Organization of Ca^{2+} stores in myeloid cells: association of SERCA2b and the type-1 inositol-1,4,5-trisphosphate receptor

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In this study, we have analysed the relationship between Ca^{2+} pumps and Ins(1,4,5)P_3-sensitive Ca^{2+} channels in myeloid cells. To study whether sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-type Ca^{2+}-ATPases are responsible for Ca^{2+} uptake into Ins(1,4,5)P_3-sensitive Ca^{2+} stores, we used the three structurally unrelated inhibitors thapsigargin, 2,5-di-t-butylhydroquinone and cyclopiazonic acid. In HL-60 cells, all three compounds precluded formation of the phosphorylated intermediate of SERCA-type Ca^{2+}-ATPases. They also decreased, in parallel, ATP-dependent Ca^{2+} accumulation and the amount of Ins(1,4,5)P_3-releasable Ca^{2+}. Immunoblotting with subtype-directed antibodies demonstrated that HL-60 cells contain the Ca^{2+} pump SERCA2 (subtype b), and the Ca^{2+}-release-channel type-1 Ins(1,4,5)P_3 receptor. In subcellular fractionation studies, SERCA2 and type-1 Ins(1,4,5)P_3 receptor co-purified. Immunofluorescence studies demonstrated that both type-1 Ins(1,4,5)P_3 receptor and SERCA2 were evenly distributed throughout the cell in moving neutrophils. During phagocytosis both proteins translocated to the periphagosomal space. Taken together, our results suggest that in myeloid cells (i) SERCA-type Ca^{2+}-ATPases function as Ca^{2+} pumps of Ins(1,4,5)P_3-sensitive Ca^{2+} stores, and (ii) SERCA2 and type-1 Ins(1,4,5)P_3 receptor reside either in the same or two tightly associated subcellular compartments.

INTRODUCTION

Intracellular Ca^{2+} stores play an important role during cellular activation. Ca^{2+} release from these stores is mediated through two families of Ca^{2+} release channels: ryanodine receptors and Ins(1,4,5)P_3 receptors. In myeloid cells, type-1, type-2 and type-3 Ins(1,4,5)P_3 receptors have been identified [1–4], whereas there is no conclusive evidence for the presence of ryanodine receptors in these cells. Ca^{2+} accumulation into stores may be mediated through a family of **S**arcoplasmic/**E**ndoplasmic **R**eticulum **C**a^{2+}-**A**TPases (SERCAs). SERCA1 and SERCA2a are exclusively expressed in muscle tissue. SERCA2b is ubiquitously distributed in non-muscle cells and is the major candidate to act as the Ca^{2+} pump of Ins(1,4,5)P_3-sensitive Ca^{2+} stores. In addition, SERCA3 is found in various non-muscle cell types (references for the distribution of SERCA-type Ca^{2+}-ATPases include [5–11]). However, Ca^{2+}-uptake mechanisms other than SERCA-type Ca^{2+}-ATPases might participate in Ca^{2+} uptake into Ins(1,4,5)P_3-sensitive Ca^{2+} stores: in some cellular systems, inhibitors of SERCA-type Ca^{2+}-ATPases do not interfere with Ca^{2+} storage in Ins(1,4,5)P_3-sensitive Ca^{2+} stores [12,13], and Ca^{2+} uptake through a H^+-dependent mechanism [12,14] or through a 140 kDa 'Ca^{2+}-ATPase-like protein' [15] has been suggested.

Even in systems where the role of SERCA-type Ca^{2+}-ATPase in intracellular Ca^{2+} accumulation is clearly established, Ca^{2+} release channels and Ca^{2+} pumps do not necessarily co-localize in the same intracellular compartment. The best studied example is the skeletal muscle sarcoplasmic reticulum (SR), where Ca^{2+} pumps are found in the longitudinal SR, while Ca^{2+} release channels are found in the terminal cysternae. Accordingly, after subcellular fractionation of muscle homogenates Ca^{2+}-ATPases and Ca^{2+} release channels are recovered in fractions of different densities. Separate sites for Ca^{2+} uptake and Ca^{2+} release have also been suggested by studies in several types of non-muscle cells [16–19].

In this study we demonstrate, in subcellular fractionation and immunofluorescence experiments, a close association between SERCA2b and the type-1 Ins(1,4,5)P_3 receptor and, in functional experiments, the role of SERCA-type Ca^{2+}-ATPases in the loading of Ins(1,4,5)P_3-sensitive Ca^{2+} stores.

MATERIALS AND METHODS

Materials

SDS/PAGE reagents were purchased from Bio-Rad. Alkaline phosphatase-coupled secondary antibodies, 5-bromo-4-chloro-3-indolyl phosphate, Nitro Blue Tetrazolium, creatine phosphokinase, creatine phosphate, thapsigargin, cyclopiazonic acid, MgATP, ionomycin, digitonin, SDS, Hepes, *N*-(hydroxyethyl)ethylenediaminetriacetic acid (HEDTA), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), antimycin A and oligomycin were obtained from Sigma (St. Louis, MO, U.S.A.). Di-isopropyl fluorophosphate (DFP) and 2,5-di-t-butylhydroquinone were from Fluka (Ronkonkoma, NY, U.S.A.), Ins(1,4,5)P_3 from L.C. Service Corp. (Woburn, MA, U.S.A.), $^{45}Ca^{2+}$ and [^{32}P]ATP were purchased from DuPont de Nemours/New England Nuclear Inc. (Dreieich, Germany). ^{35}S-labelled Protein A and ECL system were from Amersham International (Amersham, Bucks., U.K.). Acrylamide was from Kimberly Research (Manville, NJ, U.S.A.). All other reagents used were of analytical grade.

