compared with the procedure described by Borregard et al. (32). The disadvantage of this procedure was that both primary and secondary granules were trapped in a hard-packed Percoll pellet at the bottom of the tube and only the microsomal fraction could be used for further experiments.

The second step of this procedure was a subfractionation of the microsomal fraction by a discontinuous Percoll gradient, containing five different percoll layers. It was an adaptation of the method developed by Epping and Bygrave (34) (Table I).

Using a narrow piece of Teflon tubing, the different Percoll dilutions were carefully layered one under the other. The gradient was centrifuged for 10 min at 20,000 rpm (31,400 *g*, Beckman J 21 C, rotor JA-20, 4°C). Five fractions were collected by aspiration from the bottom of the tube using Teflon tubing: fraction 1 = the lower 6 ml of the gradient; fraction 2 = interphase layer 1/layer 2 (2 ml); fraction 3 = interphase layer 2/layer 3 (2 ml); fraction 4 = interphase layer 3/layer 4 (2 ml); fraction 5 = interphase layer 4/layer 5 (2 ml). Percoll was washed away as described for "Percoll procedure number 1". Fractions were resuspended in 800 μl of KCl buffer.

Table I. Subfractionation of Microsomal Fraction by a Discontinuous Percoll Gradient

	Layer	Volume	Percoll	Sucrose buffer 10×	H_2O	Microsomal suspension
		ml	%	%	%	%
Top	5	1	0	10	90	0
	4	2	10	10	80	0
	3	2	18	10	72	0
	2	2	30	10	60	0
Bottom	1	7	45	5	0	50

Assays for marker enzymes and proteins include alkaline phosphatase (plasma membrane), spectrometric assay using *p*-nitrophenylphosphate as substrate (35); galactosyl transferase (Golgi), radioassay measuring the formation of acetyl lactosamine from acetyl glucosamine and radiolabeled UDP-galactose (36); sulfatase *c* (endoplasmic reticulum), fluorimetric assay, using methylumbelliferyl sulfate as substrate (37), NADPH-cytochrome *c* reductase (endoplasmic reticulum), spectrometric assay measuring the reduction of cytochrome *c* in the presence of NADPH, modification of (38). 500 μl 50 mM phosphate buffer, pH 7.5, containing 100 μM NADPH, NaCN 1.6 mM, 30 μM cytochrome *c*, 30 μg/ml superoxide dismutase and 50 μl of sample protein were incubated for 2 min at 30°C. Controls were done in the absence of NADPH. Samples were put on ice for 5 min, centrifuged for 1 min (Microfuge) and absorption at 550 nm was measured in the supernatant. Preliminary experiments had shown that under these conditions (*a*) at the 2-min time point the increase of absorption is linear and (*b*) the reaction is linear for the measured enzyme concentrations. Vitamin B_{12} binding protein (secondary granules), radioassay measuring the specific binding of cyanocobolamin (39); β-glucuronidase (primary granules), fluorimetric assay using methylumbelliferyl-glucuronide as substrate (39); monoamine oxidase (mitochondria), radioassay measuring the deamination of [^{14}C]tryptamine to [^{14}C] indolacetic acid (40); protein concentration, colorimetric assay as described by Bradford (41), using a commercially available kit (Bio-Rad Laboratories, AG, Glattburg, Switzerland).

Measurement of free Ca^{2+} concentration. Ca^{2+} selective minielectrodes were prepared and calibrated as described by Prentki et al. (42). Homogenates or subcellular fractions were incubated in the above described KCl buffer including 1 mM $MgCl_2$. Addition of ATP-regenerating system, mitochondrial substrates, or various inhibitors are described later for each experiment. The traces shown in the figures are representative experiments that have been repeated 3–10 times.

In some control experiments Ca^{2+} pumping and Ins 1,4,5-P_3 response were measured in the presence of 10% Percoll. Neither parameter was found to be impaired by the presence of this material.

$^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake was measured as previously described (7). Homogenates or subcellular fractions were incubated in the above described KCl buffer including 1 mM $MgCl_2$. Addition of ATP-regenerating system, mitochondrial substrates, or various inhibitors are described later for each experiment.

Uptake of horseradish peroxidase. Neutrophils were isolated and treated with diisopropylfluorphosphate (DFP) as described. After DFP treatment the neutrophils were suspended in Dulbecco buffer (≈ 3.3 × 10^9 cells/ml). After 5 min preincubation, cells were incubated in the presence of 200 U/ml horseradish (HR) peroxidase for 10 min at 37°C. The reaction was stopped by the addition of 50 ml of ice-cold NaCl 0.9%. Cells were centrifuged and treated as described under "cell disruption" and "Percoll procedure number 2." Cells not incubated with

HR peroxidase served as control for the location of residual granular myeloperoxidase in the microsomal fractions. Peroxidase activity was determined using a photospectrometric assay that measured reduction of Diaminobenzidin (DAB) in the presence of H_2O_2 (43).

Electron microscopy. Homogenate or fractions were pelleted by centrifugation at 20,000 rpm for 10 min and the pellets fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min at room temperature. Following postfixation in 0.1 M OsO4 pH 7.4, pellets were dehydrated in graded ethanol and embedded in Epon for conventional electron microscopy.

