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Lung surfactant protein A (SP-A) activates a phosphoinositide/calcium signaling pathway in alveolar macrophages

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SUMMARY

Lung surfactant protein A (SP-A), the main protein component of lung surfactant which lines the alveoli, strongly enhances serum-independent phagocytosis of bacteria by rat alveolar macrophages. We tested if the effect of SP-A is due to interaction with the macrophages or to opsonization of the bacteria. In phagocytosis assays with fluorescein isothiocyanate labeled bacteria, SP-A had no opsonic effect on *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but enhanced phagocytosis by acting only on the macrophages. We characterized this activation mechanism. With single cell measurements of fura-2 loaded cells we demonstrate that SP-A raises the intracellular free calcium ion concentration 6 to 8 seconds after addition. This calcium mobilization is dose-dependent in that increased SP-A concentrations lead to a higher percentage of responding cells. Additionally, SP-A leads to a dose-dependent and transient generation of inositol 1,4,5-trisphosphate. Release of intracellular stored calcium by SP-A is a prerequisite for its stimulatory effect on phagocytosis, since SP-A-induced enhancement of phagocytosis can be impaired by prior addition of thapsigargin, a Ca^{2+}-ATPase inhibitor that leads to depletion of intracellular calcium stores.

We conclude that SP-A activates a phosphoinositide/calcium signaling pathway in alveolar macrophages leading to enhanced serum-independent phagocytosis of bacteria.

Key words: pulmonary surfactant, Ca^{2+} mobilization, inositol, phagocytosis

INTRODUCTION

The lungs are continuously exposed to infectious agents via the inhaled air. Phagocytes play a key role in host defense against bacterial invasion. In the healthy individual alveolar macrophages are the predominant phagocytes in the lower airways and the alveoli, localized in the liquid layer that covers the epithelium, in close proximity to the pulmonary surfactant.

Pulmonary surfactant is a unique macromolecular complex of phospholipids and 10% protein. It forms a monomolecular film on the luminal surface of the alveoli which dramatically reduces the surface tension between air and the lung (King and Clements, 1972).

Surfactant contains at least four lung-specific proteins, surfactant protein (SP)-A, SP-B, SP-C (Possmayer, 1988) and SP-D (Persson et al., 1990), SP-A being the predominant one of these proteins. It belongs to a family of mammalian calcium-dependent carbohydrate-binding proteins, the C-type lectins (Drickamer, 1988). SP-A recognizes high mannose structures (Haagsman et al., 1987) and its ultrastructure resembles that of the mannose binding protein from serum (Thiel and Reid, 1989) as well as the complement factor C1q (Voss et al., 1988).

SP-A has been already shown to increase antibacterial and antiviral functions of alveolar macrophages. It enhances serum-dependent phagocytosis (Tenner et al., 1989; Van Iwaarden et al., 1990) as well as serum-independent phagocytosis (Van Iwaarden et al., 1991; Manz-Keinke et al., 1992). The mechanism by which SP-A enhances serum-independent phagocytosis of bacteria is a matter of debate, two possible modes are discussed. SP-A could act either as an opsonin or it could activate macrophage functions resulting in an increased capacity for phagocytosis. Besides stimulation of phagocytosis, SP-A reveals additional immunologically relevant effects: it increases production of superoxide radicals (Van Iwaarden et al., 1990) and induces chemotactic migration (Wright and Youmans, 1993) of alveolar macrophages. These observations support the idea that SP-A enhances the host defense functions of alveolar macrophages by modulating their cell functions.

Modulation of macrophage functions by external signals has been described to occur either via cleavage of membrane phospholipids by phospholipases which initiates the cyclooxygenase pathway (Nakamiya et al., 1992), or by elevation of $[Ca^{2+}]_i$. An agonist induced calcium mobilization has been shown during activation of macrophages by platelet-activating factor (Gardner et al., 1993), endothelin (Haller et al., 1991) and α2-macroglobulin-methylamine (Misra et al., 1993). In general the source of calcium entry into the cytosol can be regulated by a number of mechanisms, i.e. through the opening of different channels leading to an influx from the extracellular space or by mobilizing intracellularly stored calcium by ryanodine- or inositol 1,4,5-trisphosphate (InsP3)-sensitive channels (Tsien and Tsien, 1990). Although macrophages have been demonstrated to

contain voltage-dependent calcium channels (Hijioka et al., 1991), agonist induced calcium mobilization in macrophages is often accompanied by transiently elevated levels of inositol phosphates (Randriamampita et al., 1991; Misra et al., 1993).