Abbreviations used: DFP, di-isopropyl fluorophosphate; HEDTA, *N*-(hydroxyethyl)ethylenediaminetriacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

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Purification of neutrophils, culture of HL-60 cells and subcellular fractionation procedures

Neutrophils were purified from the blood of healthy donors as previously described [20]. HL-60 cells were grown as described previously [21]. To obtain homogenates, cells were disrupted by nitrogen cavitation followed by a low-speed centrifugation. To obtain a 'particulate fraction', homogenates were pelleted by a centrifugation of 160000 ***g*** for 60 min. To obtain subcellular fractions, the homogenates were first separated by a discontinuous Percoll gradient. Three fractions, F1, F2, F3 (in order of decreasing density), were obtained. The light-density fraction of the Percoll gradient (F3) was further separated on a discontinuous sucrose gradient. Three fractions, S1, S2, S3 (in order of decreasing density), were obtained. For a more detailed description of the method see [21]. Protein concentrations were determined according to a modified Lowry procedure [22].

SDS/PAGE and immunochemical analysis of HL-60 protein

Proteins were separated by 3–12 %-acrylamide SDS/PAGE and transferred on to Immobilon poly(vinylidene difluoride) membrane (Millipore, U.S.A.) by standard methods. The transfers were blocked with PBS containing 0.05 % Tween-20 and 0.5 % non-fat dry milk prior to incubation with the primary antibody. ^{35}S-Protein A, alkaline phosphatase-coupled or peroxidase-coupled secondary antibodies were used to develop immunoblots. Quantitative analysis was carried out using either a densitometer or a phosphoimager (Molecular Dynamics, U.S.A.).

Antibodies used in this study

Polyclonal antibodies against the 20 N-terminal amino acids of SERCA2 (α-Nterm SERCA2) and the 15 C-terminal amino acids of the type-1 Ins(1,4,5)P_3 receptor (α-Cterm InsP_3R) were raised in our laboratory using synthetic peptides as described previously [21]. A SERCA2b-specific antibody, raised against the 12 C-terminal amino acids of SERCA2b (α-Cterm SERCA2b) was kindly provided by Dr. Franck Wuytack, Leuven, Belgium [7]. α-Nterm SERCA2 did not stain any bands in immunoblots of skeletal muscle microsomes, suggesting that it did not recognize SERCA1 (results not shown). Based on the low sequence similarity of the N-terminus of SERCA2b and SERCA3, a cross-reaction of α-Nterm SERCA2 with SERCA3 appears unlikely. The specificity of the anti-SERCA2b antibodies has been established previously [7]. α-Cterm InsP_3R did not stain any bands in immunoblots of skeletal muscle microsomes, suggesting that it did not recognize the skeletal muscle ryanodine receptor (results not shown). Given the low degree of similarity in the C-terminal region of Ins(1,4,5)P_3 receptors, α-Cterm InsP_3R is unlikely to cross-react with other Ins(1,4,5)P_3 receptor isoforms. In addition, a polyclonal anti-peptide antibody raised against the same type-1 Ins(1,4,5)P_3 receptor sequence as our antibody has been shown not to recognize type-2 Ins(1,4,5)P_3 receptor [23].

Formation of the phosphorylated intermediate

SERCA phosphoenzyme levels were assessed using the method described by Heilmann et al. [24]. Particulate fractions or subcellular fractions of HL-60 cells (0.25 mg of protein/ml) were incubated at 4 °C with 5–10 nM [γ-^{32}P]ATP (specific radioactivity approx. 5000 Ci/mmol) in a buffer containing 100 mM KCl, 1 mM $MgCl_2$, 50 μM Ca^{2+}, 20 mM Hepes, pH 6.8. For control conditions (Figure 1A), 50 μM Ca^{2+} was omitted and 1 mM EGTA was added. The reaction was stopped after 15 s by addition of 0.33 vol. of 40 % (v/v) glycerol, 260 mM Tris, pH 6.8, 4 % 2-mercaptoethanol and 12 % SDS; samples were immediately subjected to gel electrophoresis (10 % acrylamide). Phosphoproteins were visualized by autoradiography. The conditions chosen for the assessment of the phosphorylated intermediate (pH 6.8 in the sample buffer and pH 8.0 in the running buffer) precluded detection of the plasma membrane Ca^{2+}-ATPase ([25,26] see also below).

Tryptic digestion of the particulate fraction from HL-60 cells

Particulate fractions of HL-60 cells (4 mg of protein/ml) were subjected to controlled tryptic digestion by incubation for 15 min at 37 °C at the indicated protein/trypsin ratio in a buffer containing 100 mM KCl, 20 mM Hepes, pH 6.8, and 150 mM sucrose [26]. The reaction was stopped by addition of soybean trypsin inhibitor [ratio of trypsin/inhibitor: 2:1 (w/w)] and placing the samples on ice. Assays for formation of phosphorylated intermediate were performed immediately as described above.