Results

Homogenate: characterization of a mitochondrial and nonmitochondrial Ca^{2+} sequestering pool. In the presence of MgATP the homogenate was able to lower the ambient free Ca^{2+} concentration to values around 200–400 nM (Fig. 1, *solid line*). These values depended on both the initial [Ca^{2+}] in the buffer and the protein concentration in the homogenate. The addition of 2 μM Ins 1,4,5-P_3 caused a transient increase in the ambient free [Ca^{2+}]. The addition of 2 mM sodium orthovanadate, an inhibitor of a variety of ATPases, led to a slow Ca^{2+} release. In contrast, the addition of mitochondrial inhibitors (200 nM antimycin + 2 μg/ml oligomycin, 1 μM ruthenium red) did not have any effect under these conditions (Table II). These characteristics identify the nonmitochondrial intracellular Ca^{2+} pool previously described in experiments with digitonin-permeabilized neutrophils (6).

Inside-out vesicles of the plasma membrane, produced by the homogenization procedure, might have accounted for at least part of the nonmitochondrial Ca^{2+} pumping activity, because the plasma membrane of neutrophils contains an ATP-dependent Ca^{2+} pump (7). We therefore looked for the effect of 10 μM digitonin, known to permeabilize the plasma membrane of neutrophils (6), and the effect of 30 μM vanadate, known to completely inhibit the Ca^{2+} pump of the plasma membrane of neutrophils (7). At these concentrations neither agent was able to release Ca^{2+} from the nonmitochondrial Ca^{2+} pool of the homogenate (Table II). When the uptake of $^{45}Ca^{2+}$ by the homogenate was measured, the addition of 30 μM vanadate did not lead to a significant reduction (85.8±14.5% of control, n = 4) in Ca^{2+} pumping activity. This suggests that inside-out plasma membrane vesicles did not play a major role in the nonmitochondrial Ca^{2+} pumping of the homogenate.

The Ca^{2+} ionophore, ionomycin, released Ca^{2+} under these conditions (Table II), indicating that the Ca^{2+} is stored in a vesicular pool with high intravesicular [Ca^{2+}].

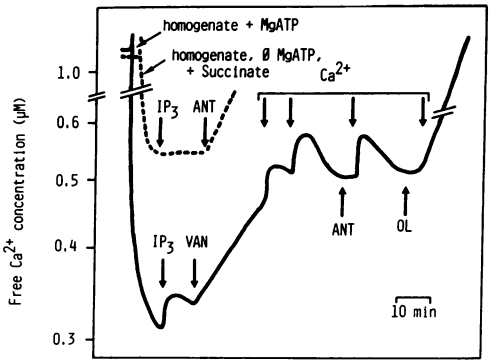

Figure 1. Regulation of ambient free Ca^{2+} concentration by a homogenate of human neutrophils, recorded with a Ca^{2+}-sensitive electrode. (*Solid line*) The homogenate (final protein concentration, ≈ 1 mg/ml) was incubated at 30°C and pH 7.0 in 400 μl of a buffer containing 100 mM KCl, 2 mM KH_2PO_4, 25 mM Hepes, 1 mM $MgCl_2$, 2 mM MgATP, 10 mM phosphocreatine, 8 U/ml creatine kinase, and 0.05% of bovine serum albumin. 2 μM Ins 1,4,5-P_3 (IP_3), 1 μM $CaCl_2$ (*Ca^{2+}*), 2 mM sodium orthovanadate (*VAN*), 200 nM antimycin A (*ANT*), or 2 μg/ml oligomycin (*OL*) were added where indicated. (*Dotted line*) The homogenate (final protein concentration, ≈ 1 mg/ml) was incubated at 30°C and pH 7.0 in 400 μl of a buffer containing 100 mM KCl, 2 mM KH_2PO_4, 25 mM Hepes, 1 mM $MgCl_2$, 5 mM succinate, 2 μg/ml oligomycin (to inhibit mitochondrial ATP-production), and 0.05% of bovine serum albumin. 2 μM Ins 1,4,5-P_3 (*IP_3*) or 200 nM antimycin A (*ANT*) were added where indicated.

The Ins 1,4,5-P_3 response in the homogenate could only be observed for a limited period of time (20–40 min after homogenization). This is possibly due to some proteolytic activity which remains despite pretreatment of neutrophils with the protease inhibitor DFP. The additional use of a combination of protease inhibitors (benzamidine, aprotinine, leupeptin, and phenylmethonsulfonylfluoride) was not possible because it led to an almost complete inhibition of Ca^{2+} pumping. In contrast to the Ins 1,4,5-P_3 response, the Ca^{2+} pumping activity was stable in both the homogenate and subcellular fractions for at least 2 h if stored at 4°C.

