Silica induces changes in cytosolic free calcium, cytosolic pH, and plasma membrane potential in bovine alveolar macrophages

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Abstract. The mineral-dust-induced activation of pulmonary phagocytes is thought to be involved in the induction of severe lung diseases. The activation of bovine alveolar macrophages (BAM) by silica was investigated by flow cytometry. Short-term incubation (<10 min) of BAM with silica gel and quartz dust particles induced increases in the cytosolic free calcium concentration ([Ca2+]i), decreases in intracellular pH (pHi), and increases in plasma membrane potential (PMP). The extent of these changes was concentration dependent, related to the type of dust and was due to Ca2+ influx from the extracellular medium. An increase in [Ca2+]i was inhibited, when extracellular Ca2+ was removed. Furthermore the calcium signal was quenched by Mn2+ and diminished by the calcium channel blocker verapamil. The protein kinase C specific inhibitor bisindolylmaleimide II (GF 109203 X) did not inhibit the silica-induced [Ca2+]i rise. In contrast, silica-induced cytosolic acidification and depolarization were inhibited by GF 109203 X but not by removal of extracellular calcium. Addition of TiO2 particles or heavy metal-containing dusts had no effect on any of the three parameters. Our data suggest the existence of silica-activated transmembrane ion exchange mechanisms in BAM, which might be involved in the specific cytotoxicity of silica by Ca2+-dependent and independent pathways.

Keywords: Phagocytes, cytotoxicity, silica dust, flow cytometry, calcium, pH, plasma membrane potential

Abbreviations

AM, alveolar macrophage;
BAM, bovine alveolar macrophage;
BSA, bovine serum albumin;

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DMSO, dimethylsulfoxide;
HBSS, Hanks’ Balanced Salt Solution;
HEPES, 4-(2-Hydroxyethyl)-1-piperazinemethanesulfonic acid;
PMP, plasma membrane potential;
ROI, reactive oxygen intermediates.

1. Introduction

The inhalation of mineral dust particles, in particular pure silica or dusts with a high quartz content, is thought to participate in the induction of lung diseases like fibrosis, emphysema, and cancer [12]. The hazardous effects of inhaled particles are mediated mainly by the release of inflammatory mediators from activated alveolar macrophages (AM). Among the mediators released by these pulmonary defense cells are superoxide anion radicals ($O_2^-$), eicosanoids, and several cytokines. The secretion of $O_2^-$ by AM leads to the generation of hydrogen peroxide, hydroxyl radicals, and singlet-oxygen. These molecules, known as reactive oxygen intermediates (ROI), have been held responsible for some of the most noxious effects of inhaled particles [6,8,10,20]. Previous work from our laboratory showed that silica or dusts derived from various environment-polluting sources stimulate within minutes of incubation the secretion of ROI by bovine AM [2].

The fundamental mechanisms of phagocyte activation by stimulants like chemotactic peptides or phorbol esters have been investigated extensively by others. Most of these studies focused on human neutrophilic granulocytes [27]. However, the signal transduction pathways in pulmonary macrophages that lead to the specific cytotoxic activity of certain dusts remain unclear. Cytotoxic quartz dusts – as well as asbestos fibers – specifically induce the secretion of a potent phagocyte activator, leukotriene B4, by the calcium-dependent 5′-lipxygenase pathway of arachidonate metabolism, while other mineral dusts only activate the calcium-independent cyclooxygenase pathway [5,16].

An increase in cytosolic free calcium ($[Ca^{2+}]_c$) may also occur during the phospholipase C/protein-kinase C-dependent stimulation of phagocytes, e.g., when the superoxide anion generating enzyme NADPH oxidase is activated [19]. The stimulation of the “respiratory burst” by chemotactic peptides or phorbol esters is paralleled by membrane depolarization [7,15,17]. Changes in cytoplasmic pH (pH$_i$) have also been observed to be closely associated with superoxide generation in macrophages. In the case of an impaired pH regulation the large burst of metabolic acid production by NADPH-oxidase might influence pH-mediated processes [21]. The transition of a resting macrophage into the activated state, hence, can be accompanied by changes in $[Ca^{2+}]_c$, plasma membrane potential (PMP), and pH$_i$.

The purpose of the present study was to investigate the signal transduction pathways in alveolar macrophages exposed to dusts that activate the release of ROI. We used flow cytometry to analyze $[Ca^{2+}]_c$, pH$_i$ and PMP as a fast and reliable screening method of cell activation.