$^{45}Ca^{2+}$ technique

For Ca^{2+}-flux measurements, HL-60 homogenates (250 μg/ml) were preincubated for 10 min at 30 °C in a buffer mimicking intracellular ionic conditions (120 mM KCl, 1 mM $MgCl_2$, 25 mM Hepes, pH 7.0) in the presence of an ATP-regenerating system (1 mM MgATP, 2.5 mM creatine phosphate, 4 units/ml creatine kinase) and mitochondrial inhibitors (0.2 μM antimycin A and 1 μg/ml oligomycin). Ca^{2+} uptake was initiated by addition of 200 nCi/ml $^{45}Ca^{2+}$ and 20 μM unlabelled Ca^{2+} (free cytosolic $[Ca^{2+}]_c = 200$ nM). Where indicated, Ca^{2+}-ATPase inhibitors were added 3 min prior to initiation of Ca^{2+} uptake. ATP-independent Ca^{2+} accumulation was subtracted and did not exceed 5 % of the total Ca^{2+} uptake. For the assessment of Ins(1,4,5)P_3-induced Ca^{2+} release, 5 μM Ins(1,4,5)P_3 was added during the last 15 s of the Ca^{2+} uptake assay. At the indicated time, 100 μl aliquots were taken in duplicate, transferred on to a 0.45 μm-pore-size filter (Millipore, HA type) and washed three times with 5 ml of a buffer containing 120 mM KCl, 1 mM $LaCl_3$ and 20 mM Hepes, pH 7.0. Filters were placed in a vial containing a liquid scintillation mixture (Ultima Gold; Packard, Meriden, CT, U.S.A.) and the radioactivity was measured using a Packard 1900 TR scintillation counter.

Immunocytochemistry and confocal microscopy

Immunofluorescence of phagocytosing neutrophils was performed as described previously [20]. Neutrophils were allowed to adhere to glass coverslips and to phagocytose opsonized heat-killed yeast in a moist chamber (37 °C). After 10 min, cells were fixed by a 60 min incubation with ice-cold paraformaldehyde (4 % in PBS, pH 7.3). The slides were washed twice (2 × 10 min) in PBS then permeabilized for 60 s in 0.3 % Triton X-100 diluted in PBS (pH 7.3), and finally washed twice (2 × 10 min) in PBS (pH 7.3). To reduce non-specific binding of the primary antibodies, 50 μl of normal pig immunoglobulin fraction (10 mg/ml; Dakopatts a/s, Copenhagen, Denmark) was added to each well on the slide and they were placed in a moist chamber at room temperature for 60 min, or overnight at 4 °C. The immunoglobulin fraction was 'poured' off the slides and the primary antibodies (described above), diluted 1:20 in PBS (pH 7.6) with 1 % (w/v) BSA, were added (10 μl/well). After 45 min incubation in a moist chamber at room temperature, the slides were washed three times (3 × 5 min) in PBS–BSA (1 %). Then, 50 μl of normal pig immunoglobulin fraction (4 mg/ml), in PBS–BSA (1 %), was

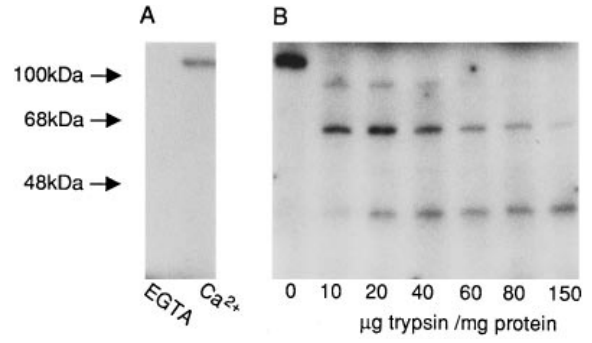

Figure 1 Characterization of a 100 kDa Ca^{2+}-ATPase in HL-60 cells

Particulate fractions of HL-60 cells (0.25 mg/ml) were incubated at 4 °C for 15 min in 100 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, pH 6.8, in the presence of 1 mM EGTA or 50 μM Ca^{2+} (**A**) and increasing concentrations of trypsin, 10 μg/mg of protein to 150 μg/mg of protein, in the presence of 50 μM Ca^{2+} (**B**). [^{32}P]ATP (25 mCi/mg of protein) was added for 15 s and proteins were separated by SDS/PAGE, ^{32}P-labelled protein was detected by autoradiography.

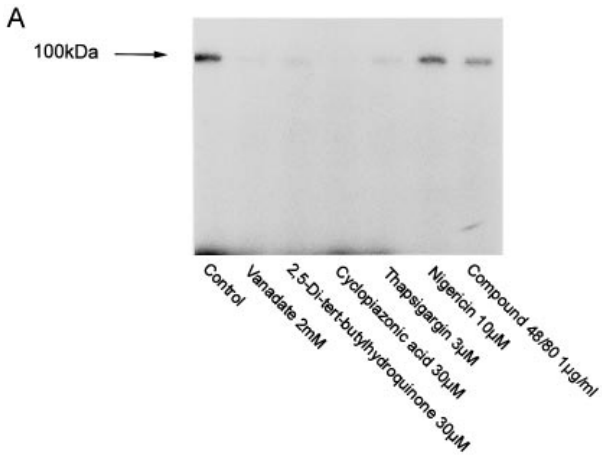

Figure 2 Inhibition of formation of phosphorylated intermediate by various Ca^{2+}-ATPase inhibitors

Particulate fraction of HL-60 cells was preincubated for 15 min with the indicated concentrations of vanadate, cyclopiazonic acid, 2,5-di-t-butylhydroquinone, thapsigargin, nigericin, or compound 48/80. The fractions were then assayed for the formation of a Ca^{2+}-ATPase phosphorylated intermediate as described in the Materials and methods section. The results are representative of three independent experiments.

added to each well and the slides were incubated in a moist chamber for another 60 min. Again the immunoglobulin fraction was poured off and 50 μl of the secondary rhodamine-conjugated pig anti-(rabbit IgG) antibody (0.012 mg/ml; Dakopatts a/s, Copenhagen, Denmark) in PBS–BSA (1 %) was added to each well, followed by a 45 min incubation. The slides were washed three times (3 × 5 min) in PBS–BSA (1 %) and twice (2 × 5 min) in PBS (pH 7.6). Finally the slides were mounted and examined.