The Ca^{2+} release in response to 2 mM vanadate (Fig. 1, *solid line*) stopped at Ca^{2+} concentrations around 500 nM. At such Ca^{2+} levels, we observed a steady state that was maintained even if several Ca^{2+} pulses were given. This value was close to the mitochondrial set-point observed in permeabilized neutrophils (6). The addition of 200 nM antimycin A, an inhibitor of the

Table II. Sensitivity of the MgATP-dependent Pool to Different Compounds

	Vanadate 30 μM	Digitonin 10 μM	Ruthenium red 1 μM	Antimycin A 200 nM + oligomycin 2 μg/ml	Vanadate 2 mM	Ionomycin 500 nM
Homogenate	Ø	Ø	Ø	Ø	+	+
Microsomes	Ø	Ø	Ø	Ø	+	+
Microsomal subfraction 5	Ø	Ø	Ø	Ø	+	+

Homogenate, microsomes, and microsomal subfraction 5 were allowed to pump Ca^{2+} to steady-state levels in the presence of MgATP in a chamber containing a Ca^{2+}-sensitive electrode, as described in legend to Fig. 1. The various compounds at the indicated concentrations were added and their capacity to release Ca^{2+} under these conditions was monitored with the Ca^{2+}-sensitive electrode. (ruth. red = ruthenium red, ant. = antimycin A, ol = oligomycin). + indicates that a Ca^{2+} release was observed (significant increase of ambient free [Ca^{2+}] within 10 min after addition of the compound, i.e., > 100 nM). Ø indicates that no Ca^{2+} release was observed (increase in ambient free [Ca^{2+}] within 5 min after addition of the compound < 10 nM). Experiments repeated at least three times.

respiratory chain of mitochondria, had no effect when added alone. However, the further addition of 2 μg/ml oligomycin, an inhibitor of the mitochondrial proton ATPase, induced Ca^{2+} release from the mitochondria. Thus in the presence of ATP, despite inhibition of the respiratory chain, mitochondrial Ca^{2+} pumping still occurs because the mitochondria can be energized by pumping protons out of the mitochondrial matrix through a proton ATPase. Indeed, in the presence of MgATP, both antimycin A and oligomycin are necessary to inhibit mitochondrial Ca^{2+} pumping (44, 45).

Experiments performed to characterize mitochondrial Ca^{2+} pumping (Fig. 1, *dotted line*) showed that in the absence of MgATP and in the presence of succinate, mitochondria pumped Ca^{2+} to steady-state levels around 500 nM. Under these conditions, Ins 1,4,5-P_3 did not release Ca^{2+}, and 200 nM antimycin alone was sufficient to completely inhibit mitochondrial Ca^{2+} pumping. In additional experiments (not shown) the homogenate was allowed to pump to steady-state levels in the absence of MgATP and in the presence of succinate (as described for Fig. 1, *dotted line*), and the effect of 2 mM vanadate and 1 μM ruthenium red was tested. As expected, vanadate, which does not inhibit mitochondrial Ca^{2+} pumping, had no effect under these conditions, whereas ruthenium red, at concentrations of 1 μM thought to be a specific inhibitor of mitochondrial Ca^{2+} influx, was fully inhibitory, just as seen for antimycin.

These findings indicate that in a homogenate of neutrophils there are two intracellular Ca^{2+} sequestering pools: first, mitochondria, which use either substrates for the respiratory chain or MgATP as source of energy, are inhibited by the combination of antimycin A and oligomycin or by ruthenium red and are insensitive to Ins 1,4,5-P_3 and millimolar concentrations of vanadate; second, a vesicular, nonmitochondrial Ins 1,4,5-P_3–sensitive pool that uses ATP as source of energy and is inhibited by millimolar concentrations of vanadate.

Subcellular distribution of nonmitochondrial Ca^{2+} pumping sites: resolution of various microsomal components. In the following sections, three different techniques of subcellular fractionation were applied to characterize the subcellular distribution of the nonmitochondrial Ca^{2+} pool: (*a*) differential centrifugation, (*b*) a Percoll gradient that separates primary granules, secondary granules, and microsomes, and (*c*) a series of two Percoll gradients that resolve the various microsomal components, i.e., endoplasmic reticulum, Golgi, plasma membrane, and endosomes. To eliminate the possibility of Ca^{2+} pumping by mitochondria, we measured Ca^{2+} uptake in the following experiments in the presence of the mitochondrial inhibitors antimycin A and oligomycin. Control experiments that were carried out in the absence of antimycin A and oligomycin but in the presence of ruthenium red gave the same results.

Differential centrifugation. The homogenate was centrifuged for 10 min at four different speeds. The supernatants of these centrifugations were designated S5 (5,000 rpm), S10 (10,000 rpm), S15 (15,000 rpm) and S20 (20,000 rpm), respectively. The activity of marker enzymes, $^{45}Ca^{2+}$ uptake, and Ca^{2+} pumping (assessed by Ca^{2+}-sensitive electrodes) in these supernatants were compared with the corresponding values in the homogenate.

