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Pulmonary Surfactant Protein A Activates a Phosphatidylinositol 3-Kinase/Calcium Signal Transduction Pathway in Human Macrophages: Participation in the Up-Regulation of Mannose Receptor Activity

Alison A. Beharka,* Joy E. Crowther,*† Francis X. McCormack,‡ Gerene M. Denning,* Jason Lees,* Eric Tibesar,* and Larry S. Schlesinger²†

Surfactant protein A (SP-A), a major component of lung surfactant, binds to macrophages and has been shown to alter several macrophage biological functions, including up-regulation of macrophage mannose receptor (MR) activity. In the present study, we show that SP-A induces signal transduction pathway(s) that impact on MR expression. The addition of human, rat, or recombinant rat SP-A to human monocyte-derived macrophages significantly raised the level of cytosolic Ca²⁺ above baseline within 10 s of SP-A addition, as measured by spectrofluorometric analysis. SP-A induced a refractory state specific for SP-A consistent with homologous desensitization of a receptor(s) linked to calcium mobilization because a second application of SP-A did not induce a rise in cytosolic Ca²⁺ whereas the addition of platelet-activating factor did. Using site-directed mutations in SP-A, we determined that both the attached sugars and the collagen-like domain of SP-A are necessary to optimize Ca²⁺ mobilization. SP-A triggered the increase in cytosolic Ca²⁺ by inducing activation of phospholipase C, which leads to the hydrolysis of membrane phospholipids, yielding inositol 1,4,5-trisphosphate and mobilizing intracellularly stored Ca²⁺ by inositol triphosphatesensitive channels. Finally, inhibition of PI3Ks, which appear to act upstream of phospholipase C in Ca²⁺ mobilization, decreased the SP-A-induced rise in MR expression, providing evidence that SP-A induction of MR activity involves the activation of a pathway in which PI3K is a component. These studies provide further evidence that SP-A produced in the lung plays a role in modulating macrophage biology, thereby contributing to the alternative activation state of the alveolar macrophage.

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nhaled particles and infectious agents represent a constant threat to the respiratory system. To defend itself, the respiratory tract has a complex system consisting of mechanical reflexes, physical barriers, and a diverse set of biochemical and cellular defenses. Acquired humoral and cellular immunity are of obvious importance in lung defense; however, it is becoming increasingly clear that components of innate immunity also play important roles in determining the response to and fate of inhaled particles. Pulmonary surfactant is a multimolecular complex, including proteins and lipids, that serves to reduce the surface tension of the alveoli, allowing expansion of the lung during inspiration. Recently, components of surfactant, specifically surfactant protein A (SP-A)³ and surfactant protein D (SP-D), have been shown to play important roles in the lung innate immune response (1, 2).

SP-A, a member of the collectin family, is the most abundant protein component (by weight) of human pulmonary surfactant (2). SP-A is produced as a 28- to 36-kDa (rat) or 35-kDa (human) polypeptide that contains a hydroxyproline-rich collagen-like domain, a carbohydrate recognition domain (CRD), and one (human) or two (rat) N-linked oligosaccharide attachment sites at Asn1 in the N terminus (rat) and Asn187 (human and rat) in the CRD (3). SP-A effects on macrophages are believed to be mediated by interactions of this protein with its receptor(s) on macrophages (1). Chronoes et al. (10) have reported a 210-kDa SP-A receptor on U937 cells and rat macrophages called SP-R210. Other SP-A-binding proteins have also been reported (reviewed in Ref. 11). Little is known about SP-A receptor.

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about the signaling pathways activated by SP-A binding to its receptor(s). Ohlmer-Schröck et al. (12) reported that human SP-A activates a phosphoinositide/calcium-signaling pathway involved in phagocytosis by rat alveolar macrophages. However, the signaling pathway(s) induced by SP-A in primary human macrophages is unknown.

Several mechanisms induce the release of small bursts of Ca²⁺ into the cytosol, which acts as a second messenger for signal transduction (13). In macrophages, the generation of D-myosin-inositol 1,4,5-triphosphate (InsP₃) is important in regulating Ca²⁺ release from the endoplasmic reticulum (ER) (reviewed in Ref. 14). Signals initiated by either G protein-linked receptors or receptors linked directly or indirectly to tyrosine kinases (14) activate phospholipase C (PLC), which hydrolyzes phosphatidylinositol (4,5)-bisphosphate to form InsP₃ and 1,2-diacylglycerol. In some cases, PI3Ks may act upstream of PLC activation because PLCγ can be activated by lipid products of PI3Ks (15, 16). Although a variety of agonists are capable of activating both PLC and PI3Ks in human macrophages (17), it is not known whether SP-A is one of them.