RESULTS

When SERCA-type Ca^{2+}-ATPases are incubated with [^{32}P]ATP in the presence of Ca^{2+}, an approx. 100 kDa phosphorylated intermediate can be visualized on autoradiograms of polyacrylamide gels (see, for example, [27]). To selectively visualize the SERCA, but not the plasma membrane Ca^{2+}-ATPase, we took advantage of the different pH sensitivities of the respective phosphoproteins. The phosphorylated intermediate of the plasma membrane Ca^{2+}-ATPase is stable only at low pH and separation by acidic SDS/PAGE is necessary for phosphoprotein detection [28]. In contrast, SERCA-type Ca^{2+}-ATPases are readily detected at higher pH values using standard Läemmli-type SDS/PAGE [24,26,27,29]. Figure 1(A) shows that HL-60 cells contain SERCA-type Ca^{2+}-ATPases which undergo Ca^{2+}-dependent formation of an approx. 100 kDa phosphorylated intermediate. Owing to their large intramembrane domains, SERCA-type Ca^{2+} pumps show a typical pattern of limited tryptic digestion. Formation of 55 and 30 kDa tryptic fragments that are still able to catalyse the autophosphorylation reaction are observed for SERCA1 and 2 [25,30]. In contrast, formation of an approx. 80 kDa tryptic fragment is observed for SERCA3 [30,31]. After limited tryptic digestion of HL-60 particulate fractions, fragments of approx. 80, 55 and 30 kDa could be observed (Figure 1B). The 55 and 30 kDa fragments most likely reflect the presence of SERCA2b (see below). The additional presence of an 80 kDa band is consistent with recent studies that suggest the expression of SERCA3 in HL-60 cells [11].

Ca^{2+}-ATPase inhibitors preclude formation of the phosphorylated intermediate, ATP-dependent Ca^{2+} uptake and Ins(1,4,5)P_3-induced Ca^{2+} release

We have previously reported that three structurally unrelated compounds, thapsigargin, cyclopiazonic acid and 2,5-di-t-butylhydroquinone effectively deplete intracellular Ca^{2+} stores in intact HL-60 cells [32,33]. To study whether these inhibitors block SERCA-type Ca^{2+} pumps in HL-60 cells, we investigated their effects on formation of the phosphorylated intermediate. As shown in Figure 2, thapsigargin, cyclopiazonic acid and 2,5-di-t-butylhydroquinone inhibited formation of the phosphorylated intermediate. An inhibition was also observed with the non-specific ATPase inhibitor vanadate, while control substances (nigericin, compound 48/80) had no relevant effects.

To study whether the Ca^{2+}-ATPases inhibited through thapsigargin, cyclopiazonic acid and 2,5-di-t-butylhydroquinone might be responsible for Ca^{2+} uptake into Ins(1,4,5)P_3-sensitive pools, we studied the effect of the inhibitors on ATP-dependent Ca^{2+} uptake and the amount of Ins(1,4,5)P_3-releasable Ca^{2+} in HL-60 homogenates. Thapsigargin, cyclopiazonic acid, 2,5-di-t-butylhydroquinone, and vanadate led to a dose-dependent inhibition of ATP-dependent Ca^{2+} accumulation (Figure 3). The amount of Ins(1,4,5)P_3-releasable Ca^{2+} was diminished with a virtually identical potency by the four different compounds, suggesting that (i) there is no pharmacological difference between the Ca^{2+} pumps responsible for the all-over Ca^{2+} uptake and those responsible for Ca^{2+} uptake into Ins(1,4,5)P_3-sensitive Ca^{2+} stores, and (ii) SERCA-type Ca^{2+} pumps are responsible for Ca^{2+} uptake into Ins(1,4,5)P_3-sensitive Ca^{2+} stores. This is in accordance with previously published results in intact HL-60 cells, where a dose-dependent inhibition of agonist-induced Ca^{2+} release by thapsigargin, cyclopiazonic acid and 2,5-di-t-butylhydroquinone was observed [32]. However, the IC_{50} for depletion of Ca^{2+} stores in intact cells was about 40-fold higher for cyclopiazonic acid (2.8 μM) and 2,5-di-t-butylhydroquinone (2.6 μM), but approx. 20-fold lower for thapsigargin (3.8 nM). These differences might reflect the fact that thapsigargin is a very hydrophobic compound (i) that penetrates freely into intact cells, and (ii) whose biological effects would be better described as inhibitor/phospholipid ratio than as concentration in an aqueous solution. Because of the very high affinity of thapsigargin for SERCA, it is also possible that the different IC_{50} in cells compared

