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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 ($[Ca^{2+}]_i$), decreases in intracellular pH (pH_i), 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 Ca^{2+} influx from the extracellular medium. An increase in $[Ca^{2+}]_i$ was inhibited, when extracellular Ca^{2+} was removed. Furthermore the calcium signal was quenched by Mn^{2+} 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 $[Ca^{2+}]_i$ rise. In contrast, silica-induced cytosolic acidification and depolarization were inhibited by GF 109203 X but not by removal of extracellular calcium. Addition of TiO_2 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 Ca^{2+}-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′-lipoxygenase pathway of arachidonate metabolism, while other mineral dusts only activate the calcium-independent cyclooxygenase pathway [5,16].

An increase in cytosolic free calcium ($[Ca^{2+}]_i$) 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 $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$, 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-Snarf-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, $\varnothing < 10$ μm (Philadelphia Quartz Company, Philadelphia, PS, USA); *quartz dust*: Sikron F 600, quartz powder, $\varnothing < 5$ 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 $\times$ g), then rinsed twice with HEPES-buffered HBSS, pH 7.4. Cells were kept at 4°C in HEPES-buffered RPMI 1640 (Gibco, 1×10^6 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 (1×10^6 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 mM 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 $\times$ 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 sorter (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$\times$ 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:

$$E_{K^+}(\mathrm{mV}) = -59 \log\left([K^+]_i/[K^+]_0\right)$$

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

Table 1
Summary of the maximum effects of silica powder QUSO G 32 or titanium dioxide dust on $[Ca^{2+}]_i$, pH_i, 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 (% max. response)	pH_i	PMP [mV]
Unstimulated	0 ± 4	7.35 ± 0.05	-74 ± 6
+ Silica (QUSO G 32) (200 μg/ml)	$+26 \pm 8$	7.03 ± 0.08	-28 ± 13
+ " + EGTA (2.5 mM)	-8 ± 4	7.11 ± 0.08	-32 ± 12
+ " + Verapamil (15 μM)	$+12 \pm 3$	n.d.	n.d.
+ " + GF 109203X (1.25 μM)	$+29 \pm 5$	7.26 ± 0.02	-72 ± 8
+ " + BSA (0.25%)	$+2 \pm 6$	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, $[Ca^{2+}]_i$ 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 Ca^{2+} increase is mainly mediated by Ca^{2+} influx from the external medium. Stimulation of the cells in the absence of Ca^{2+} prevented the transient rise in $[Ca^{2+}]_i$ (Fig. 2B). Further stimulation in the presence of Ca^{2+} and Mn^{2+} ions led to a profound decrease of the Fluo-3 fluorescence (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 stimulation with silica powder. The pretreatment did not inhibit the SiO_2-induced Ca^{2+} influx (Fig. 2C). In the presence of verapamil, a specific inhibitor of voltage-gated L-type Ca^{2+} channels, influx of calcium was clearly diminished (Table 1). 15 μM verapamil reduced 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 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: $\Delta pH = -0.32 \pm 0.08$) and persisted for 10 min. The interrelation between $[Ca^{2+}]_i$ increase and pH_i decrease was investigated by the depletion of extracellular 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 ($\Delta pH = -0.09 \pm 0.02$, $p < 0.01$, 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