We hypothesized that SP-A binding to human macrophages activates a signal transduction pathway that uses Ca²⁺ as a second messenger. In this study, we examined Ca²⁺ mobilization in human monocyte-derived macrophages (MDM) in response to SP-A. Using wild-type and variant SP-A proteins with site-directed mutations, we investigated whether the attached carbohydrates and/or collagen-like domain of SP-A are necessary for activation. Using inhibitors, we studied the possible link between components of the Ca²⁺/InsP₃ signal transduction pathway and SP-A up-regulation of MR expression. Our data indicate that SP-A induces Ca²⁺ mobilization by activating PLC, which hydrolyzes membrane phospholipids and yields InsP₃, and that activation of PI3K(s), potentially through a PLC-signaling pathway, plays a role in SP-A up-regulation of MR.

### Materials and Methods

#### Buffers, reagents, and media

RPMI 1640 medium with l-glutamine was purchased from Invitrogen Life Technologies. RPMI 1640 medium was used alone or with 20 mM HEPES (Sigma-Aldrich) and 1 mg/ml human serum albumin (HSA) (Calbiochem) at pH 7.2. Fura 2-AM was purchased from Molecular Probes. BAPTA-AM, wortmannin, LY294002, U73122, and U73343 were purchased from Calbiochem. Thapsigargin, polyoxymin B sul fate (PMB), and LPS from Escherichia coli 055:B5 strain were purchased from Sigma-Aldrich. Platelet-activating factor (PAF) was a kind gift from Dr. L. Stoll (University of Iowa, Iowa City, IA). PE-conjugated mouse anti-human MR mAb and PE-conjugated anti-mouse IgG1x Ab were purchased from BD Pharmigen. Anti-SP-R210 Ab was a generous gift from Dr. Z. A. Chronacos (University of Texas, Tyler, TX).

#### SP-A proteins

The SP-A proteins used in this study were produced and purified as previously described (18) and shown schematically in Beharka et al. (8). In brief, bronchoalveolar lavage was used to obtain SP-A from patients with alveolar proteinosis protein (aAPP) SP-A and from healthy volunteers (native human SP-A) (19). Native rat SP-A was purified from silica-pretreated rat alveolar macrophages (19). Native rat SP-A was produced and purified as described previously (17). Recombinant rat SP-A (SP-Ahyp), which is deficient in hydroxyproline content and hence designated SP-Ahyp, was produced from SF-9 insect cells following infection with a recombinant baculovirus containing a 1.6-kb cDNA for rat SP-A (18). SP-Ahyp proteins devoid of oligosaccharides at one or both of the consensus sequences for N-linked glycosylation were generated by amino acid substitutions at the Asn¹⁸ (SP-Ahyp) or Asn¹⁸¹ site (SP-Aوار) or at both the Asn¹ and Asn¹⁸¹ sites (SP-Aوارر) (18). Carbohydrate-deficient SP-A proteins retained structural and biologic functions, including partial oligomerization, aggregation of phospholipid liposomes, binding to immobilized carbohydrate, inhibition of lipid secretion from type II cells, and competition for receptor occupancy on type II cells (18). The synthesis of the mutant SP-Ahyp protein containing a nested deletion of the proximal collagen-like domain (Gly⁸⁵Gly⁴⁴TM2), a truncation of the protein at the neck region resulting in a protein lacking the collagen-like and the N-terminal regions (TM1-2-3), and an amino acid substitution at the Asn¹⁸¹ site (TM1-2-3/Asn¹⁸¹) were made as described previously (20, 21). SP-A proteins were suspended in buffer containing 10 mM HEPES + 20 mM NaCl and kept at −20°C until use. Purity of the SP-A proteins was assessed by SDS-PAGE and silver staining. Endotoxin levels in SP-A preparations were determined using an Limulus amoebocyte lysate kit (BioWhittaker) and ranged from undetectable to 300 pg/µg protein, with an average of 15 pg/µg protein.