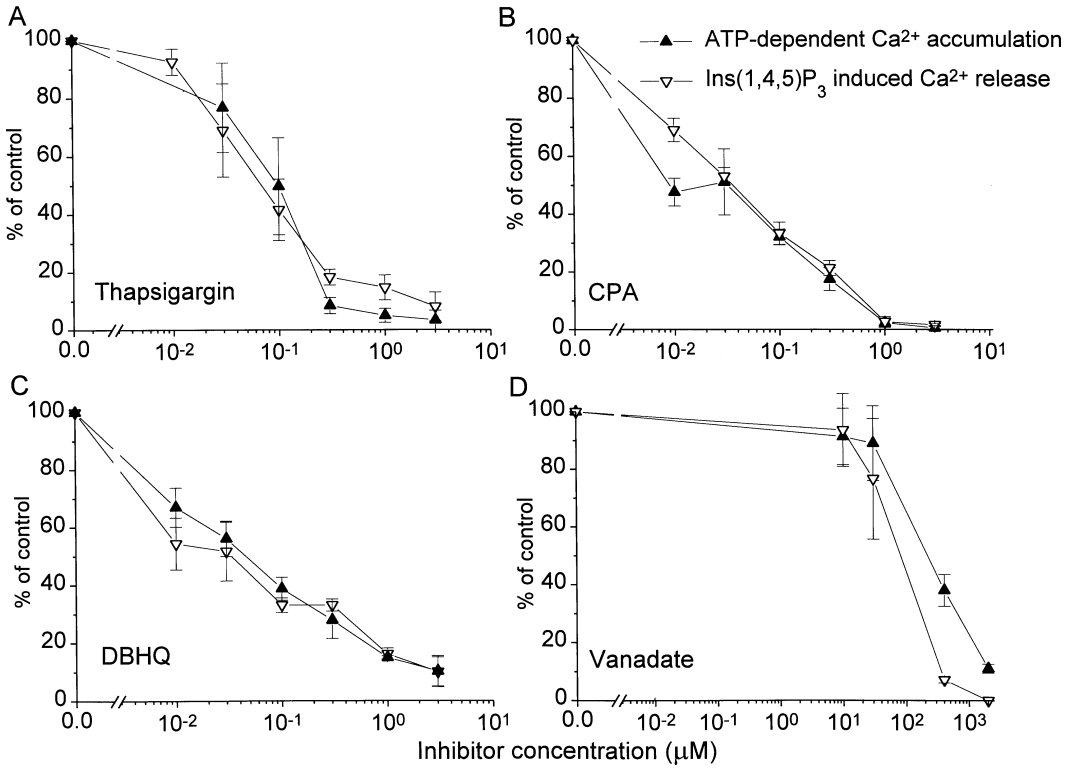

Figure 3 Effect of SERCA inhibitors on ATP-dependent Ca^{2+} accumulation and amount of Ins(1,4,5)P_3-releasable Ca^{2+}

HL-60 homogenates were, after a 3 min preincubation with the respective inhibitor at the indicated concentration, allowed to accumulate $^{45}Ca^{2+}$ for 10 min in the presence of ATP. ATP-dependent Ca^{2+} accumulation (▲) and amount of Ca^{2+} that could be released by 5 μM Ins(1,4,5)P_3 (▽) are shown as a function of the inhibitor concentration. The values were normalized as follows: the Ca^{2+} content of homogenates loaded in the absence of Ca^{2+}-ATPase inhibitors (2.5 to 3.2 nmol of Ca^{2+}/mg of protein) was defined as 100%. The amount of Ca^{2+} released by 5 μM Ins(1,4,5)P_3 in the absence of Ca^{2+}-ATPase inhibitors (1.4 to 1.6 nmol of Ca^{2+}/mg of protein) was defined as 100%. IC_{50} values were 68.5 ± 29.3 nM, 37.8 ± 2.2 nM, 30.3 ± 17.0 nM, and 177.3 ± 30.1 μM, for ATP-dependent Ca^{2+} accumulation and 91.7 ± 37.8 nM, 38.1 ± 10.3 nM, 46.8 ± 15.7 nM, and 61.5 ± 36.2 μM for Ins(1,4,5)P_3-induced Ca^{2+} release, for thapsigargin, cyclopiazonic acid (CPA), 2,5-di-t-butylhydroquinone (DBHQ) and vanadate respectively. Results are given as means ± S.E.M. of three independent experiments performed in duplicate.

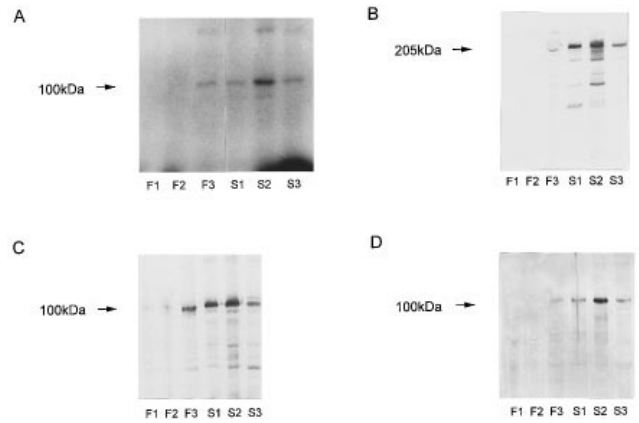

Figure 4 Distribution of SERCA2b and type-1 Ins(1,4,5)P_3 receptor in subcellular fractions of HL-60 cells

HL-60 cell homogenates were separated by a discontinuous Percoll gradient. Three subcellular fractions were obtained and are referred to, in order of decreasing density, as F1, F2 and F3. The light-density fraction F3 was further separated by a discontinuous sucrose gradient leading to another three subcellular fractions S1, S2 and S3 (referred to in order of decreasing density). (**A**) Ca^{2+}-dependent formation of a phosphorylated intermediate; (**B**, **C** and **D**) immunoblots using antibodies against type-1 Ins(1,4,5)P_3 receptor, SERCA2b and SERCA2 respectively. The antibodies are described in the Materials and methods section. Blots are representative from two to three independent experiments performed in duplicates.

with homogenate may simply be related to the relative amounts of SERCA in the two preparations.

Distribution of SERCA and Ins(1,4,5)P_3 receptor in subcellular fractions of HL-60 cells

We have previously developed a subcellular fractionation procedure to obtain subcellular fractions highly enriched in inositol-trisphosphate binding [21]. The procedure consists of a nitrogen cavitation, followed by Percoll- and sucrose-density gradients. As compared with the starting homogenate, Ins(1,4,5)P_3 binding and calreticulin content were enriched approx. 7-fold through the Percoll gradient (F3) and further enriched to approx. 25-fold through the sucrose gradient (S2).