In the present study we examined the mechanism of SP-A induced enhancement of phagocytosis and the signaling pathway responsible. As no qualitative differences between human and rat SP-A have been observed (Van Iwaarden et al., 1991; Wright and Youmans, 1993) we used human SP-A throughout the studies with rat alveolar macrophages. We show that SP-A enhances serum-independent phagocytosis of alveolar macrophages by interaction with macrophages and we characterize the activation mechanism. We demonstrate that interaction between SP-A and macrophages leads to InsP3 elevation and increase in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

SP-A

Proteinosis SP-A, purified from lung lavage fluid of proteinosis patients and recombinant SP-A, expressed in Chinese hamster ovary cells transfected with the human SP-A gene α3, were gifts from K. P. Schäfer (Byk Gulden Pharmaceuticals, Dept of Molecular Biology, Konstanz, Germany). Proteinosis and recombinant SP-A were isolated and purified as described (Hawgood et al., 1987). Throughout all tests the same preparation of proteinosis SP-A was used. Purity of the SP-A preparation was assessed by SDS-PAGE. Possible contamination with lipopolysaccharide (LPS) was tested in two different functional tests. RAW cells (mouse macrophage line) secret tumor necrosis factor (TNF) when incubated with LPS (for details see Espevik and Nissen-Meyer, 1986) but SP-A does not induce any TNF secretion under identical conditions. Furthermore, SP-A stimulates phagocytosis of bacteria by alveolar macrophages while LPS does not influence phagocytosis rates. Unless otherwise mentioned, proteinosis SP-A was used in all experiments.

Others

Homologous serum was obtained by heart punctation of Wistar rats and stored at −80°C. Anti-SP-A-antiserum from rabbit was a gift from K. P. Schäfer (Byk Gulden Pharmaceuticals, Dept of Molecular Biology, Konstanz, Germany). Fluorescein isothiocyanate (FITC), pluronic F-127 and fura 2-dextran were obtained from Molecular Probes (Eugene, Oregon, USA). Fura-2 acetoxymethyl ester (AM), lipopolysaccharide (LPS) from *Salmonella abortus equi*, probenecid, thapsigargin, 1,1,2-trichlorotrifluoroethane and trioctylamine were purchased from Sigma (Munich, Germany). Phospholipase C inhibitor U-73122 and the inactive structural analog U-73343 were from Biomol (Hamburg, Germany). Dulbecco's phosphate buffered saline (PBS) was provided by Gibco (Eggenstein, Germany) and Hanks' balanced salt solution (HBSS) from Flow (Bonn, Germany).

Alveolar macrophages

Rat alveolar macrophages were isolated by lung lavage of Wistar rats (250-300 g body weight) as previously described (Manz-Keinke et al., 1991) and suspended in HBSS containing 20 mM Hepes (HBSS/Hepes). Viability of the cells was determined by trypan blue dye exclusion and always exceeded 95%.

Bacteria

Escherichia coli K12 (MC 4100) and *Staphylococcus aureus* (Cowan I) were kindly provided by W. Boos (University of Konstanz, Germany). *Pseudomonas aeruginosa* (DSM No. 288) was kindly provided by R. Conrad (University of Konstanz). Bacteria were harvested after 16 hours (when turned into stationary phase). Detailed cultivation conditions were as previously described (Manz-Keinke et al., 1992).

Phagocytosis assay

For phagocytosis assays bacteria were labeled with FITC and suspended in HBSS/Hepes as previously described (Manz-Keinke et al., 1992). Unless mentioned otherwise macrophages were suspended, and macrophages and bacteria were used in a ratio of 1:10 in 100 μl HBSS/Hepes and incubated for 60 minutes at 37°C, 5% CO_2. During the phagocytosis assay and preincubation times the cells were permanently shaken gently to provide mixing and to prevent adhering to the surface of the test tubes. Under these conditions the macrophages remained in suspension and did not attach to the plastic surface. Phagocytosis was stopped by adding 1 ml of ice-cold buffer and washing the macrophages three times by centrifugation for 10 minutes at 400 ***g***. Macrophages were examined by fluorescence light microscopy; cells with one or more bacteria bound or ingested were counted as positive. One hundred macrophages per sample were analyzed. Each sample was carried out in duplicate.

To prove complete ingestion of the bacteria some samples were counted in the presence of ethidium bromide. Addition of ethidium bromide to quench fluorescence associated with FITC-labeled bacteria allows discrimination of bound from internalized bacteria (Nichols et al., 1993). We determined that 85%±3% of the macrophages counted as positive in the absence of ethidium bromide had actually ingested the bacteria.

In preliminary experiments with adherent macrophages we needed higher SP-A concentrations to induce a $[Ca^{2+}]_i$ rise than we needed in experiments with suspended macrophages to enhance phagocytosis rates or to induce formation of inositol 1,4,5-trisphosphate (InsP3). Therefore, we tested the responsiveness of adherent macrophages to different SP-A concentrations in a phagocytosis test. With adherent macrophages a 2-3 fold higher concentration of SP-A was necessary to enhance phagocytosis comparable to our previously published data for macrophages in suspension (Manz-Keinke et al., 1992). This phenomenon of different responsiveness of adherent and suspended alveolar macrophages has also been observed by Mueller et al. (1989).