### Human macrophages

Blood was obtained from healthy adult volunteers using an approved Institutional Review Board protocol for human subjects at the University of Iowa and the Veterans Affairs Medical Center. PBMC from single donors were isolated from heparinized blood on Ficoll-sodium diatrizoate (Pharmacia) and cultured in Teflon wells (Savillex) in the presence of 20% autologous serum at 37°C for 5 days (22). On the day of each experiment, PBMC containing MDM were removed from Teflon wells, washed extensively, and counted. For Ca²⁺ experiments, 3 × 10⁶ MDM were adhered to 25-mm acid-cleaned glass coverslips in single wells for 2 h in the presence of medium containing RPMI 1640 medium + 20 mM HEPES + 10% autologous serum. For measurement of InsP₃, 1 × 10⁶ MDM were adhered to 10-mm acid-cleaned glass coverslips for 2 h in single wells.

#### Cell line culture

1937 cells, obtained from the American Type Culture Collection, were maintained at 5% CO₂ and subcultured 5% CO₂ in RPMI 1640 medium with heat-inactivated 10% FBS (HyClone), 1 mM glutamine, 20 mM HEPES, and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively). Cells were maintained in 175-cm² flasks and passaged every 3–4 days by scraping the flask with a cell scraper. Cells were not used after passage 30.

#### Measurement of cytosolic calcium ([Ca²⁺]cyt)

Measurement of [Ca²⁺]cyt was performed as described previously (23, 24). Briefly, MDM on 25-mm round glass coverslips were washed and loaded with fura 2 by addition of the cell-permeate form, fura 2-AM, in RPMI 1640 medium for 30 min at 37°C in 5% CO₂. MDM were then washed with HEPES-buffered saline containing glucose and stimulated by the addition of SP-A proteins, LPS or PAF, as indicated (<5% v/v). Measurements of the apparent [Ca²⁺]cyt were done using two different spectrophotometers. In the first set of experiments, [Ca²⁺]cyt was measured using the Photoscan II spectrophotometer (Photon Technology International) with a Nikon microscope (Nikon) at the Cell Fluorescence Core Facility (Veterans Affairs Medical Center). The second set of experiments used an ImageMaster Ratio Fluorescence imaging system (Photon Technology International) at the Central Microscopy Research Facility (University of Iowa). In both cases, the final Ca²⁺ concentrations were determined from the ratio of emission intensities (emission wavelength (λₑ) 510 nm) to excitation wavelengths of (λₑ) 340 and 380 nm using a Photon Technology International software package. Background fluorescence intensities for each λₑ were obtained using non-fura 2-loaded cells and were subtracted from the raw data. The ratios of the corrected fluorescence intensities were then converted to [Ca²⁺] values using the formula [Ca²⁺] = Kᵣ × (R/Rmin)/(Rmax/Rmin) – R, where the maximum and minimum ratios (Rmax and Rmin respectively), as well as the apparent dissociation constant (Kᵣ), were empirically derived from [Ca²⁺] curves generated using the instrument. Positive (PAF), vehicle (Tris buffer; DMSO), and HSA controls were done for each experiment. Measurements were repeated using MDM isolated from a minimum of three different human SP-A donors.

In the second method, MDM were adhered to 24-well tissue culture plates in RPMI 1640 medium + 10% autologous serum, then washed and repleted in Dulbecco’s PBS plus 10 mM HEPES plus 1 mg/ml human serum albumin plus 0.1% glucose (DPBS-HHG) containing 10 µM fluo-4 AM (Molecular Probes) for 1 h at 37°C. Cells were then washed three times to remove unhydrolyzed dye and repleted with DPBS-HHG. Calcium measurements were made using a BMG FluorStar microplate fluorometer (BMG Lab Technologies) with excitation and emission wavelengths of 485 and 520 nm, respectively. Readings were taken every 3 s for 2 min to establish baseline, then 10 µg/ml SP-A were added and readings taken every 3 s for 10 min. A second bolus of SP-A (10 µg/ml) or PAF (100 nM)-positive control was added at 3–60 min after the first addition of SP-A and fluorescence changes monitored. The maximal increase in fluorescence over baseline was determined for each SP-A or PAF addition. Responses of SP-A-treated cells to a second treatment with SP-A were expressed as percentage of initial response.
Competitive binding assay with inhibitors