As shown in Fig. 2, the marker for primary granules (β-glucuronidase) and secondary granules (vitamin B_{12}–binding protein) disappeared almost entirely at relatively low centrifugation speeds 5,000 and 10,000 rpm, respectively. Markers for the endoplasmic reticulum (sulfatase C) and the plasma membrane (alkaline phosphatase) sedimented at higher centrifugation

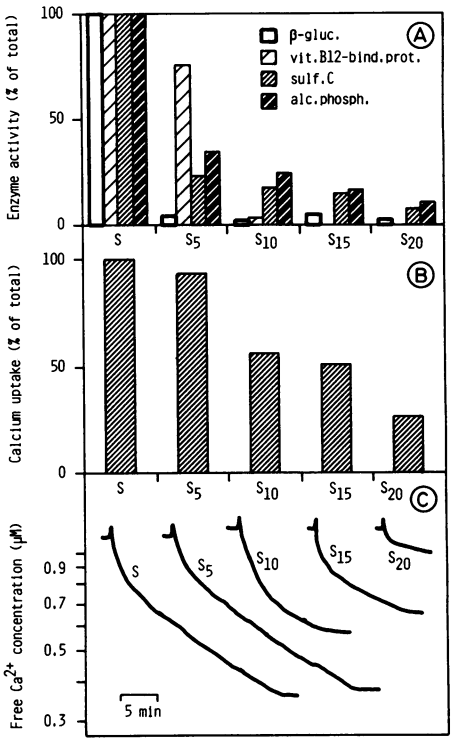

Figure 2. Activity of marker enzymes (*A*), uptake of $^{45}Ca^{2+}$ (*B*), and changes in ambient free Ca^{2+} concentration (*C*) in fractions obtained by differential centrifugations at various speeds. Activity of marker enzymes and $^{45}Ca^{2+}$ uptake in the different supernatants are shown as percent of the total activity in the homogenate (typical experiment repeated three times). Changes in ambient free Ca^{2+} concentration were measured with a Ca^{2+}-sensitive electrode as described in Fig. 1. Both $^{45}Ca^{2+}$ uptake and measurement of free [Ca^{2+}] were performed using a buffer that contained 100 mM KCl, 2 mM KH_2PO_4, 25 mM Hepes, 1 mM $MgCl_2$, 2 mM MgATP, 10 mM phosphocreatine, 8 U/ml creatine kinase, and 0.05% of bovine serum albumin. Antimycin A (200 nM) and oligomycin (2 μg/ml) were included in the incubation medium to inhibit mitochondrial Ca^{2+} pumping. The final protein concentration was ≈ 1 mg/ml. *S* represents the homogenate, *S5, S10, S15,* and *S20* the supernatants of centrifugations at 5,000, 10,000, 15,000 and 20,000 rpm for 10 min, respectively.

speed. Ca^{2+} pumping activity sedimented at even higher centrifugation speeds than microsomal markers. For example, when 80 and 90% of these markers were in the pellet, 50 and 30%, respectively, of the total Ca^{2+} pumping activity remained in the supernatant. The resolution of the different organelles was quite poor with this technique, and a more detailed analysis of the role of the different granules and microsomal components in Ca^{2+} homeostasis could only be achieved with Percoll gradients. Nevertheless, the results obtained with the differential centrifugation demonstrate that most of the Ca^{2+} pumping activity sediments with light organelles and that there is no major role for granules in Ca^{2+} pumping. In addition these results provide an important control because they were obtained with a technique of separation in which Percoll was not used.

Separation of granules and microsomes. A Percoll gradient that separates primary granules (fraction at the bottom of the gradient = fraction 1), secondary granules (fraction in the middle of the gradient = fraction 2), and microsomes (fraction at the top of the gradient = fraction 3), described by Borregard et al. (32), was used. As shown in Fig. 3, Ca^{2+} pumping activity and

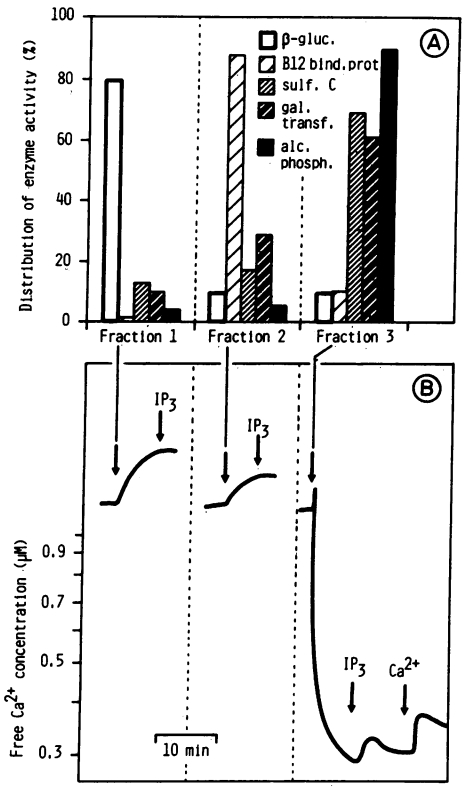

Figure 3. Primary granules, secondary granules, and microsomes: regulation of ambient free Ca^{2+} concentration and response to Ins 1,4,5-P_3. (*A*) shows the distribution of marker enzymes (percent of total activity in the gradient) in three different fractions (typical experiment repeated three times). Changes in ambient free Ca^{2+} concentration (*B*) were measured as described in Fig. 1. Antimycin A (200 nM) and oligomycin (2 μg/ml) were included in the incubation medium. The final protein concentration was ≈ 0.8 mg/ml. Where indicated, 2 μM Ins 1,4,5-P_3 (*IP_3*) and 1 μM $CaCl_2$ (*Ca^{2+}*) were added.

responses to Ins 1,4,5-P_3 were absent in both primary and secondary granules. In contrast, the microsomal fraction was able to lower ambient [Ca^{2+}] to values ~ 300 nM within 10 min. Like in the homogenate, this Ca^{2+} pumping was dependent on the presence of MgATP and could be inhibited by 2 mM Vanadate (Table II). Ins 1,4,5-P_3 was able to release Ca^{2+} from this fraction. The response to Ins 1,4,5-P_3 was only observed if the homogenization and fractionation procedure were performed very rapidly and at 4°C.