Determination of inositol 1,4,5-trisphosphate (InsP3)

A specific binding protein assay (DuPont, Bad Homburg, Germany) was used in principle according to the manufacturer's instructions. Macrophages (2×10^7/ml) were incubated in 50 μl volumes with SP-A at various concentrations for the indicated times at room temperature. Incubation was stopped by adding 150 μl of ice-cold 2 M trichloroacetic acid. After centrifugation for 15 minutes at 4,000 ***g*** at 4°C, supernatants were removed and allowed to reach room temperature. The water-soluble components were extracted by adding a double volume of a 3:1 (v/v) mixture of 1,1,2-trichlorotrifluoroethane and trioctylamine. The InsP3 content of the extract was determined in the binding-protein assay. Each sample was carried out in duplicate.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in single cells was measured using the calcium-sensitive dye fura-2. Fura-2/AM loading medium was prepared by mixing equal volumes of fura-2/AM (2 mM in dimethyl sulfoxide) with pluronic F-127 (12.5% in dimethyl sulfoxide); 4 ml of macrophage suspension (106 cells/ml) were loaded in HBSS/Hepes plus probenecid (final concentration 5 mM) for 30 minutes at 37°C with 20 μl of fura-2/AM loading medium. Subsequently the cells were incubated on glass coverslips for 30 minutes for adherence, followed by 60 minutes incubation at 37°C to assure hydrolysis of the dye.

In our system sequestration of fura-2 from the cytoplasm into vesicles was not completely blocked in the presence of 5 mM probenecid. This finding is in contrast to results described by Steinberg et al. (1987) who reported nearly maximal inhibition of sequestration in mouse peritoneal macrophages with probenecid at this concentration (Berridge, 1993) and could be due to differences between the two macrophage populations. Sequestration results in a portion of fluorescent indicator dye being incapable of responding to changes in cytosolic $[Ca^{2+}]$. Therefore $[Ca^{2+}]_i$ is not expressed in

absolute values but as the ratio of fluorescence emission at 350/380 nm excitation (R 350/380). In an attempt to circumvent sequestration we loaded the cells with fura-2/AM at lower temperature. However, this treatment resulted in a reduced loading efficiency without affecting sequestration. Direct microinjection of fura-2 or fura-2 dextran led to an artefactual persistent increase in $[Ca^{2+}]_i$ that prevented the measurement of SP-A-induced $[Ca^{2+}]_i$ changes.

Calcium measurements in single cells were carried out using an inverted Zeiss IM microscope equipped with quartz optics. For fluorescence illumination a 50 W mercury lamp was used. Excitation filters were 340 or 350 and 380 nm band pass (LOT, Darmstadt, Germany). The 380 nm filter was paired with a 30% neutral density filter to optimize camera adjustment. Cells were viewed with a 63× Neofluar, NA 1.2 (Zeiss, Oberkochen, Germany). Fluorescence emission was directed through a dichroic mirror (FT 460 Zeiss) and a 470 nm LP emission filter (Zeiss). Images were recorded with a low light SIT camera (Heimann, Wiesbaden, Germany) on a VHS video recorder (Panasonic, Osaka, Japan).

Photometric intensity measurements of recorded images and microscopic calibration were done as previously described (Schlatterer et al., 1992). Acquisition of consecutive image pairs was done with time intervals of 3.2 seconds. The ratios of fluorescence emission at 350 and 380 nm (R 350/380) excitation were used to calculate basal $[Ca^{2+}]_i$ levels in individual cells on the basis of calibration curves obtained with defined calcium standards according to Schlatterer et al. (1992).

For imaging experiments, image pairs were recorded by the low light camera at time intervals of 12 seconds. The images were digitized in real time to 512×512 pixel and 256 gray scales using an AD-card (Data Translation) and stored on hard disk. Ratio images were obtained by dividing the two corresponding images on a pixel to pixel basis. Ratio images (R 340/380) are displayed as false colour images.

RESULTS

We previously described that human recombinant SP-A as well as SP-A isolated from lung lavage material from proteinosis patients enhance serum-independent phagocytosis of bacteria by alveolar macrophages (Manz-Keinke et al., 1992). Now we were interested in the underlying mechanisms. Two possibilities could be responsible for enhanced phagocytosis: either opsonization of the bacteria by SP-A or activation of the macrophages induced by SP-A or both. First we tested the ability of SP-A to enhance phagocytosis by directly interacting with the macrophages.

Interaction between SP-A and macrophages is necessary for enhanced phagocytosis

Macrophages were preincubated with SP-A for different time periods, then SP-A was removed from the cells by washing. Subsequently bacteria were added to the pretreated macrophages and their phagocytosis rate was determined. Preincubation of the macrophages with SP-A was sufficient to cause enhancement of phagocytosis rates similar to those obtained when SP-A was present throughout the test. Fig. 1 shows the effect of various preincubation times on this activation. Preincubation of macrophages with SP-A for 30 to 60 minutes was necessary to lead to enhanced phagocytosis. In contrast, enhanced phagocytosis induced by serum depended on its continued presence throughout the incubation. Thus, in contrast to serum, SP-A preincubation primes macrophages for increased activity. This effect is clearly different from that of the opsonizing compounds (immunoglobulins and complement factors) in serum.