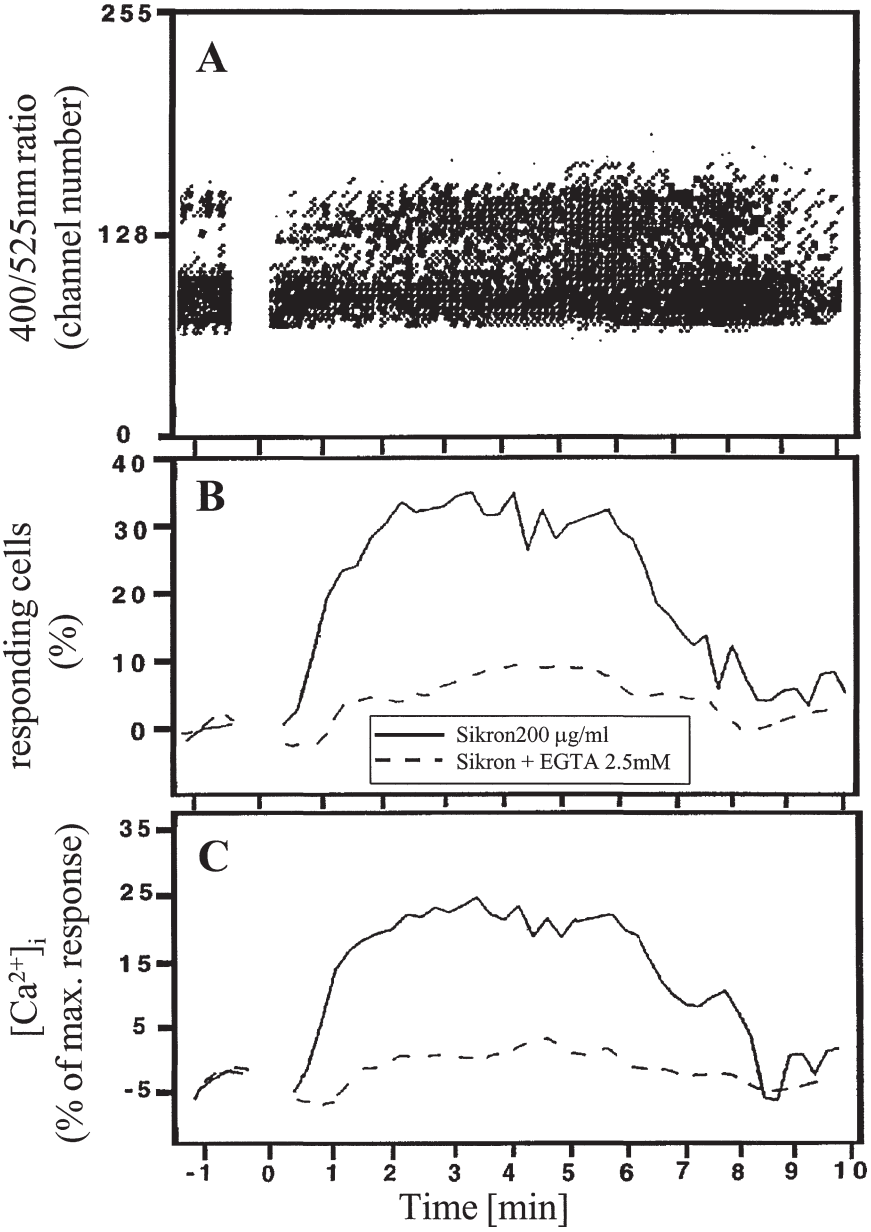

Fig. 1. Kinetics of $[Ca^{2+}]_i$ of Indo-1-stained BAM after stimulation with quartz dust Sikron F 600. Baseline fluorescence was monitored for 1 min. Cells were then stimulated by the addition of a 100× concentrated quartz suspension ($t = 0$ min) and subsequently measured for the indicated time. In (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.