SP-A was fluorescently labeled using an Alexa Fluor 647 protein labeling kit (Molecular Probes). Cells were adhered to 4-well plates, washed, and rested overnight in RPMI 1640 medium + 10% serum. Macrophages were lifted using rubber policeman and resuspended in DPBS-HHG. Macrophages in suspension at 1E5/tube were incubated in triplicate tubes with 10 μg/ml unlabeled SP-A, 10–100 nM wortmannin, 3–9 μM Ly294002, 5 μM U73122, 5 μM U73343, or medium controls for 30 min at 37°C, then incubated with 1 μg of fluorescent-labeled SP-A at 4°C for 2 h. For conditions of SP-A preincubation, cells were pelleted once to remove medium containing unbound SP-A before the addition of labeled SP-A. Cells were washed, fixed in 2% paraformaldehyde, and fluorescence indicative of SP-A uptake was measured using a Beckman FACScan Calibur flow cytometer. Background fluorescence (macrophages with no SP-A) was subtracted from all test conditions and values set relative to the positive control (macrophages + SP-A, no inhibitors).

Measurement of InsP₃

Two different methods were used to determine whether SP-A induced InsP₃ formation. In the first method, adherent MDM were incubated with 20 μg/ml APP SP-A for 0, 15, 60 s, and 10 min (time dependence) or with 0, 2.5, 5, 10, or 40 μg/ml APP SP-A for 15 s (time dependence). The reaction was stopped, and the cells were lysed by the addition of 0.2× volume of ice-cold 20% perchloric acid on ice for 20 min. After centrifugation for 15 min at 2000 × g at 4°C, supernatants were removed and neutralized with ice-cold 1.5 M KOH containing 60 mM HEPES. KC1O₄ was sedimented by centrifugation at 2000 × g for 15 min. Determination of InsP₃ in the supernatant was done using a competitive RIA (Amersham Biosciences), according to the manufacturer’s instructions.

In the second method, adherent MDM were incubated for 48 h in RPMI 1640 medium containing 1 μCi/ml myo-[3H]inositol (Amersham Biosciences), and turnover of inositol phosphates was measured as described previously (23, 25). Briefly, cells were washed with HEPES-buffered saline supplemented with 10 mM glucose (HBS-G) and incubated in HBS-G for 20 min at 37°C, followed by incubation for 20 min with HBS-G containing 10 mM lithium chloride. Finally, cells were treated with SP-A as in the first assay. Inositol phosphates were extracted overnight at 4°C with 0.5 M perchloric acid. The acid extract was neutralized with 2.5 M KOH and 0.5 M HEPES (pH 7.4) and centrifuged to remove the precipitate. The inositol phosphate species were then collected using anion-exchange column chromatography (Dowex AG, 1–8 ×, 100–200 mesh, formate form; Bio-Rad) as described previously (23, 25).

Flow cytometry

PBMC were incubated with inhibitors (thapsigargin, wortmannin, U73343, or BAPTA), vehicle control (DMSO), or medium for 30 min followed by SP-A proteins (10 μg/ml) or HSA protein control (10 μg/ml) in Teflon wells for 2 h. As a positive control for up-regulation of MR, PBMC were incubated with inhibitors (thapsigargin, wortmannin, U73343, or medium controls for 30 min at 37°C, then incubated with 1 μg of fluorescent-labeled SP-A at 4°C for 2 h. For conditions of SP-A preincubation, cells were pelleted once to remove medium containing unbound SP-A before the addition of labeled SP-A. Cells were washed, fixed in 2% paraformaldehyde, and fluorescence indicative of SP-A uptake was measured using a Beckman FACScan Calibur flow cytometer. Background fluorescence (macrophages with no SP-A) was subtracted from all test conditions and values set relative to the positive control (macrophages + SP-A, no inhibitors).

Statistics

Data were subjected to an analysis of normality. Normally distributed data were analyzed by ANOVA followed by Student’s t test using SPSS statistical program software. Statistical significance was defined as p < 0.05.