SP-A has no opsonic activity on *E. coli*, *S. aureus* and *P. aeruginosa*

The finding that SP-A enhanced phagocytosis by directly acting on the macrophages did not exclude the possibility that it might as well act as an opsonin on the bacteria used in our assays. We therefore preincubated the bacteria with SP-A for 60 minutes, removed SP-A by centrifugation and incubated these pretreated bacteria together with the macrophages in the phagocytosis assay. Fig. 2 shows that removal of SP-A from the bacteria prior to incubation with the macrophages resulted in phagocytosis rates similar to rates without any SP-A (to be compared with control value with buffer in Fig. 1). When SP-A was still present during the phagocytosis assay with the macrophages phagocytosis was enhanced. In contrast, experiments with homologous serum clearly revealed its opsonizing effects; no difference in the phagocytosis rates was observable, whether after preincubation of the bacteria the serum was removed or not prior to the phagocytosis assay. We therefore conclude that, in contrast to serum, the effect of SP-A on phagocytosis is not due to any opsonic activity on the bacteria tested.

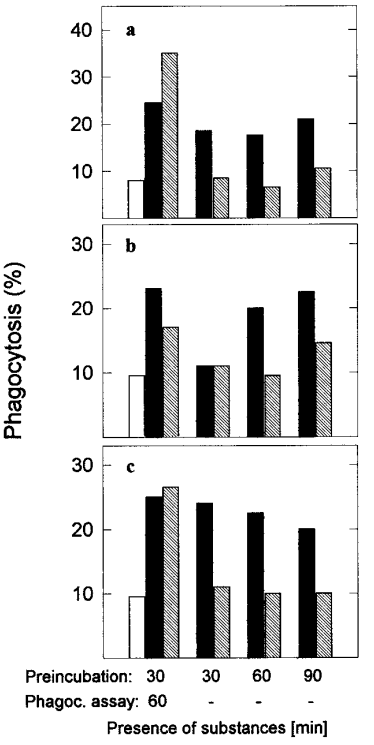

Fig. 1. Preincubation of macrophages with SP-A leads to enhanced phagocytosis. Macrophages were preincubated with SP-A (filled bars) or 10% homologous serum (hatched bars) for 30, 60 or 90 minutes at 37°C. After preincubation for the indicated times SP-A or serum was removed by centrifugation (three times for 10 minutes at 400 ***g***). Subsequently the macrophages were incubated with bacteria in the phagocytosis assay for another 60 minutes. In control experiments macrophages were preincubated either with buffer (open bar), or SP-A, or 10% homologous serum for 30 minutes prior to the addition of the bacteria. The phagocytosis assay in controls was performed by incubating the macrophages together with the bacteria for 60 minutes, while the substances remained in the test. The results of one typical experiment out of three for each species are shown. (a) *E. coli*, (b) *S. aureus*, (c) *P. aeruginosa*. SP-A concentrations used were 10 μg/ml for *S. aureus*, and 20 μg/ml for *E. coli* and *P. aeruginosa*.

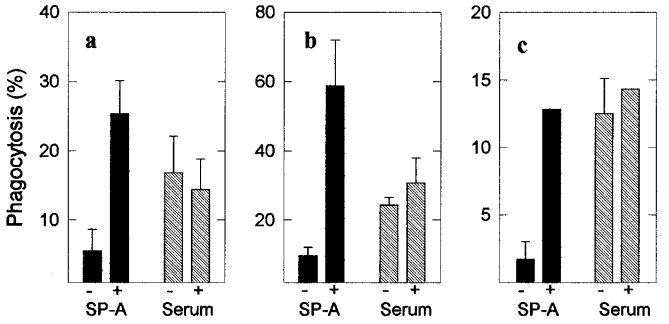

Fig. 2. SP-A has no opsonic effect on *E. coli*, *S. aureus* and *P. aeruginosa*. Bacteria were preincubated with SP-A (20 μg/ml) (filled bars) or with 10% homologous serum (hatched bars) for 60 minutes at 37°C. After preincubation SP-A or serum was either removed by centrifugation (three times for 10 minutes at 10,000 ***g***) from the bacteria (bars: –SP-A; –serum) or not (bars: +SP-A; +serum). The pretreated bacteria were used in the phagocytosis assay for another 60 minutes. Phagocytosis rates in control experiments: *E. coli* 5.8±1.3; *S. aureus* 12.2±3.8; *P. aeruginosa* 7.7±2.6. (a) *E. coli*, (b) *S. aureus*, (c) *P. aeruginosa*. Macrophages and bacteria were used in a ratio of 1:30. Means and standard deviations of three experiments for each bacteria species are shown. For *P. aeruginosa* (bars: +) two experiments were carried out.

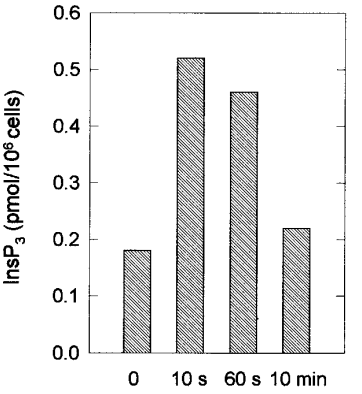

Fig. 3. Time course of the InsP3 formation in response to SP-A: 10^6 macrophages (in suspension) were incubated with SP-A (60 μg/ml) at room temperature for the indicated time periods. Incubation was stopped by adding ice-cold TCA and InsP3 content of the samples was determined as described in Materials and Methods. One out of four separate experiments is shown.