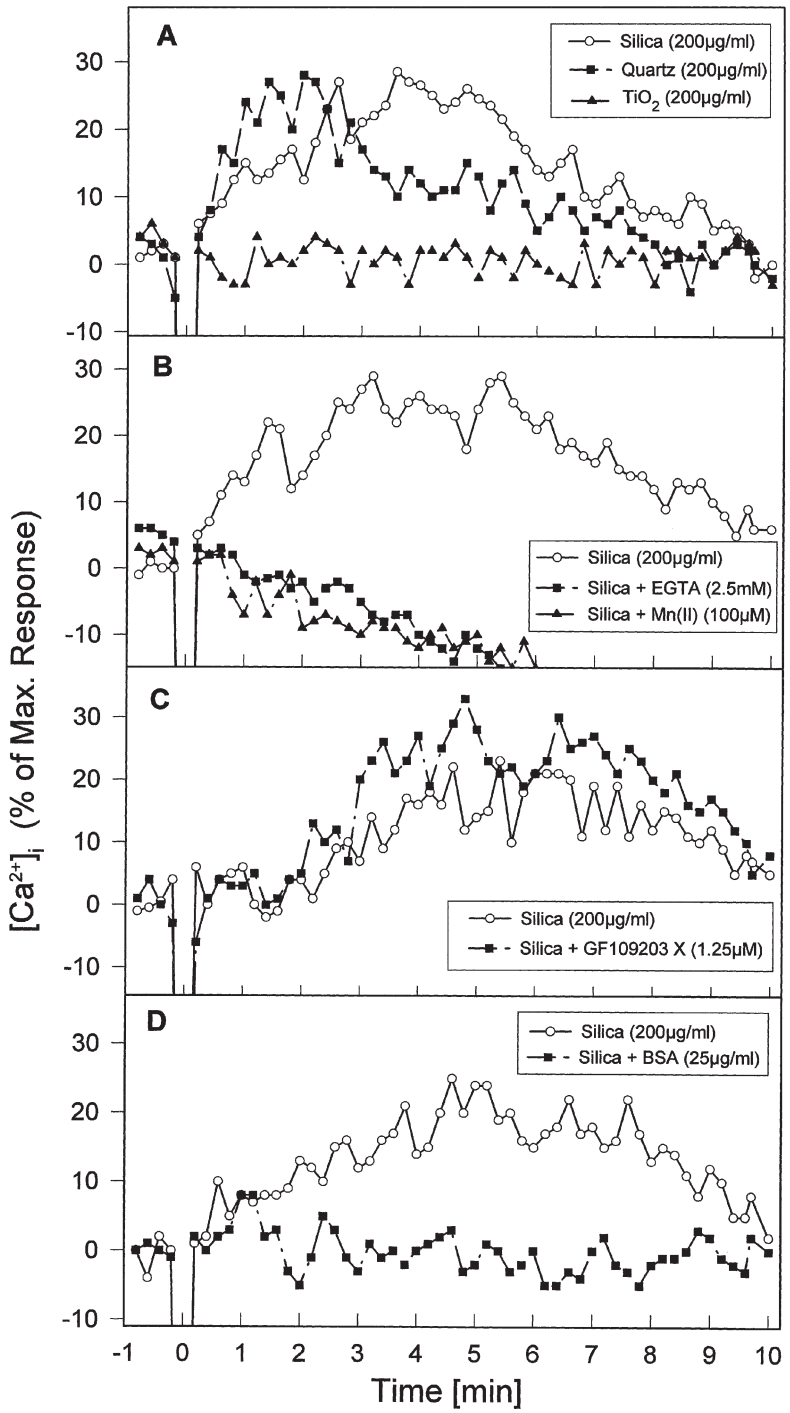

Fig. 2. Time course of $[Ca^{2+}]_i$ after stimulation of Fluo-3 stained BAM with dusts (200 μg/ml). Cells were stimulated with quartz dust (Sikron F 600), silica powder (QUSO G 32) or TiO_2 (A), or silica powder with or without 10 min preincubation with 2.5 mM EGTA or 100 μM $MnCl_2$ (B) or 1.25 μM 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.