2. Materials and methods

2.1. Reagents

Indo-1 AM was purchased from Becton & Dickinson, Heidelberg, Germany. Bis-Oxonol (DiBaC$_4$ [3]) and Carboxy-SnafF-1 AM acetate were from Molecular Probes, Eugene, OR, USA.
Fluo-3 AM, Verapamil, Nigericin, and 4-Bromo-A23187 were from Sigma Chemie, Taufkirchen, Germany. GF 109203 X was obtained from Calbiochem, Frankfurt/Main, Germany. All other chemicals used were from Sigma Chemie or Merck, Darmstadt, Germany.

Dusts: silica powder: QUSO G 32, microfine precipitated silica gel, ∅ < 10 µm (Philadelphia Quartz Company, Philadelphia, PS, USA); quartz dust: Sikron F 600, quartz powder, ∅ < 5 µm (Palas, Karlsruhe, Germany); titanium dioxide: anatas modification (Sigma Chemie); heavy metal-containing dusts: the dust samples, obtained from several environmental pollution-related sources, were purified and characterized as published previously [2]. Stock solutions were made by dissolving 20 mg of dust/ml in sterile 0.9% saline. To determine the extent to which the geometrical shape of the particle surface can contribute to the activating properties of dusts, bovine serum albumine (BSA) was used to modify the surface of dust particles without changing size and shape. Dusts were coated with BSA by suspending it in 0.9% saline containing 0.25% BSA (Gibco Life Technologies, Eggenstein, Germany) and incubated for 30 min.

2.2. Preparation of macrophages

Bovine alveolar macrophages (BAM) were collected by broncho-alveolar lavage as described elsewhere [2]. BAM were purified by density gradient centrifugation (70/35% Percoll, Pharmacia, Uppsala, Sweden, 7 min, 3000 × g), then rinsed twice with HEPES-buffered HBSS, pH 7.4. Cells were kept at 4°C in HEPES-buffered RPMI 1640 (Gibco, 1 × 10⁶ cells/ml) to avoid cell–cell adherence. Routinely, the storage was extended up to the next morning (12 h). After isolation the cells were tested for viability by trypan-blue exclusion assay (>95% viable cells) and for purity by non-specific esterase staining [26] (>96% positive cells).

2.3. Staining

BAM (1 × 10⁶ cells/ml) were incubated in HEPES-buffered RPMI 1640 for 3 h at 37°C and then stained with indo-1 [11,25], Fluo-3 [14], bis-Oxonol [13], or Snarf-1 [22]. Stock solutions of fluorescent dyes (1 nM in DMSO) were stored in aliquots for 10 ml cell suspension at −20°C. Cells were loaded with 10 µM Indo-1, 5 µM Fluo-3 (each for 30 min), or Snarf-1 acetate (5 µM, 20 min) at 37°C and then washed twice with RPMI (200 × g, 5 min). Staining of BAM with bis-Oxonol (100 nM, 37°C) was carried out for exactly 10 min without any washing steps. Bis-Oxonol stained cells were counterstained for membrane damaged cells with propidium iodide (PI, 5 µg/ml, 10 min).

2.4. Flow cytometry

Experiments with Indo-1 were carried out on an EPICS 751 cell sortor (Coulter Electronics, Hialeah, FL, USA) with gateway software. Fluo-3, Snarf-1, and bis-Oxonol measurements were done on a FACScan flow cytometer (Becton & Dickinson, Heidelberg, Germany) with LYSIS II software. Baseline fluorescence and cellular response remained stable for at least 2 h if cells were kept at ambient temperature in the dark. Prior to the analysis, BAM were warmed to 37°C for 10 min. All further steps were performed at 37°C. Baseline values were recorded for 1 min, the data acquisition was interrupted for 30 s, a 100× stock suspension of dust particles was added to the cells and the tube was agitated for 3 s. Then the measurement was continued for up to 60 min. Maintenance of membrane integrity after dust-addition was confirmed by PI exclusion (1 µg/ml, 5 min). During the period of analysis no detectable increase of the percentage of PI stained cells was found.
In some cases the light-scattering properties of dust particles interfered with that from the macrophages. Therefore macrophages were further identified by gating on high autofluorescence in an unused fluorescence channel (630 nm for Fluo-3 and bis-Oxonol and 530 nm for Snarf-1 acetate).