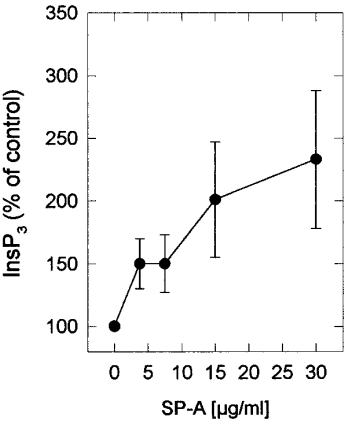

Fig. 4. Generation of InsP3 is dependent on SP-A concentration. Macrophages (in suspension) were incubated with different SP-A concentrations for 60 seconds at room temperature. Stopping the incubation and determination of the InsP3 content of the samples was accomplished as described in Material and Methods. The data shown are calculated as percentile change from the control. Means and standard deviations of three to five experiments for each data point are shown.

SP-A leads to activation of the InsP3 pathway

Since we found that SP-A enhanced phagocytosis by activating the macrophages, most likely a signal transduction system is involved. The mode of macrophage activation by platelet-activating factor and α2-macroglobulin has been shown to proceed via generation of InsP3 and intracellular calcium mobilization (Randriamampita et al., 1991; Misra et al., 1993). Thus we studied whether SP-A exerted its effect also along this signal route and determined the concentration of InsP3 before and after the addition of SP-A.

A typical time course of InsP3 formation in response to SP-A is shown in Fig. 3. An InsP3 content significantly above basal values was always detectable at the earliest time point measured, i.e. 10 seconds. In two experiments maximal InsP3 formation was reached at that time, whereas in two other experiments the InsP3 concentration further increased up to 60 seconds after addition of SP-A. InsP3 formation returned to basal values 10 minutes after stimulation. This general time course was seen in all experiments. The InsP3 formation was dependent on the SP-A concentration. The dose-dependency is shown in Fig. 4. Increased SP-A concentrations led to enhanced InsP3 formation. SP-A concentrations as low as 4 μg/ml were already effective. These results show that SP-A activates the formation of InsP3 in alveolar macrophages.

SP-A increases $[Ca^{2+}]_i$ in alveolar macrophages

We tested the effect of SP-A on $[Ca^{2+}]_i$ in adherent macrophages with single cell measurements of fura-2 loaded cells. The time course of $[Ca^{2+}]_i$ of three individual macrophages stimulated with SP-A is shown in Fig. 5. Within 6 to 8 seconds after addition of SP-A a $[Ca^{2+}]_i$ increase was measured (Fig. 5a). In all cells measured the observed $[Ca^{2+}]_i$ increase lasted several minutes. Fig. 5b shows that $[Ca^{2+}]_i$ had reached a plateau phase roughly 15 minutes after addition of SP-A which was above prestimulation levels and lasted until 50 minutes. These observations are consistent with findings of Randriamampita et al. (1993). They recorded an increase of $[Ca^{2+}]_i$ which persisted for at least 15 minutes for both P388D1 macrophages and astrocytoma cells in response to an extract of stimulated Jurkat lymphocytes. Basal $[Ca^{2+}]_i$ levels in the individual cells ranged from 20 to 120 nM. Addition of SP-A increased $[Ca^{2+}]_i$ up to 100% to 500% of the basal values (97 cells measured). Stimulation with recombinant SP-A led to similar $[Ca^{2+}]_i$ increases as proteinosis SP-A (data not shown). Furthermore, the specificity of the SP-A effect was proved with anti-SP-A-antiserum. SP-A that had been adsorbed by anti-SP-A-antiserum coated onto colloidal gold particles showed no effect on $[Ca^{2+}]_i$ (see Fig. 5c) while untreated SP-A added 10 minutes afterwards enhanced $[Ca^{2+}]_i$.

False colour images (Fig. 6) show the individual and heterogenous changes of the $[Ca^{2+}]_i$ of three different macrophages after addition of SP-A. The macrophage on the