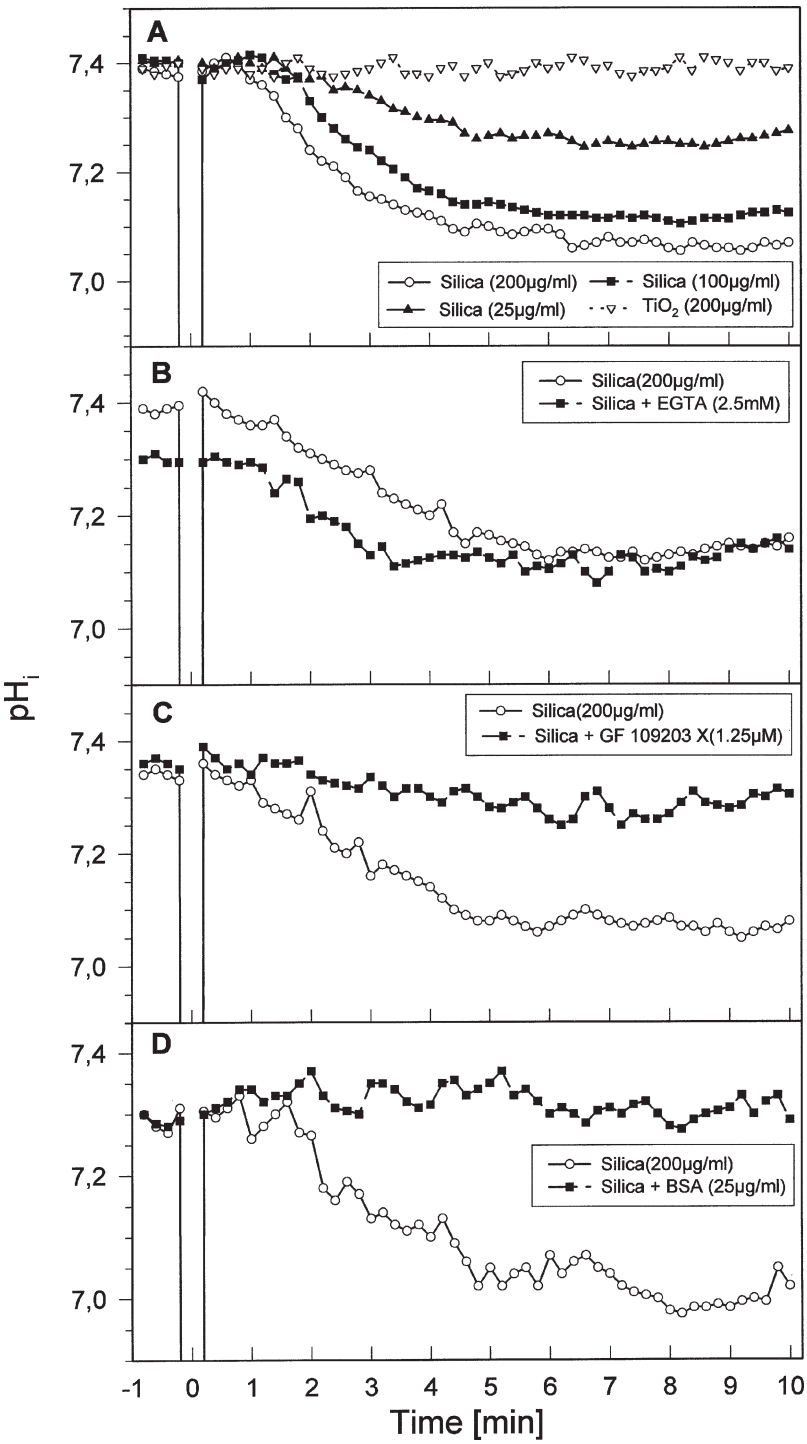

Fig. 3. Kinetics of pH_i after stimulation of BAM with silica (QUSO G 32) or TiO_2 (A), silica with or without preincubation EGTA (B), GF 109203 X (C), and with untreated or BSA-coated silica (D). Data show tracings of single runs and are representative for at least 6 experiments. Experimental details as in Fig. 2.

maximal 7–10 min after stimulation for silica and quartz. The silica powder showed a greater effect (maximal PMP $= -28 \pm 13$ mV) than the quartz dust (maximum PMP $= -37 \pm 9$ mV). After reaching the peak PMP the cells began to repolarize gradually (at 15 min to about -55 mV). TiO_2 (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 SiO_2-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 SiO_2 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 SiO_2 particles resulted in a marked, dose dependent, and transient $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ for 120 min. From the fact that the extent of the increased $[Ca^{2+}]_i$ was not diminished after complexing extracellular Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} kinetic profiles following stimulation with the same stimulus. Attached cells maintained an increased free cytosolic Ca^{2+} level whereas cells in suspension showed a rapid decrease in $[Ca^{2+}]_i$.

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 $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} ions, (ii) the known calcium antagonist verapamil is able to inhibit a substantial portion of the $[Ca^{2+}]_i$ change, and (iii) the indicator fluorescence is quenched totally when extracellular Mn^{2+} 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 O_2^- and H_2O_2 [2] only silica-dusts affected a change in $[Ca^{2+}]_i$. Moreover, the silica-induced $[Ca^{2+}]_i$-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 Ca^{2+}-transient is mediated by specific, protein kinase C-independent, Ca^{2+}-channels that open after contact of BAM cell-membrane with silica. Whether there is a Ca^{2+}-dependent as well as a Ca^{2+}-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 $[Ca^{2+}]_i$. Addition of PKC specific inhibitor GF 109203 X [28] is without any effect on $[Ca^{2+}]_i$, whereas the cytosolic acidification and the depolarization are clearly