In this study we have used the same fractions to analyse the subcellular distribution of the phosphorylated Ca^{2+}-ATPase intermediate (Figure 4A), and SERCA2 (Figures 4C and 4D) and type-1 Ins(1,4,5)P_3 receptor (Figure 4B). To obtain statistics, we normalized the specific protein content of the different fractions with respect to the most intensely stained fraction (S2 of the sucrose gradient), which was defined as 100 (Table 1). Clearly, SERCA2 and type-1 Ins(1,4,5)P_3 receptor showed a very similar distribution within the different fractions. There appeared to be a slightly different distribution of the phosphorylated intermediate and SERCA2 (Table 1, fraction S1). As the phosphorylated intermediate signal comes from both, SERCA2 and

Table 1 Relative content of phosphorylated intermediate, SERCA2b, type-1 Ins(1,4,5)P_3 receptor, and Ins(1,4,5)P_3-binding sites in subcellular fractions

Data shown in this Table represent statistics of a quantification of experiments shown in Figure 4 (columns 1, 2 and 3) or results from a previous study (column 4; [21]). Immunoblots and autoradiograms were quantified as described in the Materials and methods section. The protein content of the various fractions were normalized with respect to the most intensely stained fraction (S2 of the sucrose gradient), which was defined as 100. Data are mean ± S.E.M. [SERCA2b and Ins(1,4,5)P_3 binding; $n = 3$ and $n = 4$, respectively] and mean ± range [phosphorylated intermediate and type-1 Ins(1,4,5)P_3 receptor; $n = 2$]; all experiments were performed in duplicate.

	Phosphorylated intermediate (%)	SERCA2b (%)	Type-1 Ins(1,4,5)P_3 receptor (%)	Ins(1,4,5)P_3 binding (%)
F1	3 ± 3	2 ± 2	6 ± 8	1 ± 1
F2	7 ± 7	3.5 ± 3.5	3.5 ± 5	2 ± 1
F3	31 ± 11	33.5 ± 18	28 ± 24	24 ± 3
S1	27 ± 4	66 ± 7	63 ± 5	83 ± 20
S2	100	100	100	100
S3	32 ± 1	31 ± 7	38 ± 7	20 ± 11

SERCA3, this might indicate a partially different subcellular distribution of the two Ca^{2+}-ATPases. For comparison, we have also added previous results [21], showing Ins(1,4,5)P_3 binding in these fractions. The distribution of Ins(1,4,5)P_3 binding was very similar to the distribution of the type-1 Ins(1,4,5)P_3 receptor.

Immunofluorescence of Ins(1,4,5)P_3 receptor and Ca^{2+}-ATPase during chemotaxis and phagocytosis

We have recently shown that translocation of calreticulin and SERCA2 to the periphagosomal space occurs during phagocytosis in human neutrophils, suggesting a transport of intracellular Ca^{2+} stores to their site of action [34]. To investigate whether this Ca^{2+} store translocation includes Ins(1,4,5)P_3-sensitive Ca^{2+} stores, we performed confocal immunofluorescence studies using the antibody against the type-1 Ins(1,4,5)P_3 receptor and against SERCA2. Immunofluorescence staining of adherent, non-phagocytosing neutrophils showed an equal distribution of both SERCA2 and type-1 Ins(1,4,5)P_3 receptor throughout the entire cell (Figures 5A and 5C). During phagocytosis, both SERCA2 and type-1 Ins(1,4,5)P_3 receptor showed a similar translocation towards the periphagosomal space (Figures 5B and 5D).

DISCUSSION

Our results demonstrate a close association between SERCA2 and the type-1 Ins(1,4,5)P_3 receptor: (i) inhibition of SERCA-type Ca^{2+} pumps leads to an inhibition of Ins(1,4,5)P_3-induced Ca^{2+} release with identical dose–inhibition curves; (ii) an ex-