Results

SP-A induces Ca²⁺ mobilization in MDM

To determine the influence of SP-A on Ca²⁺ mobilization, we measured the level of [Ca²⁺]ᵢ, cyt in adherent fura-2 loaded MDM using a Photoscan II spectrofluorometer. In these studies, the basal [Ca²⁺]ᵢ, cyt values for MDM were ~75–150 nM and were similar between experiments. A trace of a typical experiment using an optimal concentration of APP SP-A (10 μg/ml) is shown in Fig. 1A. Exposure of MDM to SP-A resulted in a significant increase in [Ca²⁺]ᵢ, cyt concentration over baseline (p < 0.05). The effect was rapid and transient. Cytosolic Ca²⁺ levels began to rise within 10 s of SP-A addition, and the maximal concentration was reached in ~100 s with an amplitude of 125 ± 21 nM over baseline. The maximal level of [Ca²⁺]ᵢ, cyt was sustained for <100 s, after which the level of [Ca²⁺]ᵢ, cyt declined over a period of 5 ± 1 min. However, even after 20 min, [Ca²⁺]ᵢ, cyt levels never returned completely to baseline (data not shown). The kinetics of the rise in [Ca²⁺]ᵢ, cyt observed after addition of SP-A were similar to those observed with an optimal concentration (50 nM) of PAF (Fig. 1B), except the magnitude of the response to SP-A was less. PAF, a known activator of the InsP₃/Ca²⁺ pathway in macrophages (27), was used as the positive control in all experiments.

FIGURE 1. SP-A increases cytosolic Ca²⁺ concentration. MDM were loaded with fura-2 and stimulated with 10 μg/ml APP SP-A alone (A), LPS (0.5 μg/ml) for 400 s followed by PAF (10 nM) (B), or APP SP-A (10 μg/ml) and PMB (10 μg/ml) (C). The [Ca²⁺]ᵢ, cyt response was analyzed by Photoscan II spectrofluorometer. APP SP-A increased [Ca²⁺]ᵢ, cyt after a time lag of 5–10 s. Addition of PMB did not significantly reduce the APP SP-A increase in [Ca²⁺]ᵢ, cyt. LPS stimulation did not result in Ca²⁺ mobilization at the concentrations used, and MDM were still capable of responding to the positive control, PAF. Representative of three to eight independent experiments.

LPS does not affect the SP-A-induced rise in \([Ca^{2+}]_{cyt}\).

The preparations of SP-A used contained variable levels of LPS. However, our data provide evidence that the increase in \([Ca^{2+}]_{cyt}\) is due solely to SP-A and not to contaminating LPS because neutralization of LPS through the addition of PMB (5 \(\mu\)g/ml) before stimulation with APP SP-A did not significantly diminish the APP SP-A-induced \([Ca^{2+}]_{cyt}\) response (Fig. 1C). Furthermore, addition of LPS alone, either at levels found in the SP-A preparations (<300 pg/ml; data not shown) or higher levels used as controls (0.5 \(\mu\)g/ml; Fig. 1B), did not mobilize \([Ca^{2+}]_{cyt}\). Failure of LPS to mobilize \(Ca^{2+}\) was not due to an inability of the MDM to respond because the addition of PAF after LPS treatment resulted in a significant \([Ca^{2+}]_{cyt}\) response (Fig. 1B).

**SP-A induced \(Ca^{2+}\) mobilization is concentration dependent.**

The SP-A-induced elevation in \([Ca^{2+}]_{cyt}\) was concentration dependent (Fig. 2). APP SP-A induced a rise in \([Ca^{2+}]_{cyt}\) when added at 0.5 \(\mu\)g/ml (threshold concentration), and the increase over baseline was significant at 1 \(\mu\)g/ml (Fig. 2). The dose response was linear at concentrations of up to 5 \(\mu\)g/ml and then plateaued, with no additional effect at higher concentrations.

SP-R210 has been reported as a receptor for SP-A that is present on rat alveolar and bone marrow-derived macrophages and the human myeloid cell line U937 (10). To determine whether signaling through SP-R210 was responsible for the SP-A-induced rise in \([Ca^{2+}]_{cyt}\), we blocked binding of SP-A to SP-R210 through the addition of 10 \(\mu\)g/ml rabbit anti-SP-R210 (10) or control rabbit IgG to MDM for 20 min before stimulation with 5 \(\mu\)g/ml APP SP-A. SP-R210 did not affect the subsequent rise in \([Ca^{2+}]_{cyt}\) by APP SP-A (174 ± 35 vs 167 ± 21 nM for MDM treated with anti-SP-R210 Ab or isotype control, respectively, \(n = 3\)). Expression of SP-R210 and C1qR, but not the putative SP-AR identified by Strayer et al. (28) and Stevens et al. (29), has been demonstrated on U937 cells (10). Addition of APP SP-A did induce a rise in \([Ca^{2+}]_{cyt}\) in U937; however, a 10-fold greater concentration of SP-A was required for an optimal response in these cells compared with human MDM (50 \(\mu\)g/ml APP SP-A for U937 vs 5 \(\mu\)g/ml for MDM, \(n = 3\)).