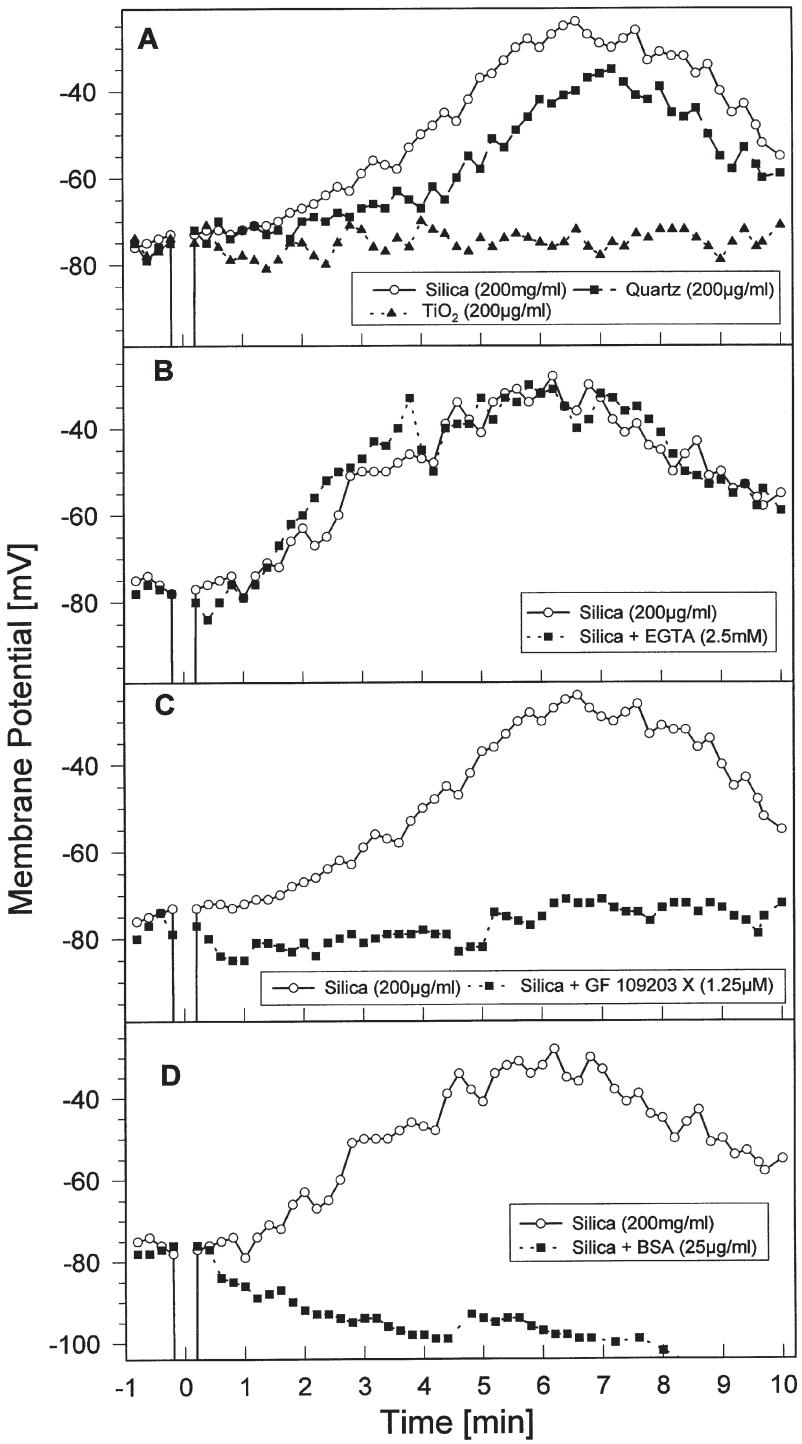

Fig. 4. Kinetics of apparent potassium potential of BAM after stimulation with silica (QUSO G 32) or TiO_2 (A), silica with or without preincubation with EGTA (B), 1.25 μM GF 109203 X (C), and with untreated or BSA-coated silica (D). Data show tracings of single runs and are representative for at least 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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