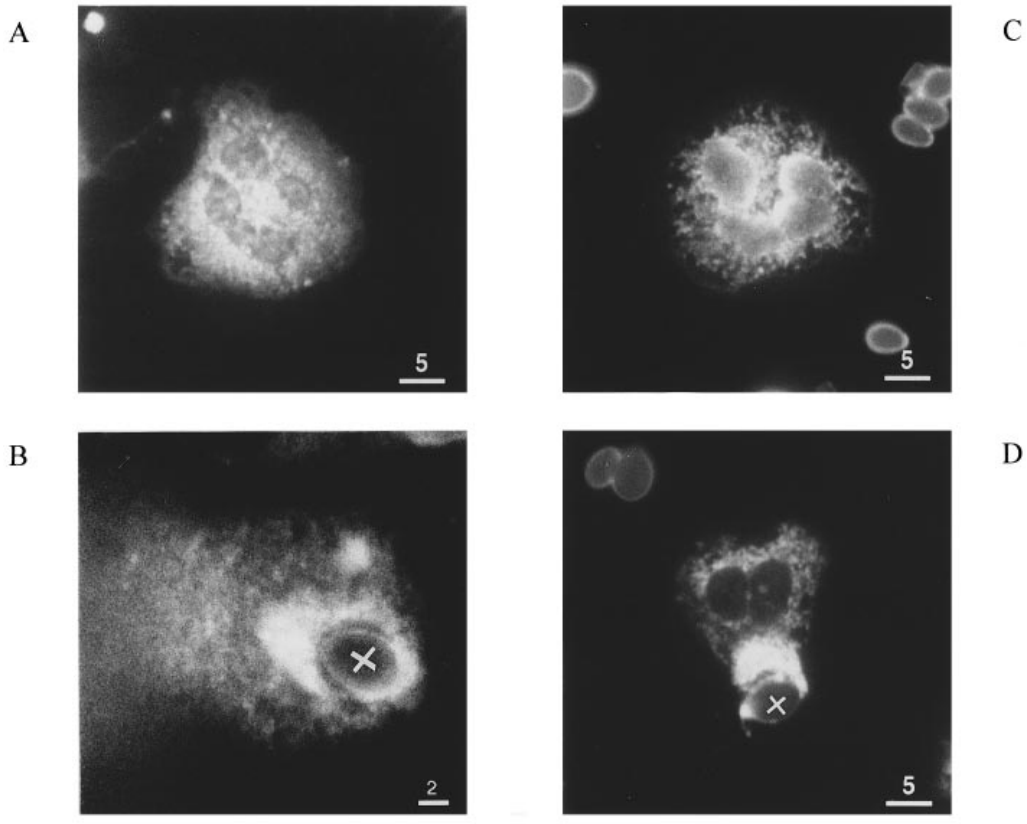

Figure 5 Distribution of Ca^{2+}-ATPase and Ins(1,4,5)P_3 receptor in resting and phagocytosing neutrophils

Non-phagocytosing (**A** and **C**) and phagocytosing (**B** and **D**) adherent neutrophils were permeabilized with 0.3% Triton and incubated either with antibodies against the type-1 Ins(1,4,5)P_3 receptor (**A** and **B**) or with antibodies against SERCA2 (**C** and **D**). Each panel shows rhodamine fluorescence from 1-μm-thick confocal serial sections through the centre. The white cross indicates the position of phagocytosed yeast particles. The numbers above the scale bars indicate their length (μm). The examples shown are representative of the cells from a total of three experiments. In control experiments without primary antibody, only a faint background fluorescence and no periphagosomal enrichment was observed.

tensive subcellular purification procedure yielded a co-purification of both proteins; and (iii) both proteins showed the same immunofluorescence pattern during chemotaxis and phagocytosis of neutrophils.

The Ca^{2+}-ATPase inhibitors thapsigargin, cyclopiazonic acid and 2,5-di-t-butylhydroquinone at appropriate concentrations are relatively selective for SERCA-type Ca^{2+}-ATPases, but do not enable us to distinguish between SERCA subtypes [35,36]. Thus, the functional data presented in Figure 3 do not provide evidence with respect to the involved SERCA and Ins(1,4,5)P_3 receptor isoforms. However, the data clearly demonstrate the function of SERCA-type Ca^{2+}-ATPases as Ca^{2+} pumps of Ins(1,4,5)P_3-sensitive Ca^{2+} stores in myeloid cells. This is particularly relevant with respect to reports from other cell types, that suggest Ca^{2+} uptake into Ins(1,4,5)P_3-sensitive Ca^{2+} stores through SERCA-unrelated mechanisms [12–15].

Previous studies in other non-muscle cells have raised the possibility that sites for Ca^{2+} uptake and for Ca^{2+} release might be localized in separate intracellular compartments [16–19]. The endoplasmic reticulum network, thought to contain intracellular Ca^{2+} stores, is broken into so-called microsomes during cell homogenization. Proteins that are differentially located within this network, should therefore be differentially recovered after subcellular fractionation. We have previously described [21] such a separation of Ca^{2+} store markers [calreticulin and Ins(1,4,5)P_3 binding] from other endoplasmic reticulum proteins that are unrelated to its Ca^{2+} store function (sulphatase C and cytochrome *c* reductase). Thus, our gradients are able to detect endoplasmic reticulum subfractions. However, in the present study, SERCA2 and type-1 Ins(1,4,5)P_3 receptor show a virtually identical subcellular distribution. Thus, our results strongly suggest that in HL-60 cells, SERCA2 and type-1 Ins(1,4,5)P_3 receptor reside within the same, or within closely associated compartments.

Changes in the subcellular localization of the Ins(1,4,5)P_3 receptor in response to cellular activation has been observed in *X. laevis* oocytes [37] and in lymphocytes [38]. Previous studies from our group revealed a dynamic redistribution of the Ca^{2+} store markers calreticulin and SERCA2 during phagocytosis in human neutrophils [34]. In this study we show that, in phagocytosing neutrophils, the type-1 Ins(1,4,5)P_3 receptor is similarly translocated to the periphagosomal space. Thus, it appears that entire Ins(1,4,5)P_3-sensitive Ca^{2+} stores, and not only Ca^{2+}-ATPase and calreticulin are translocated. This adds weight to the concept that the observed phenomenon corresponds indeed to translocation of organelles, rather than individual proteins. The parallel translocation of SERCA2 and type-1 Ins(1,4,5)P_3 receptor clearly argues in favour of a co-localization of the two proteins on the translocated organelles.

However, myeloid cells do not only contain SERCA2 and type-1 Ins(1,4,5)P_3 receptor, but also SERCA3 ([11]; see also Figure 1), type-2 Ins(1,4,5)P_3 receptor and type-3 Ins(1,4,5)P_3 receptor [2–4]. Although it is possible that these proteins localize to the same type of Ca^{2+} store, other possibilities have to be taken into consideration: distinct SERCA3/type-3 Ins(1,4,5)P_3 receptor Ca^{2+} stores might exist or a separation of Ca^{2+} release and Ca^{2+} uptake sites might be present on the level of these proteins.