**The rise in \([Ca^{2+}]_{cyt}\) is refractory to a second treatment of SP-A.**

The SP-A-induced rise in \([Ca^{2+}]_{cyt}\) was not seen after a second stimulation with SP-A at the same concentration as the first (10 \(\mu\)g/ml), even after 3 min (Fig. 3A). When 20 \(\mu\)g/ml APP SP-A (Fig. 3B) or 40 \(\mu\)g/ml APP SP-A (Fig. 3C) were used as the secondary stimulus, \([Ca^{2+}]_{cyt}\) increased within 20 s of APP SP-A addition but never reached levels obtained by the first SP-A stimulus. However, the refractory state was specific to SP-A, as MDM exposed to APP SP-A retained their ability to respond to a second unrelated agonist, PAF (Fig. 3D), and MDM exposed to PAF were capable of responding to SP-A (Fig. 3E) to the same extent as MDM treated with SP-A alone.

We examined the length of this refractory period in MDM and found full recovery of the calcium response to a second stimulation with SP-A by 60 min (Fig. 4). The reduced rise in \([Ca^{2+}]_{cyt}\), seen upon a second stimulation with SP-A at time points of <60 min was not due to a reduction in the ability of SP-A to bind to MDM because preincubation of MDM with 10 \(\mu\)g/ml SP-A for 15 or 30 min at 37°C did not inhibit binding of subsequently added SP-A (Fig. 4). These data suggest that SP-A induces a refractory state in

**FIGURE 2.** SP-A-dependent increase in cytosolic \(Ca^{2+}\) is concentration dependent. The \([Ca^{2+}]_{cyt}\) response of MDM was analyzed by Photoscan II spectrophotometer for 10 min after addition of the indicated concentrations of APP SP-A (as described in the legend to Fig. 1). The data show the maximum increase in \([Ca^{2+}]_{cyt}\) (nM) reached over baseline. The amount of \(Ca^{2+}\) mobilized was concentration dependent. Results are expressed as mean ± SEM of three to eight independent experiments. *Significantly higher levels of \([Ca^{2+}]_{cyt}\) compared with unstimulated controls; \(p < 0.05\) by Student’s \(t\) test.

**FIGURE 3.** SP-A-treated MDM are refractory to a second stimulation with SP-A. MDM exposed to 10 \(\mu\)g/ml APP SP-A became refractory to a second stimulation with the same concentration of APP SP-A (10 \(\mu\)g/ml) (A). Secondary exposure to two times (20 \(\mu\)g/ml) (B) or four times (40 \(\mu\)g/ml) (C) the concentrations of APP SP-A resulted in a rise in \([Ca^{2+}]_{cyt}\), but the rise was significantly lower than that achieved with the first exposure. MDM exposed to APP SP-A retained their ability to respond to a second unrelated agonist, PAF (D), and MDM stimulated with PAF retained their ability to respond to APP SP-A (E). Results are expressed as ratiometric calcium concentration in relative units (see Materials and Methods for equation) over time. Representative of three independent experiments.
pressed as percentage of binding to MDM incubated with medium alone.

bound protein determined by flow cytometry. Binding data (

other sources (Fig. 5). Native human SP-A and native rat SP-A
concentration of APP SP-A (10

cytoplasmic Ca\(^{2+}\)). For measurements of

macrophages that is specific for SP-A and thus resembles homol-
gous receptor desensitization with regard to mobilization of in-
tracellular Ca\(^{2+}\).

SP-A source affects the degree of Ca\(^{2+}\) mobilization

We next determined the influence of the source of SP-A protein on
Ca\(^{2+}\) mobilization. When added to MDM at 10 µg/ml, the increase in [Ca\(^{2+}\)\(_{\text{cyt}}\)] by APP SP-A was significantly higher than from all other sources (Fig. 5). Native human SP-A and native rat SP-A
duced similar \((p > 0.05)\) levels of [Ca\(^{2+}\)\(_{\text{cyt}}\) that were signifi-
cantly higher \((p < 0.005)\) than that induced by the wild-type SP-
A\(^{\text{hyp}}\). Additionally, the amount of SP-A required to achieve a threshold [Ca\(^{2+}\)\(_{\text{cyt}}\) response varied with SP-A type (0.5, 1, 1, and 2 µg/ml for APP, human native, native rat, and SP-A\(^{\text{hyp}}\), respectively). These results indicate that the source of SP-A (APP, and native, recombinant) affects the degree of Ca\(^{2+}\) mobilization.