**Determination of \([Ca^{2+}]_i\):** Indo-1 was used for ratiometric measurements of \([Ca^{2+}]_i\), and for the determination of the percentage of responders (for experimental details see [25]). All cells with an Indo-1 ratio of more than two standard deviations above baseline were regarded as responders. Fluo-3 measurements were done on the FACScan cytometer (excitation 488 nm, emission 530 nm). \([Ca^{2+}]_i\) changes were expressed as percentages of the maximum value which was measured after the addition 10 µM calcium ionophore 4-Br-A23187.

**Determination of cytosolic pH:** Cytosolic pH (pH\(_i\)) was analyzed ratiometrically with Snarf-1 acetate (excitation 488 nm, emissions 570 nm, 630 nm) by the method of Dieter [4]. 1 ml of HBSS with a reversed molar Na\(^+\)/K\(^+\) ratio (K-HBSS, pH varying from 6.0 to 8.0) was added to 100 µl suspension of dye-loaded cells. Then potassium ionophore Nigericin (2 µg/ml) was added leading to an equilibrium of pH\(_i\) = pH\(_0\). The fluorescence ratios were fitted to pH values by a polynomic second degree regression analysis.

**Determination of plasma membrane potential:** Plasma membrane potential (PMP) was determined with the hydrophobic dye bis-Oxonol (excitation 488 nm, emission 530 nm). PI was measured simultaneously at above 630 nm. To avoid diluting effects by adsorption of the dye in the tubings, the cuvette system and tubing of the cytometer were equilibrated with 100 µM bis-Oxonol in HBSS before and between the measurements. Before analysis PI stained cells were excluded by gating. Calibration of the fluorescence intensity to PMP was carried out by mixing 250 µl BAM suspension (HBSS), 250 µl bis-oxonol suspension (400 nM) and 500 µl of HBSS and K-HBSS (K\(^+\) concentration 5 to 37.5 mM). After 10 min (37°C) fluorescence was measured and correlated to the apparent K\(^+\) potential of the cells, calculated by the Nernst equation:

\[
E_{K^+}(\text{mV}) = -59 \log([K^+]_i/[K^+]_0)
\]

assuming the intracellular potassium concentration [K\(^+\)]\(_i\) to be 100 mM [13]. In the range of 5–40 mM extracellular K\(^+\) this calibration resulted in a linear relationship of fluorescence intensity and E\(_K\).

2.5. **Statistics**

All experiments were performed with cells isolated from at least three different animals and were repeated at least twice with each batch of cells. Data are shown as traces of single experiments representative for results obtained in all of the measurements or as arithmetic mean ±1 standard deviation (SD).

3. **Results**

3.1. **Alterations in cytosolic free calcium concentration \([Ca^{2+}]_i\) after stimulation with dusts**

Addition of quartz dust and silica gel powder to BAM induces a transient rise in \([Ca^{2+}]_i\) in a fraction of the total BAM population. This is shown in Fig. 1A by the increase of the Indo-1 fluorescence ratio after addition of the quartz dust Sikron F 600. About 35% of the cells elevated their \([Ca^{2+}]_i\) (Fig. 1B). The overall increase of \([Ca^{2+}]_i\) was up to 30% of the maximum response induced by the
Summary of the maximum effects of silica powder QUSO G 32 or titanium dioxide dust on 
\([\text{Ca}^{2+}]_i\), pH, and PMP. Incubation procedures are described in Fig. 1. Data are mean of 
at least 6 experiments ±1 standard deviation and were measured in the interval of 3–7 min 
after stimulation

|                      | [Ca\(^{2+}\)]\(_i\), change | pH,          | PMP [mV] |
|----------------------|------------------------------|--------------|-----------|
| Unstimulated         | 0 ± 4                        | 7.35 ± 0.05  | −74 ± 6   |
| + Silica (QUSO G 32) (200 µg/ml) | +26 ± 8                    | 7.03 ± 0.08  | −28 ± 13  |
| + " + EGTA (2.5 mM)  | −8 ± 4                       | 7.11 ± 0.08  | −32 ± 12  |
| + " + Verapamil (15 µM) | +12 ± 3                     | n.d.         | n.d.      |
| + " + GF 109203X (1.25 µM) | +29 ± 5                     | 7.26 ± 0.02  | −72 ± 8   |
| + " + BSA (0.25%)    | +2 ± 6                       | 7.33 ± 0.11  | <−75\(^*\) |
| TiO\(_2\) (200 µg/ml) | 0 ± 5                        | 7.31 ± 0.13  | −72 ± 5   |

n.d. = not determined, \(^*\) = out of calibration range.