Table III shows the distribution of the mitochondrial marker enzyme monoamine oxidase and the distribution of mitochondrial Ca^{2+} pumping in the absence of MgATP and the presence of succinate, a substrate for the respiratory chain of mitochondria. These results clearly demonstrate that (*a*) there is a very good correlation between the distribution of mitochondrial Ca^{2+} pumping and the distribution of the mitochondrial marker enzyme monoamine oxidase, and (*b*) there is no correlation between the MgATP-dependent Ca^{2+} pool (100% in fraction 3, as shown in Fig. 3) and mitochondria (~ 75% in fraction 2).

Subfractionation of microsomes. To search for a correlation between various microsomal organelles and Ca^{2+} pumping, we developed a two-step Percoll procedure. The first step rapidly separated microsomes from granules, and the second step partially separated the different microsomal organelles. Five fractions were obtained and designated fraction 1 to fraction 5, from the bottom to the top. The separation procedure, performed immediately after homogenization, required ~ 80 min to complete. Because the Ins 1,4,5-P_3 response could be observed for no longer than 40 min after homogenization, no attempt was made to determine the Ins 1,4,5-P_3 response in these fractions.

Table III. Distribution of the Mitochondrial Marker Enzyme Monoamine Oxidase and Mitochondrial Ca^{2+} Uptake in a Gradient That Separates Primary Granules (Fraction 1), Secondary Granules (Fraction 2), and Microsomes (Fraction 3)

	Fraction 1	Fraction 2	Fraction 3
Monoamine oxidase (% gradient distribution)	1±2	78±14	21±12
Mitochondrial Ca^{2+} uptake (% gradient distribution)	2±1	72±7	26±1

Values are given as percentage of the activity in the whole gradient (mean±SD, $n = 4$ for monoamine oxidase and $n = 3$ for mitochondrial Ca^{2+} uptake). Mitochondrial Ca^{2+} uptake was measured as uptake of $^{45}Ca^{2+}$ in the absence of MgATP at 37°C and pH 7.0 in a buffer containing 100 mM KCl, 2 mM KH_2PO_4, 25 mM Hepes, 1 mM $MgCl_2$, 5 mM succinate, 2 μg/ml oligomycin (to inhibit mitochondrial ATP production), and 0.05% of bovine serum albumin.

As shown in Fig. 4, the maximal activity of the endoplasmic reticulum marker, sulfatase C, was found in fraction 2; the maximal activities of the marker for plasma membrane, alkaline phosphatase, and the Golgi, galactosyl transferase, were found in fraction 4 (Fig. 4 *A*). This distribution of microsomal marker enzymes corresponds well to the results obtained by Epping and Bygrave, when they performed a similar Percoll gradient with liver cell homogenate (34). The maximal activity of the nonmitochondrial Ca^{2+} pumping, whether determined by uptake of $^{45}Ca^{2+}$ or by the Ca^{2+}-sensitive electrode, was found in fraction 5 (Fig. 4, *B* and *C*).

The Ca^{2+} uptake in this fraction was dependent on the presence of MgATP and could be inhibited by 2 mM vanadate. As in the homogenate, neither 10 μM digitonin nor 30 μM vanadate inhibited Ca^{2+} pumping (Table II).

Table IV shows the mean±SD of the distribution of the three different markers and the $^{45}Ca^{2+}$ uptake in these fractions.

Evaluation of the data using the least squares regression method (Table V) shows a negative correlation between the endoplasmic reticulum marker and the Ca^{2+} uptake ($r = -0.49$) and a positive correlation between the marker for plasma membrane and Ca^{2+} uptake ($r = 0.54$) and the marker for Golgi and Ca^{2+} uptake ($r = 0.64$).

Because the negative correlation between the Ca^{2+} pumping activity and the endoplasmic reticulum was an unexpected finding, we compared in additional experiments the distribution of sulfatase C in the five fractions with another endoplasmic reticulum marker, NADPH-cytochrome C reductase. Calculations with least square regression method showed a good correlation between the sulfatase C and NADPH-cytochrome C reductase ($r = 0.69$, $P \leq 0.01$, $n = 20$) and a negative correlation between Ca^{2+} pumping and NADPH-cytochrome C reductase ($r = -0.52$).