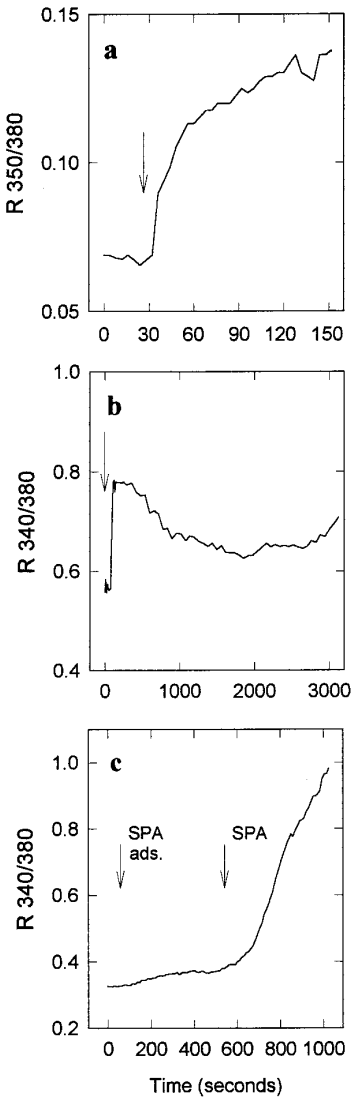

Fig. 5. Time course of the change in $[Ca^{2+}]_i$ in single macrophages during stimulation with SP-A. Single cell measurement of adherent macrophages loaded with fura-2/AM. The ratio of fura-2 fluorescence emission either at 350/380 nm or 340/380 nm excitation (R350/380 and R340/380) is plotted versus time. At the indicated time (arrow) SP-A (30 μg/ml in (a) or 120 mg/ml in (b) and (c)) was added. (a) The increase in $[Ca^{2+}]_i$ was detectable after a time lag of 6 to 8 seconds. (b) $[Ca^{2+}]_i$ was registered over a period of 50 minutes. (c) SP-A adsorbed onto anti-SP-A-antiserum coated onto colloidal gold particles did not effect $[Ca^{2+}]_i$, while the addition of SP-A 10 minutes later aroused $[Ca^{2+}]_i$ increase.

right shows a slow rise in $[Ca^{2+}]_i$ over the time period registered while the $[Ca^{2+}]_i$ of the two other macrophages already drops again. Also the typical spreading behaviour of the macrophages is seen.

The percentage of macrophages responding with an increase in $[Ca^{2+}]_i$ depends on the SP-A concentration

We then determined the dose-dependency of the $[Ca^{2+}]_i$ rise induced by SP-A. The data of these experiments are shown in Table 1. Increased concentrations of SP-A did not augment the observed change in $[Ca^{2+}]_i$ in individual cells, but resulted in an increase in the number of responding macrophages. Nearly 70% of the macrophages responded with a $[Ca^{2+}]_i$ increase upon stimulation with 120 μg SP-A/ml.

Table 1. The percentage of macrophages responding to SP-A with an increase in $[Ca^{2+}]_i$ depends on the SP-A concentration

SP-A (μg/ml)	Percentage of cells responding with calcium increase	*n*
15	0	21
30	36	25
60	56	18
120	69	23

The $[Ca^{2+}]_i$ response of single rat alveolar macrophages was analyzed after adding the indicated SP-A concentrations. The response was followed for 3 minutes. Data are from 3-6 independent experiments. *n*, number of cells analyzed.

Table 2. Pretreatment with thapsigargin decreases the stimulative SP-A effect on phagocytosis

Preincubation	% Phagocytosis (±s.d.)	*n*
Buffer	66.4±5.1	5
Thapsigargin (0.1 μM)	67	2
Thapsigargin (1 μM)	56.7±13	3
Thapsigargin (10 μM)	31.3±6.0	3

Macrophages were preincubated with thapsigargin or buffer for 60 minutes before adding bacteria plus SP-A (20 μg/ml) (with thapsigargin remaining in the test). In control experiments (preincubation with buffer) phagocytosis rate without addition of SP-A was 20.1±6.2 (*n*=5), in experiments with addition of 10 μM thapsigargin it was 19.9±4.1 (*n*=4). Addition of thapsigargin after preincubation of the macrophages with SP-A revealed no effect of thapsigargin on the phagocytosis rate (64.7±1.6, *n*=3). *S. aureus*/stat was used in all phagocytosis assays. s.d., standard deviation; *n*, number of experiments.

Thapsigargin suppresses the stimulative effect of SP-A on phagocytosis

Our data suggest that calcium mobilization caused by SP-A is most probably induced by InsP3 generation. This pathway of $[Ca^{2+}]_i$ increase usually involves liberation from intracellular stores. Therefore, any impairment of the liberation of intracellularly stored calcium should affect the enhancement of phagocytosis by SP-A. Thapsigargin, an irreversible inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase is a useful agent to deplete intracellular calcium pools (Thastrup et al., 1989). We preincubated macrophages with thapsigargin for one hour before adding SP-A. Thapsigargin at a concentration of 10 μM suppressed the SP-A enhancement of phagocytosis (Table 2). In contrast, when SP-A was added to the macrophages 10 minutes before thapsigargin, the SP-A mediated enhancement of phagocytosis was unimpaired. These results further prove the involvement of intracellular calcium stores in the activation of macrophages by SP-A. Their depletion prevents the SP-A mediated enhancement of serum-independent phagocytosis.