The attached carbohydrates and collagen-like domain of SP-A are both involved in SP-A-induced Ca\(^{2+}\) mobilization

We next explored which of the domains or structural elements of
SP-A mediated the Ca\(^{2+}\) response. To study the potential influence of the carbohydrate moieties of SP-A in mediating its effect on
Ca\(^{2+}\) mobilization, we used SP-A\(^{\text{hyp}}\) proteins devoid of oligosac-
charides at one (Asn\(^{1}\) site (SP-A\(^{\text{thr1}}\)) or Asn\(^{187}\) site (SP-A\(^{\text{ser187}}\)) or both (Asn\(^{1}\) and Asn\(^{187}\) (SP-A\(^{\text{thr1ser187}}\)) of the consensus sequence sites for glycosylation. The absence of carbohydrates at these sites on the variant proteins used in these studies has been demonstrated previously (7, 18).

In parallel experiments, we investigated the importance of the collagen-like domain of SP-A for induction of intracellular Ca\(^{2+}\).

FIGURE 4. The MDM refractory period to SP-A is <60 min and is not a result of decreased SP-A binding to MDM. For measurements of

[Ca\(^{2+}\)\(_{\text{cyt}}\)], MDM were loaded with fluo-4 and stimulated with 10 µg/mlAPP SP-A for 3–60 min, followed by a second stimulation with the same concentration of APP SP-A (10 µg/ml). The [Ca\(^{2+}\)\(_{\text{cyt}}\) response was ana-
lized by Fluorostar microplate fluorometer. [Ca\(^{2+}\)\(_{\text{cyt}}\)] results are ex-
pressed as mean percent of initial response ± SEM of two independent experiments. To determine SP-A binding, MDM were incubated with 10 µg/ml APP SP-A or medium controls for 15–30 min at 37°C, then incu-
bated with 1 µg of fluorescent-labeled SP-A ± at 4°C for 2 h, washed, and bound protein determined by flow cytometry. Binding data (■) are ex-
pressed as percentage of binding to MDM incubated with medium alone ± SD and are representative of two independent experiments.

FIGURE 5. The [Ca\(^{2+}\)\(_{\text{cyt}}\)] response varies with SP-A type. The

[Ca\(^{2+}\)\(_{\text{cyt}}\)] response of MDM was analyzed by Photoscan II spectrofluorometer after the addition of the indicated sources of SP-A at 10 µg/ml. Ad-
dition of APP SP-A gave the greatest response. Addition of native human or native rat SP-A resulted in similar rises in [Ca\(^{2+}\)\(_{\text{cyt}}\]. Addition of SP-
A\(^{\text{hyp}}\) produced from SF-9 insect cells (hyp) resulted in a lower response than the other SP-As tested. Results are expressed as mean ± SEM of three to eight independent experiments. * , Significantly different from baseline, \(p < 0.05\).

FIGURE 6. SP-A carbohydrate moieties are involved in SP-A-induced mobilization of Ca\(^{2+}\). To determine which carbohydrate(s) of SP-A are
necessary for increased [Ca\(^{2+}\)\(_{\text{cyt}}\), MDM labeled with fura 2 were stimu-
lated with the following SP-A proteins at 10 and 40 µg/ml: SP-A\(^{\text{hyp}}\) (hyp), recombinant protein devoid of oligosaccharide at the Asn\(^{1}\) (thr\(^{1}\)), Asn\(^{187}\) (ser\(^{187}\)), or both the Asn\(^{1}\) and Asn\(^{187}\) sites (thr\(^{1}\)ser\(^{187}\)). The [Ca\(^{2+}\)\(_{\text{cyt}}\) response was analyzed by ImageMaster Ratio Fluorescence imaging system. When the proteins were added at 10 µg/ml, none was capable of inducing a level of [Ca\(^{2+}\)\(_{\text{cyt}}\)] greater than baseline. At 40 µg/ml, the proteins all induced small, short duration rises in [Ca\(^{2+}\)\(_{\text{cyt}}\]. Only the rise in [Ca\(^{2+}\)\(_{\text{cyt}}\)] induced by the SP-A\(^{\text{hyp}}\) protein was significantly greater than baseline. Results are expressed as mean ± SEM of three to eight independent ex-
periments. * , Significant compared with baseline [Ca\(^{2+}\)\(_{\text{cyt}}\), \(p < 0.05\).
(Fig. 7). Truncated SP-A proteins lacking the first half of the collagen-like domain but still possessing both carbohydrate attachment sites (TM2), lacking the collagen-like domain and the N-terminal segment (Asn\(^1\) to Ala\(^{127}\)) but still possessing the carbohydrate attachment site at Asn\(^{197}\) (TM1-2-3), or lacking the collagen-like domain, N-terminal segment, and both carbohydrate attachment sites (TM1-2-3\(^{ser187}\)) were added to fura 2-labeled MDM. None of the truncated mutants was capable of inducing a Ca\(^{2+}\) response similar in strength or duration to that achieved by APP SP-A or SP-A\(^{hyp}\). However, similar to the response seen using carbohydrate-deficient SP-A proteins, all of the collagen-deficient mutants induced a very low-level increase of Ca\(^{2+}\), which was similar between the mutants. Taken together, these studies demonstrate a critical role for both the carbohydrates and collagen-like domain in the Ca\(^{2+}\) response.