Localization of endosomes in the microsomal subfractions (HR peroxidase uptake). In different cell types, endosomes have been found in the light fractions of gradients (46–48). Because

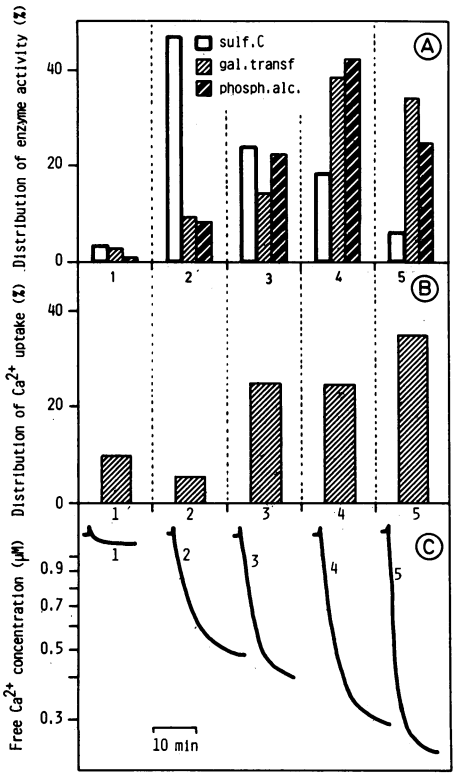

Figure 4. Microsomal subfractions: $^{45}Ca^{2+}$ uptake and capacity to lower ambient free Ca^{2+} concentration. (*A*) The distribution of the marker enzymes (% of total activity in the gradient) in five different fractions is shown (typical experiment repeated five times). (*B*) shows the distribution of the $^{45}Ca^{2+}$ uptake (% gradient distribution) in the same fraction (typical experiment repeated three times). Changes in ambient free Ca^{2+} concentration (*C*) were measured as described in Fig. 1. Both $^{45}Ca^{2+}$ uptake and measurement of free [Ca^{2+}] were performed using a buffer that contained 100 mM KCl, 2 mM KH_2PO_4, 25 mM Hepes, 1 mM $MgCl_2$, 2 mM MgATP, 10 mM phosphocreatine, 8 U/ml creatine kinase, and 0.05% of bovine serum albumin. Antimycin A (200 nM) and oligomycin (2 μg/ml) were included in the incubation medium to inhibit mitochondrial Ca^{2+} pumping. The final protein concentration was ≈ 0.5 mg/ml. Statistics on distribution of enzymes and $^{45}Ca^{2+}$ uptake are given in Table IV and Table VI.

these vesicles might play a role in intracellular Ca^{2+} regulation, we investigated their subcellular distribution in the above described microsomal subfractions. No specific biochemical marker exists for these organelles, however they can be identified by the localization of endocytosed material at early time points of endocytosis (46–52). Using a protocol similar to that described for macrophages (50), we loaded endosomes with HR peroxidase and performed the two-step Percoll gradient described above to obtain microsomal subfractions. Residual myeloperoxidase activity from neutrophil granules was found only in the lower microsomal subfractions and was subtracted from the total peroxidase activity. The results (Table VI) show that most of the endosomes are found in the light microsomal subfractions with a maximum in fraction 5. Therefore, interestingly, a very good correlation was found between the distribution endosomes and Ca^{2+} uptake in this gradient (r = 0.96, least squares regression method; see also Table V).

Comparative electron microscopy of homogenate, microsomes, and microsomal subfraction 5. Fig. 5 *A* shows a typical homogenate containing abundant granules and relatively few microsomal structures. Fig. 5 *B* shows the whole microsomal fraction (fraction 3 of Percoll procedure 1) containing mostly heterogenous vesicular material. The microsomal subfraction 5 (Fig. 5 *C*) shows vesicular material less heterogenous than that observed in the whole microsomal fraction. No rough endoplasmic reticulum or mitochondria and hardly any granules could be seen in this fraction.

Discussion

In this study, we analyzed the subcellular distribution of nonmitochondrial Ca^{2+} pumping sites in human neutrophils. A homogenate of neutrophils that had a pattern of Ca^{2+} pumping similar to digitonin permeabilized neutrophils (6) provided the starting material for three different separation techniques. Mitochondria, which showed a remarkable ability to pump Ca^{2+} in the homogenate despite their paucity in neutrophils, were appropriately inhibited. The following conclusions were drawn:

(*a*) Virtually all Ca^{2+} pumping activity and the Ins 1,4,5-P_3-induced Ca^{2+} release was located in the microsomal fraction.

Table IV. Distribution of Marker Enzymes and $^{45}Ca^{2+}$ Uptake in Microsomal Subfractions

		Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Alkaline phosphatase	Distribution	5.4±4.4	17±7.7	24±11.7	30±9.8	23.3±11.5
	Enrichment	0.5±0.5	3.6±2.3	5.8±3.1	7.1±3.3	6.6±2.8
	Recovery	3.6±2.9	9.7±4.9	12.4±5.4	18.7±9.5	15.5±9.3
Galactosyl transferase	Distribution	7.9±4.6	14.8±6.5	20.2±6.4	31.2±4.8	25.6±14.1
	Enrichment	ND	ND	ND	ND	ND
	Recovery	ND	ND	ND	ND	ND
Sulfatase C	Distribution	13.1±10.6	33.1±9.7	27.2±5.2	14.2±6.0	10.6±7.5
	Enrichment	0.8±0.4	3±1.3	4.4±3.7	1.6±1.1	1.48±1.1
	Recovery	7.8±5.7	18.6±7.2	13.2±4.5	6.3±3.9	3.9±3.6
Uptake $^{45}Ca^{2+}$	Distribution	5.4±3.8	6.0±5.1	20.7±13.8	19.3±5.0	46.4±22.7
	Enrichment	0.4±0.4	0.5±0.4	2.2±1.5	1.9±0.4	4.7±0.9
	Recovery	2±1.2	2.6±2.3	8.7±6.7	7.8±2.0	19.7±9.9