calcium ionophore 4-Br-A23187 (Fig. 1C). The slope of the calcium transient was different for the 
different type of dusts: For the quartz dust it was maximal at 2–4 min and lasted typically 6–8 min, 
whereas the silica gel powder caused maximum effect after 5–7 min and lasted 8–10 min. Then, 
\([\text{Ca}^{2+}]_i\) returned to basal levels and did not increase again for at least 1 h (not shown). No response 
was found with TiO\(_2\) powder (Fig. 2A, summary of the results in Table 1), buffer alone, or the heavy 
metal-containing dusts (not shown).

Dust induced \(\text{Ca}^{2+}\) increase is mainly mediated by \(\text{Ca}^{2+}\) influx from the external medium. Stim-
ulation of the cells in the absence of \(\text{Ca}^{2+}\) prevented the transient rise in \([\text{Ca}^{2+}]_i\) (Fig. 2B). Further stimulation in the presence of \(\text{Ca}^{2+}\) and Mn\(^{2+}\) ions led to a profound decrease of the Fluo-3 fluo-
rescence (Fig. 2B). Since calcium channels might be opened by PKC-mediated processes, cells were 
incubated with the PKC-specific inhibitor bisindolylmaleimide-II (GF 109203 X [28]) prior to stimu-
lation with silica powder. The pretreatment did not inhibit the SiO\(_2\)-induced \(\text{Ca}^{2+}\) influx (Fig. 2C).

In the presence of verapamil, a specific inhibitor of voltage-gated L-type \(\text{Ca}^{2+}\) channels, influx of 
calcium was clearly diminished (Table 1). 15 µM verapamil reduced \(\text{Ca}^{2+}\) influx by 41\% (\(p < 0.01\)). At higher doses (300 µM), as used by other authors [1], verapamil damaged the cells. Stimulation 
with BSA-coated silica did not elicit a \(\text{Ca}^{2+}\) response (Fig. 2D).

3.2. Change of intracellular pH

In resting cells the steady state pH\(_i\) was determined to be about 7.3–7.4 depending on the cell 
preparation. Stimulation with silica generated a sustained acidification of the cytosol (Fig. 3A). The acidification was concentration dependent (at 200 µg/ml silica: ΔpH = −0.32 ± 0.08) and persisted for 10 min. The interrelation between \([\text{Ca}^{2+}]_i\) increase and pH\(_i\) decrease was investigated by the 
depletion of extracellular \(\text{Ca}^{2+}\) with 2.5 mM EGTA. Although the initial pH in the presence of EGTA 
was slightly lower than the initial pH, the treatment did not affect the final pH\(_i\) after stimulation 
(Fig. 3B). Incubating the cells with silica in the presence of GF 109203 X caused a significant 
inhibition of the silica-induced acidification (ΔpH = −0.09 ± 0.02, \(p < 0.01\), Fig. 3C). When BAM 
were challenged with TiO\(_2\), heavy metal-containing dusts, or BSA-coated silica no pH changes were 
observed (Figs 3A and 3D).