Additionally we tested the effect of phospholipase C inhibitor. U-73122 has been shown to inhibit agonist-induced InsP3-increases in several cell systems (Smallridge et al., 1992). Incubation with the inhibitor efficiently prevented the SP-A effect on phagocytosis. In contrast, U-

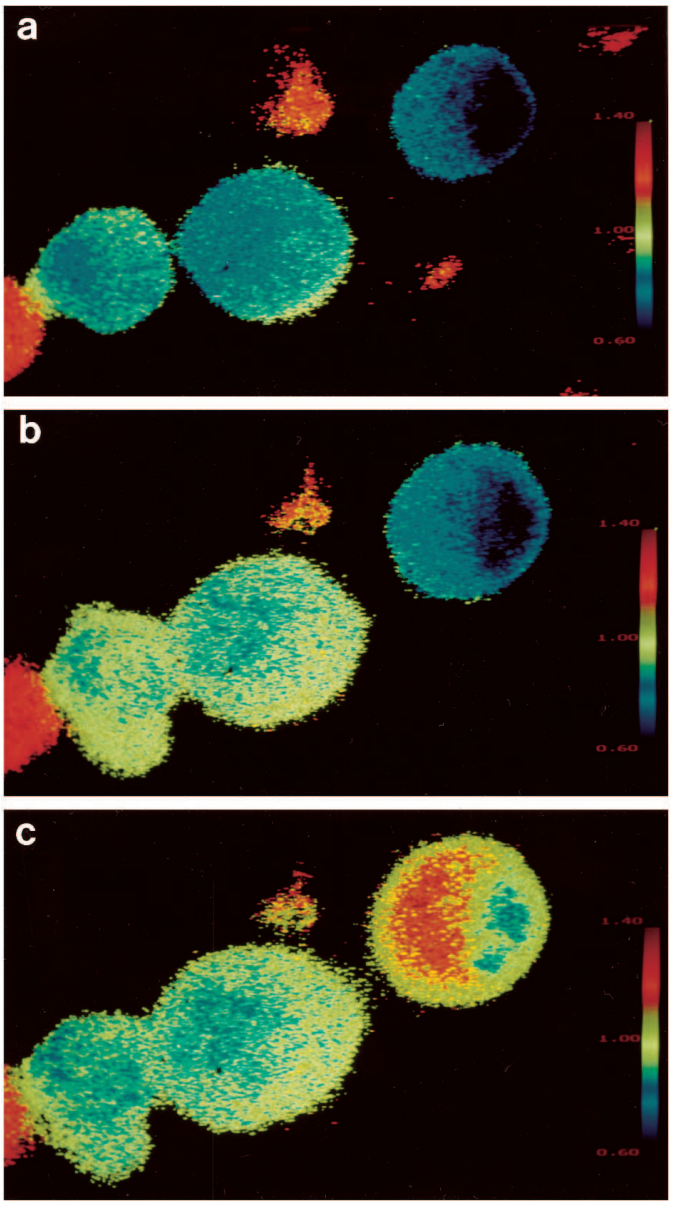

Fig. 6. SP-A initiates changes in $[Ca^{2+}]_i$ in macrophages. Adherent alveolar macrophages loaded with Fura-2/AM (a) 17 seconds, (b) 144 seconds, and (c) 374 seconds after addition of SP-A (120 µg/ml). False colour images of the ratio 340/380.

73343, a structurally analogous but inactive substance, showed no influence (Table 3). The effect of the phospholipase C inhibitor indicates the involvement of InsP3 in the SP-A effect. As the substances used are dissolved in DMSO we tested DMSO in control experiments. DMSO had no effect on the phagocytosis.

The specificity of the SP-A effect was tested with anti-SP-A-serum. We incubated the macrophages with SP-A in the presence of anti-SP-A-antiserum, removed serum and SP-A by centrifugation and tested the macrophages in the phagocytosis assay. Macrophages pretreated with SP-A and anti-serum showed no enhanced phagocytosis while macrophages pretreated with SP-A alone showed enhanced phagocytosis (Table 3).

Table 3. Effect of macrophage pretreatment with different substances on the stimulative SP-A effect on phagocytosis

Preincubation	SP-A (±s.d.)	% Phagocytosis (±s.d.)
Buffer	–	14.0±3.6
Buffer	+	42.3±6.7
U-73122	–	19.3±5.8
U-73122	+	22.0±5.6
U-73343	–	14.5±2.1
U-73343	+	38.5±0.7
DMSO	–	16.3±7.1
DMSO	+	37.7±1.5
Antiserum	–	13.3±3.2
Antiserum	+	14.5±2.1

Macrophages were preincubated with substances or buffer for 2 minutes at 37°C. After addition of SP-A (10 µg/ml), preincubation was continued for 30 minutes before adding *S. aureus*/stat. The phagocytosis tests were stopped after 60 minutes. Phospholipase C inhibitor U73122 (1 µM) and the inactive analog U73343 (1 µM) were dissolved in DMSO. In control tests 7.5 µl DMSO were applied to the 150 µl test volume. Antiserum (10%) was removed after preincubation by centrifugation. Data are the average of three experiments. s.d., standard deviation.

DISCUSSION

SP-A activates alveolar macrophages via InsP3 formation and $[Ca^{2+}]_i$ increase

We showed that SP-A led to a dose-dependent and transient generation of InsP3. At 10 seconds after addition of SP-A InsP3 formation was significantly increased, the peak of the response occurred 10 to 60 seconds after stimulation and InsP3 declined to basal values within 10 minutes. This rapid formation of InsP3 is in line with data reported for peritoneal macrophages (Prpic et al., 1988). Involvement of InsP3 was further proved in phagocytosis experiments. Phospholipase C inhibitor U-73122, which blocks the agonist-induced production of InsP3 inhibits the stimulative effect of SP-A on phagocytosis.