Distribution (percentage of the total activity in the gradient), enrichment (specific activity in the fraction divided by the specific activity in the postnuclear supernatant), and recovery (percentage of activity in the microsomal starting material) of microsomal marker enzymes and $^{45}Ca^{2+}$ uptake in five microsomal subfractions. Values are the mean±SD of five (alkaline phosphatase, galactosyl transferase, sulfatase C) or three (uptake $^{45}Ca^{2+}$) different experiments. $^{45}Ca^{2+}$ uptake was measured as described in the legend to Fig. 2.

Table V. Correlation of Different Markers with Uptake of $^{45}Ca^{2+}$ in Microsomal Subfractions

Organelle	Marker enzyme	Correlation with $^{45}Ca^{2+}$ uptake
Endoplasmic reticulum	Sulfatase C	−0.49
Endoplasmic reticulum	NADPH cytochrome C red.	−0.52
Golgi	Galactosyl transferase	0.62
Plasma membrane	Alkaline phosphatase	0.47
Endocytic vesicles	Uptake of HR peroxidase	0.96

Data used to calculate correlation coefficients by the least square regression method are those described in Tables IV (marker enzymes) and VI (HR peroxidase uptake).

Neither primary nor secondary granules participated in intracellular Ca^{2+} homeostasis in neutrophils.

(*b*) There was a negative correlation between the distribution of Ca^{2+} pumping activity and the distribution of endoplasmic reticulum markers.

(*c*) The maximal Ca^{2+} uptake occurred in light vesicular subfractions of microsomes.

Although separation of organelles by differential centrifugation was quite poor in comparison to Percoll gradients, the qualitative results were similar. These results rule out the possibility that Percoll selectively damaged a putative Ca^{2+} transport system of neutrophil granules.

During neutrophil maturation, granules become the predominant organelle and concomitantly there is a progressive loss of endoplasmic reticulum and ribosomal material (53, 54), which reflects the low activity of synthetic processes in the mature cells. In mature neutrophils, hardly any endoplasmic reticulum elements can be detected by electron microscopy (53, 54). Therefore neutrophils are an interesting model to investigate the role of nonmitochondrial Ca^{2+} pools, other than the endoplasmic reticulum, in intracellular Ca^{2+} homeostasis.

Due to the predominance of granules in neutrophils, it was important to investigate whether granules were able to pump Ca^{2+} and respond to Ins 1,4,5-P_3. Our finding that granules do not participate in Ca^{2+} homeostasis differs with another study in neutrophils (27). However, there are two major criticisms concerning this previous study. First, the granule preparation was obtained by differential centrifugation. This technique does not resolve properly the different neutrophil organelles (see Fig. 2). Therefore the possibility exists that the granule preparation used in this previous report was contaminated by mitochondria and microsomal structures. Second, mitochondrial Ca^{2+} pumping was insufficiently inhibited; only inhibitors of the respiratory chain (antimycin A or sodium azide) and no inhibitor of the mitochondrial proton ATPase (oligomycin) were used in the presence of ATP (see Fig. 1 and references 44, 45).

We attempted to localize more precisely the organelles responsible for Ca^{2+} pumping in the microsomal fraction by developing a two-step Percoll procedure that separated endoplasmic reticulum markers from Golgi and plasma membrane markers.

In other cell types endoplasmic reticulum is generally believed to be an important site for nonmitochondrial intracellular Ca^{2+} pumping (24–26, 55–58). As pointed out above, neutrophils are very poor in endoplasmic reticulum content. In studies with permeabilized cells, however, neutrophils possess intracellular Ca^{2+} pumping activity (6) comparable to cells much richer in total endoplasmic reticulum content (59–61). Therefore the role of endoplasmic reticulum in intracellular Ca^{2+} homeostasis of neutrophils was uncertain. In fact, in our microsomal subfractions of neutrophils the amount of endoplasmic reticulum was inversely related to the quantity of Ca^{2+} uptake. These data do not exclude the possibility that endoplasmic reticulum of neutrophils pumps Ca^{2+}, but they clearly demonstrate that this possible pumping is of no quantitative importance.

Even if there was some correlation between Ca^{2+} pumping and plasma membrane distribution, two observations strongly argue against pumping by plasma membrane inside-out vesicles in our fractions. The Ca^{2+} pumping activity of plasma membrane vesicles is around 100-fold more sensitive to vanadate (7) than the Ca^{2+} pumping activity of permeabilized cells (6) or the homogenate and light vesicular fraction from the Percoll gradient in the present study. A high vanadate sensitivity of plasma membrane and low vanadate sensitivity of intracellular Ca^{2+} pumping organelles has been observed not only in neutrophils but also in other tissues (62–68). In addition, the homogenate and the light vesicular fraction were not sensitive to digitonin at concentrations known to permeabilize the plasma membrane of neutrophils (6).