3.3. Dust-induced changes in plasma membrane potential

Quiescent BAM showed an apparent potassium potential of about −74 mV. Following addition of 
SiO\(_2\) after a lag phase of about 2 min the cells substantially depolarized. The depolarization was
Fig. 1. Kinetics of \([\text{Ca}^{2+}]_i\) of Indo-1-stained BAM after stimulation with quartz dust Sikron F 600. Baseline fluorescence was monitored for 1 min. Cells were then stimulated by the addition of a 100\(\times\) concentrated quartz suspension \((t = 0 \text{ min})\) and subsequently measured for the indicated time. In (A) data are plotted as the 400/525 nm fluorescence emission ratio of single cells in a bivariate array vs. time. In (B) the percentage of responders and in (C) the percentage of maximal response is plotted vs. time. Hatched lines in (B) and (C) show the kinetics of cells from the same preparation in the presence of 2.5 mM EGTA.
Fig. 2. Time course of $[\text{Ca}^{2+}]_i$ after stimulation of Fluo-3 stained BAM with dusts (200 µg/ml). Cells were stimulated with quartz dust (Sikron F 600), silica powder (QUSO G 32) or TiO$_2$ (A), or silica powder with or without 10 min preincubation with 2.5 mM EGTA or 100 µM MnCl$_2$ (B) or 1.25 µM GF 109203 X (C). In (D) untreated silica or BSA-coated silica (final concentration of BSA 25 µg/ml) were added. One representative measurement out of at least 6 similar experiments is shown as the percentage of maximum response. Stimulation procedure as in Fig. 1.
Fig. 3. Kinetics of pH, after stimulation of BAM with silica (QUSO G 32) or TiO₂ (A), silica with or without preincubation EGTA (B), GF 109203 X (C), and with untreated or BSA-coated silica (D). Data show tracings of single runs and are representative for at least 6 experiments. Experimental details as in Fig. 2.
maximal 7–10 min after stimulation for silica and quartz. The silica powder showed a greater effect (maximal PMP = −28±13 mV) than the quartz dust (maximum PMP = −37±9 mV). After reaching the peak PMP the cells began to repolarize gradually (at 15 min to about −55 mV). TiO₂ (Fig. 4A) as well as other heavy metal-containing dusts (not shown) failed to induce any response.

Depletion of extracellular calcium did not influence the SiO₂-induced depolarization (Fig. 4B). Conversely, preincubation of BAM with the PKC inhibitor GF 109203 X nearly completely inhibited the depolarization (Fig. 4C). Cells slightly hyperpolarized, when stimulated with BSA-coated SiO₂ particles (Fig. 4D).

4. Discussion

In the present study several mineral dusts were investigated for their potential to alter cellular parameters involved in the signal transduction pathways of activated phagocytes. As shown previously [2], these dusts induced the release of reactive oxygen intermediates by bovine alveolar macrophages (BAM).

Incubating BAM with SiO₂ particles resulted in a marked, dose dependent, and transient [Ca²⁺]ₗ rise. Similar observations were reported by Tuomala et al. [29] with polymorphonuclear leukocytes. Conversely, Chen et al. [3] observed after stimulation of rat AM with quartz dust sustained elevated cytoplasmic levels of [Ca²⁺]ₗ for 120 min. From the fact that the extent of the increased [Ca²⁺]ₗ was not diminished after complexing extracellular Ca²⁺ with EGTA these authors concluded that calcium was released from internal stores. The discrepancy between these results and our data might be due to the different animal species investigated (rat vs. bovine) or to the way cells were exposed to dust samples. Chen and coworkers studied Ca²⁺ metabolism in AM cultivated as adherent monolayers whereas AM was analyzed in suspension in this study and by Tuomala at al. [29]. Szöllösi et al. [24] reported that adherent cells and cells in suspension exhibit different Ca²⁺ kinetic profiles following stimulation with the same stimulus. Attached cells maintained an increased free cytosolic Ca²⁺ level whereas cells in suspension showed a rapid decrease in [Ca²⁺]ₗ.

Our data suggests that there is an influx of extracellular calcium ions. This is supported by the finding that (i) there is no elevation of [Ca²⁺]ₗ in the absence of extracellular Ca²⁺ ions, (ii) the known calcium antagonist verapamil is able to inhibit a substantial portion of the [Ca²⁺]ₗ change, and (iii) the indicator fluorescence is quenched totally when extracellular Mn²⁺ ions are added. This fluorescence quenching has been used to discriminate between calcium influx and mobilization from internal stores [18] (Figs 2B and 2D). Gamalei et al. [9] proposed that the presence of extracellular reactive oxygen intermediates (ROI) is sufficient for the induction of calcium fluxes. This proposition does not agree with our findings: Although all investigated dusts induced similar amounts of extracellular O₂⁻ and H₂O₂ [2] only silica-dusts affected a change in [Ca²⁺]ₗ. Moreover, the silica-induced [Ca²⁺]ₗ-rise is markedly diminished if the dusts are coated with BSA, whereas the secretion of ROI is not affected by this modification (unpublished data). Therefore the Ca²⁺-transient is mediated by specific, protein kinase C-independent, Ca²⁺-channels that open after contact of BAM cell-membrane with silica. Whether there is a Ca²⁺-dependent as well as a Ca²⁺-independent pathway for the dust-induced activation of NADPH oxidase as proposed by Tuomala et al. [29] can not be concluded from our data.