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REFERENCES

1 Yamada, N., Makino, Y., Clark, R. A., Pearson, D. W., Mattei, M.-G., Guénet, J.-L., Ohama, E., Fujino, I., Miyawaki, A., Furuichi, T. and Mikoshiba, K. (1994) Biochem. J. **302**, 781–790
2 Sugiyama, T., Furuya, A., Monkawa, T., Yamamoto Hino, M., Satoh, S., Ohmori, K., Miyawaki, A., Hanai, N., Mikoshiba, K. and Hasegawa, M. (1994) FEBS Lett. **354**, 149–154
3 Sugiyama, T., Yamamoto Hino, M., Miyawaki, A., Furuichi, T., Mikoshiba, K. and Hasegawa, M. (1994) FEBS Lett. **349**, 191–196
4 Yamamoto Hino, M., Sugiyama, T., Hikichi, K., Mattei, M.-G., Hasegawa, K., Sekine, S., Sakurada, K., Miyawaki, A., Furuichi, T., Hasegawa, M. and Mikoshiba, K. (1994) Receptors-Channels **2**, 9–22
5 Brandl, C. J., Green, N. M., Korczak, B. and MacLennan, D. H. (1986) Cell **44**, 597–607
6 Anger, M., Samuel, J.-L., Marotte, F., Wuytack, F., Rappaport, L. and Lompré, A.-M. (1993) FEBS Lett. **334**, 45–48
7 Wuytack, F., Eggermont, J. A., Raeymaekers, L., Plessers, L. and Casteels, R. (1989) Biochem. J. **264**, 765–769
8 Burk, S. E., Lytton, J., MacLennan, D. H. and Shull, G. E. (1989) J. Biol. Chem. **264**, 18561–18568
9 Gunteski-Hamblin, A.-M., Greeb, J. and Shull, G. E. (1988) J. Biol. Chem. **263**, 15032–15040
10 Lytton, J. and MacLennan, D. H. (1988) J. Biol. Chem. **263**, 15024–15031
11 Wuytack, F., Dode, L., Baba-Aissa, F. and Raeymaekers, L. (1995) Biosci. Rep., **15**, 299–306
12 Thevenod, F. and Schulz, I. (1988) Am. J. Physiol. **255**, G1–G40
13 Robinson, I. M., Cheek, T. R. and Burgoyne, R. D. (1992) Biochem. J. **288**, 457–463
14 Bode, H. P., Eder, B. and Trautmann, M. (1994) Eur. J. Biochem. **222**, 869–877
15 Burgoyne, R. D., Cheek, T. R., Morgan, A., O'Sullivan, A. J., Moreton, R. B., Berridge, M. J., Mata, A. M., Coyler, J., Lee, A. G. and East, J. M. (1989) Nature (London) **343**, 72–74
16 Villa, A., Sharp, A. H., Racchetti, G., Podini, P., Bole, D. G., Dunn, W. A., Pozzan, T., Snyder, S. H. and Meldolesi, J. (1992) Neuroscience **49**, 467–477
17 Nori, A., Villa, A., Podini, P., Witcher, D. R. and Volpe, P. (1993) Biochem. J. **291**, 199–204
18 Rossier, M. F. and Putney, J. W. J. (1991) Trends Neurosci. **14**, 310–314
19 Islam, M. S. and Berggren, P.-O. (1993) Biochem. J. **293**, 423–429
20 Jaconi, M. E. E., Lew, D. P., Carpentier, J. L., Magnusson, K. E., Sjogren, M. and Stendahl, O. (1990) J. Cell Biol. **110**, 1555–1564
21 Van Delden, C., Favre, C. J., Spat, A., Cerny, E., Krause, K. H. and Lew, D. P. (1992) Biochem. J. **281**, 651–656
22 Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356
23 Parys, J. B., De Smedt, H., Missiaen, L., Bootman, M. D., Sienaert, I. and Casteels, R. (1995) Cell Calcium **17**, 239–249
24 Heilmann, C., Spamer, C. and Gerok, W. (1983) Biochem. Biophys. Res. Commun. **114**, 584–592
25 Stewart, P. S., MacLennan, D. H. and Shamoo, A. E. (1976) J. Biol. Chem. **251**, 712–719
26 Spamer, C., Heilman, C. and Gerok, W. (1987) J. Biol. Chem. **262**, 7782–7789
27 Heimann, P., Spamer, C. and Gerok, W. (1984) J. Biol. Chem. **259**, 11139–11144
28 Sarkadi, B., Enyedi, A., Földes-Papp, Z. and Gardos, G. (1986) J. Biol. Chem. **261**, 9552–9557
29 Heilmann, C., Spamer, C. and Gerok, W. (1985) J. Biol. Chem. **260**, 788–794
30 Kovacs, T., Corvazier, E., Papp, B., Magnier, C., Bredoux, R., Enyedi, A., Sarkadi, B. and Enouf, J. (1994) J. Biol. Chem. **269**, 6177–6184
31 Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K. S. and Casteels, R. (1994) J. Biol. Chem. **269**, 1410–1416
32 Demaurex, N., Lew, D. P. and Krause, K. H. (1992) J. Biol. Chem. **267**, 2318–2324
33 Favre, C. J., Lew, D. P. and Krause, K.-H. (1994) Biochem. J. **302**, 155–162
34 Stendahl, O., Krause, K. H., Krischer, J., Jerström, P., Theler, J. M., Clark, R. A., Carpentier, J. L. and Lew, D. P. (1994) Science **265**, 1439–1441
35 Lytton, J., Westlin, M. and Hanley, M. R. (1991) J. Biol. Chem. **266**, 17067–17071
36 Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2466–2470
37 Kume, S., Muto, A., Aruga, J., Nakagawa, T., Michikawa, T., Furuichi, T., Nakade, S., Okano, H. and Mikoshiba, K. (1993) Cell **73**, 555–570
38 Khan, A. A., Steiner, J. P., Klein, M. G., Schneider, M. F. and Snyder, S. H. (1992) Science **257**, 815–818