Table VI. Total Activity, Specific Activity, and Distribution of HR Peroxidase in Microsomal Subfractions

	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Total activity of peroxidase	0.13±0.03 (0.23±0.09)	0.14±0.05 (0.21±0.09)	0.09±0.02 (0.18±0.07)	0.13±0.07 (0.26±0.04)	0.47±0.23 0.6±0.09
Control (residual granular peroxidase)	0.16±0.11 (0.17±0.06)	0.1±0.03 (0.14±0.03)	0.03±0.02 (0.06±0.04)	0.01±0.01 (0.01±0.02)	0.02±0.03 (0.04±0.06)
Total control (uptake of HR peroxidase)	0 (0.06)	0.04 (0.07)	0.06 (0.012)	0.12 (0.25)	0.45 (0.56)
Distribution of HR peroxidase	0	5.9%	8.9%	17.9%	67.1%

Neutrophils were incubated with HR peroxidase for 10 min at 37°C. Peroxidase activity was measured in microsomal subfractions (see Fig. 4 and Table IV). Cells that were not incubated with HR peroxidase served as control for residual granular peroxidase. Values are expressed as total activity (U/ml) and in parentheses specific activity (U/mg protein). Distribution of HR peroxidase indicates percentage of total gradient activity in each fraction. Results are the means±SD of four experiments.

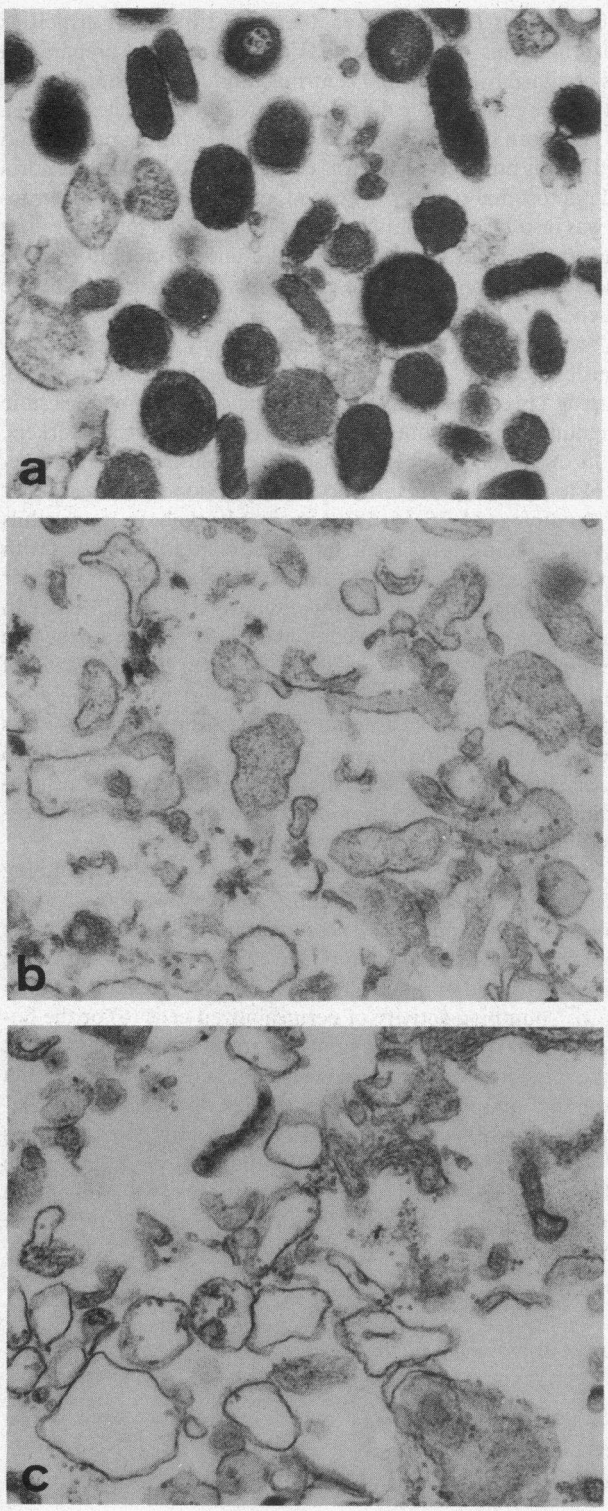

Figure 5. Electron microscopy of (*a*) homogenate, (*b*) total microsomes (fraction 3 of Fig. 3), and (*c*) microsomal subfraction 5 (fraction 5) of Fig. 4). × 33,000. See text for description.

Because the main Ca^{2+} pumping organelles in neutrophils seem unlikely to be the endoplasmic reticulum or plasma membrane vesicles, other possibilities must be considered:

(*a*) A very good correlation between maximal Ca^{2+} pumping activity and markers for endosomes was found. Human neutrophils are active in ligand-dependent and -independent endocytosis, and the subsequently created vesicles could be involved in intracellular Ca^{2+} pumping.

(*b*) Our data is compatible with a role for Golgi elements in Ca^{2+} homeostasis in neutrophils. This would be consistent with results obtained in the lactating mammary glands (69), where a purified Golgi fraction was demonstrated to possess Ca^{2+} transporting activity.

(*c*) It is worth considering the possibility that there may be yet undefined specialized structures that regulate intracellular free Ca^{2+} concentration in neutrophils.

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