Silica-induced pH changes and depolarization are mediated by other signal transducing pathways as silica-induced alterations in [Ca²⁺]ₗ. Addition of PKC specific inhibitor GF 109203 X [28] is without any effect on [Ca²⁺]ₗ, whereas the cytosolic acidification and the depolarization are clearly
Fig. 4. Kinetics of apparent potassium potential of BAM after stimulation with silica (QUSO G 32) or TiO$_2$ (A), silica with or without preincubation with EGTA (B), 1.25 $\mu$M GF 109203 X (C), and with untreated or BSA-coated silica (D). Data show tracings of single runs and are representative for at least 6 experiments. Experimental details as in Fig. 2.
inhibited. On the other hand, the Ca\(^{2+}\)-influx is not necessary for acidification or depolarization, for both processes occur in the absence of extracellular free Ca\(^{2+}\). Like Ca\(^{2+}\)-influx, depolarization and acidification are also independent from NADPH oxidase activation. Both depolarization and acidification changes are clearly diminished when BSA-coated dusts were used as stimulus.

The alterations observed by flow cytometry are not the effect of impaired membrane integrity since cell viability, detected with PI, did not decrease during the investigation. The effects also are not due to indicator- or dust-related artifacts, for all of the processes could be inhibited specifically by either PKC inhibition or removal of extracellular Ca\(^{2+}\).

The presented data demonstrate that contact of BAM with silica induces in parallel the induction of a transient calcium influx as well as a more sustained cytoplasmic acidification and a transient depolarization. Although the functional role of the observed effects still remains to be explained by further experiments, one or more of them are potentially involved in the generation of inflammatory mediators responsible for the specific cytotoxicity of silica.

Our experiments demonstrate that flow cytometry is a suitable tool for the rapid screening of early responses of phagocytes to airborne dusts. If measurements were extended by analyzing the respiratory burst activity of the cells [23], low cell requirements and the possibility of investigating mixed cell populations by multiparametric analysis makes this an attractive approach for monitoring dust-induced biological phenomena in human alveolar macrophages.

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**References**

[1] K.I. Andrabi, N. Kaal, S. Mudessar, J.B. Bilawari and N.K. Gangali, Collagen-stimulated superoxide production: evidence for coupled mobilization of calcium, *Biochem. Med. Metab. Biol.* **45** (1991), 235–243.

[2] I. Berg, T. Schlüter and G. Gercken, Increase of bovine alveolar macrophage superoxide anion and hydrogen peroxide release by dusts of different origin, *J. Toxicol. Environ. Health* **39** (1993), 101–111.

[3] J. Chen, L.C. Armstron, S. Liu, J.E. Gerriets and J.A. Last, Silica increases cytosolic free calcium ion concentration of alveolar macrophages in vitro, *Toxicol. Appl. Pharmacol.* **53** (1991), 14–23.

[4] P. Dieter, Relationship between intracellular pH changes, activation of protein kinase C, and NADPH oxidase in macrophages, *FEBS Letters* **298** (1992), 17–21.

[5] K.E. Driscoll, J.M. Higgins, M.J. Leytart and L.L. Crosby, Differential effects of mineral dusts on the in vitro activation of alveolar macrophage eicosanoid and cytokine release, *Toxicol. in Vitro* **4** (1990), 284–288.

[6] I. Emerit, M. Keck, J. Feingold and M.A. Michelson, Activated oxygen species at the origin of chromosome breakage and sister-chromatid exchange, *Mutation Res.* **103** (1982), 165.

[7] M.P. Fletcher and J.C. Gasson, Enhancement of neutrophil function by granulocyte-macrophage colony-stimulating factor involves recruitment of a less responsive subpopulation, *Blood* **71** (1990), 652–658.

[8] I. Fridovich, Superoxide radical: an endogenous toxicant, *Ann. Rev. Pharmacol. Toxicol.* **23** (1983), 239–257.

[9] I.A. Gamalei, A.B. Kaulin and K.M. Kirpichnikowa, Changes in the membrane potential, the cytoplasmic Ca\(^{2+}\) concentration and in the transport of fluorescein anions across the macrophage plasma membrane under the influence of O\(^{-}\) and H\(_2\)O\(_2\) in small concentrations, *Titologiya* **33** (1991), 79–85.

[10] A.W. Girotti, Mechanism of lipid peroxidation, *J. Free Radical Biol. Med.* **1** (1985), 87–95.