Furthermore we investigated, whether addition of SP-A to macrophages results in changes in $[Ca^{2+}]_i$. We used single cell measurements of fura-2 loaded, adherent cells for these experiments. Measurements of $[Ca^{2+}]_i$ in populations of cells suffer from the disadvantage that changes which do not occur simultaneously in the population may not be detected (Krusk and Maxfield, 1987). In our single cell measurements SP-A induced a dose-dependent increase in $[Ca^{2+}]_i$ starting within 6 to 8 seconds after stimulation. Increasing concentrations of SP-A led to a higher percentage of responding macrophages. Findings in other cell systems suggest that such a dose-dependent cell-recruitment might be a more general phenomenon. It has been recently shown that cholecystokinin-octapeptide dose-dependently increased the percentage of pancreatic acinar cells responding by calcium mobilization (Willems et al., 1993). Our data indicate that SP-A can activate alveolar macrophages by mobilization of $[Ca^{2+}]_i$. This mobilization can be at least partly due to an InsP3 mediated calcium release.

Since SP-A binding to macrophages depends on the presence of calcium ions we could not carry out calcium measurements in the absence of extracellular calcium. Therefore, we used a complementary approach to examine the source of calcium. To investigate the involvement of intracellular stores during activation of macrophages by SP-A, we depleted intra-

cellular stores by the use of thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase pump (Thastrup et al., 1989), and examined its effect on phagocytosis. Incubation of macrophages with thapsigargin before stimulation with SP-A impaired the enhancing effect of SP-A on phagocytosis. In contrast, when macrophages were incubated with SP-A before addition of thapsigargin the phagocytosis rate was similar to that without thapsigargin. This result confirms that SP-A is only able to enhance phagocytosis when an intracellular calcium store has not been depleted previously.

SP-A enhances serum-independent phagocytosis by activating the macrophages

We show in this study that the percentage of macrophages responding with $[Ca^{2+}]_i$ increase depends on the SP-A concentration. This recruitment corresponds to the increase in phagocytosis rates: the percentage of macrophages phagocyting bacteria increases in response to increasing SP-A concentrations (Manz-Keinke et al., 1992).

The interaction between macrophages and SP-A is sufficient to enhance phagocytosis. This effect is time dependent; macrophages have to be stimulated with SP-A for 30 to 60 minutes to react with an enhanced phagocytosis capacity. The mechanism for how InsP3 induced calcium mobilization leads to enhanced phagocytosis capacity is not known. Kruskal and Maxfield (1987) described activation of peritoneal macrophages by contact with a glass surface leading to immediate spreading and 'frustrated' phagocytosis. This was preceded by a rise in $[Ca^{2+}]_i$, derived from intracellular pools (Kruskal and Maxfield, 1987). Although SP-A also evokes a $[Ca^{2+}]_i$ increase in macrophages, its mechanism of activation of phagocytosis is different, because the effect depends on incubation for at least 30 minutes.

We suggest that a rise in $[Ca^{2+}]_i$ could influence receptors in the cell membrane necessary for bacteria binding or uptake. A time period of 60 minutes is too short for genomic effects. An increase of receptor proteins by de novo protein synthesis requires e.g. 12 hours or more (Cooper et al., 1984). An alternative would be an increase of binding or uptake sites on the cell surface by recruitment from intracellular membrane stores.

There are conflicting results concerning a possible role of SP-A as an opsonin. We could detect no opsonic activity of proteinosis SP-A with three bacteria species tested. This is in line with results of van Iwaarden et al. (1990) with *S. aureus* and with our previous data on human recombinant SP-A (Manz-Keinke et al., 1992). However, SP-A was reported to act as an opsonin on Herpes simplex virus (Van Iwaarden et al., 1991) and on *Pneumocystis carinii* (Zimmerman et al., 1992). Recently an opsonic effect of proteinosis SP-A on *Streptococcus pneumoniae* and *S. aureus* was described, while these authors found no enhanced attachment of bacteria after preincubation of the macrophages with SP-A for 30 minutes (McNeely and Coonrod, 1993). One possible explanation for this finding is that incubation of the macrophages for only 30 minutes would be too short to induce increased phagocytosis. On the other hand the differing results concerning binding of SP-A to *S. aureus* might be due to variations in the bacteria strains used.

Although SP-A can act as an opsonin on some organisms, our findings suggest that the main mechanism by which SP-A enhances phagocytosis is due to macrophage activation.

Interaction of SP-A and macrophages

Specific binding of SP-A to the macrophages has been described and appears necessary for affecting cell functions, whereas the binding mechanism of SP-A is under debate at the moment. Our previous results suggest that the globular domain of SP-A mediates the binding to carbohydrate structures on the macrophage surface (Manz-Keinke et al., 1991; Ohmer-Schröck et al., 1993). On the other hand, Pison et al. (1992) postulated binding via the collagen-like domain to a receptor that binds the complement factor C1q. However, in a series of experiments we could not detect enhanced phagocytosis of alveolar macrophages in the presence of C1q (unpublished observation). These conflicting results suggest that SP-A might interact with macrophages via various mechanisms and that more than one binding site might exist.

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