[11] G. Grynkiewicz, M. Ponie and R.Y. Tsien, A new generation of Ca\(^{2+}\) indicators with greatly improved fluorescence properties, *J. Biol. Chem.* **266** (1985), 3440–3450.

[12] A.G. Heppleston, Pulmonary toxicology of silica, coal, and asbestos, *Environ. Health Perspect.* **55** (1984), 111–127.

[13] I. Holopainen, M.O.K. Enkvist and K.E.D. Aakerman, Glutamate receptor agonists increase intracellular Ca\(^{2+}\) independently of voltage-gated Ca\(^{2+}\) channels in rat cerebellar granule cells, *Neurosci. Letters* **98** (1989), 57–62.
[14] J.P.Y. Kao, J.M. Alderton, R.Y. Tsien and R.A. Steinhardt, Active involvement of Ca\(^{2+}\) in mitotic progression of swiss 3T3 fibroblasts, *J. Cell Biol.* **111** (1990), 183–196.

[15] S. Kitigawa and R.B. Johnston, Relationship between membrane potential changes and superoxide-releasing capacity in resident and activated mouse peritoneal macrophages, *J. Immunol.* **135** (1985), 3417–3423.

[16] D.C. Kuhn and L.M. Demers, Influence of mineral dust surface chemistry on eicosanoid production by the alveolar macrophage, *J. Toxicol. Environ. Health* **35** (1992), 39–50.

[17] M.A. Martin, W.M. Nauseef and A.D. Clark, Depolarization blunts oxidative burst of human neutrophils. Parallel effects of monoclonal antibodies, depolarizing buffers, and glykolytic inhibitors, *J. Immunol.* **140** (1988), 3928–3935.

[18] J.E. Merritt, R. Jacob and T.J. Hallam, Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils, *J. Biol. Chem.* **277** (1992), 73–79.

[19] F. Morel, J. Doussiere and F.V. Vignais, The superoxide-generating oxidase of phagocytic cells, *Eur. J. Biochem.* **201** (1991), 523–546.

[20] G.A. Murrell, M.J. Francis and L. Bromley, Modulation of fibroblast proliferation by oxygen free radicals, *Biochem. J.* **265** (1990), 659–665.

[21] A. Nanda, S. Grinstein and J.T. Curnutte, Abnormal activation of H\(^{+}\) conductance in NADPH oxidase-deficient neutrophils, *Proc. Natl. Acad. Sci. USA* **90** (1993), 760–764.

[22] P.S. Rabinovich and C.H. June, Intracellular ionized calcium, membrane potential, and pH, in: *Flow Cytometry: A Practical Approach*, M.G. Ormerod, ed., IRL Press, Oxford, 1990, pp. 161–185.

[23] G. Rothe and G. Valet, Flow cytometric assays of oxidative burst activity in phagocytes, *Methods Enzymol.* **233** (1994), 539–548.

[24] J. Szilösi, B.G. Feuerstein, W.C. Hyun, M.K. Das and L.J. Marton, Attachment of A 172 human glioblastoma cells affects calcium signaling: A comparison of image cytometry, flow cytometry, and spectrofluorometry, *Cytometry* **12** (1991), 707–716.

[25] A. Tárnok, Improved kinetic analysis of cytosolic free calcium in pressure-sensitive neuronal cells by fixed-time flow cytometry, *Cytometry* **23** (1996), 82–89.

[26] A. Tárnok and I. Brammer, Population kinetics and nuclear/cytoplasmic ratio of mononuclear phagocytes in the X-irradiated rhabdomyosarcoma R1H, in: *Advances in Analytical Cellular Pathology*, G. Burger, M. Oberholzer and P.G. Vooijs, eds, Elsevier, Amsterdam, 1990, pp. 281–283.

[27] M. Theilen, B. Dewald and M. Baggioni, Neutrophil signal transduction and activation of the respiratory burst, *Physiol. Rev.* **73** (1993), 797–821.

[28] D. Toullec, P. Pianetti, H. Coste, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, L. Duhamel, D. Charon and J. Kirilovsky, The bisindolylmaleimide GF 109203 X is a potent and selective inhibitor of protein kinase C, *J. Biol. Chem.* **266** (1991), 15771–15781.

[29] M. Tuomala, M.R. Hirvonen, M. Holopainen and K. Savolainen, Stimulation of human polymorphonuclear leukocytes by consecutive doses of quartz and interactions of quartz with fMLP, *Toxicol. Appl. Pharmacol.* **118** (1993), 224–232.