**SP-A induces Ca\(^{2+}\) mobilization from intracellular stores**

The rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by agonists is accomplished through two mechanisms: the release of Ca\(^{2+}\) from intracellular stores, of which the ER is the largest contributor, and by influx across the plasma membrane (30). We next evaluated the relative contribution of these two mechanisms to the SP-A-induced calcium response. Thapsigargin is an irreversible ER Ca\(^{2+}\)-ATPase inhibitor whose application leads to depletion of intracellular Ca\(^{2+}\) stores (31). Fura 2-loaded MDM were pretreated with thapsigargin (0.1, 1, or 10 \(\mu\)M) for 60 min. Preliminary experiments confirmed that this pretreatment did not adversely affect cell viability (data not shown). Pretreatment with thapsigargin resulted in the dose-dependent loss of APP SP-A-induced Ca\(^{2+}\) mobilization (Table I). Thapsigargin at 10 \(\mu\)M essentially prevented any increase in Ca\(^{2+}\) in response to APP SP-A. These results provide evidence for the involvement of intracellular Ca\(^{2+}\) stores in the initial SP-A-induced rise in \([\text{Ca}^{2+}]_{\text{cyt}}\). However, the data do not exclude the possibility that calcium influx plays a role later in the Ca\(^{2+}\) response.

**FIGURE 7.** The collagen-like domain of SP-A is involved in the SP-A-induced mobilization of Ca\(^{2+}\). The \([\text{Ca}^{2+}]_{\text{cyt}}\) response of MDM was analyzed by Photoscan II spectrofluorometer after the addition of the indicated sources of SP-A at 10 \(\mu\)g/ml. Wild-type SP-A\(^{hyp}\) (hyp) induced a rise in \([\text{Ca}^{2+}]_{\text{cyt}}\), which was significantly above baseline. Recombinant rat proteins containing a deletion of the proximal collagen-like domain (TM2) and protein lacking the collagen-like and the N-terminal regions (TM1-2-3) and devoid of oligosaccharide at the Asn\(^{197}\) site (TM1-2-3\(^{ser187}\)) induced small, short duration rises in \([\text{Ca}^{2+}]_{\text{cyt}}\); however, the level of \([\text{Ca}^{2+}]_{\text{cyt}}\) achieved was not significantly different from baseline. Results are expressed as mean ± SEM of three to eight independent experiments. *, Significantly different from baseline, \(p < 0.05\).

### Table I. SP-A-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) is decreased by inhibition of PLC and PI3K and reduction of intracellular calcium stores

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Percentage of Inhibition of SP-A-Induced ([\text{Ca}^{2+}]_{\text{cyt}}) (Mean ± SEM)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thapsigargin (0.1 (\mu)M)</td>
<td>62 ± 5*</td>
<td>3</td>
</tr>
<tr>
<td>Thapsigargin (1 (\mu)M)</td>
<td>83 ± 8*</td>
<td>5</td>
</tr>
<tr>
<td>Thapsigargin (10 (\mu)M)</td>
<td>97 ± 3*</td>
<td>3</td>
</tr>
<tr>
<td>U73122 (5 (\mu)g/ml)</td>
<td>73 ± 10</td>
<td>3</td>
</tr>
<tr>
<td>U73343 (5 (\mu)g/ml)</td>
<td>12 ± 22</td>
<td>3</td>
</tr>
<tr>
<td>Wortmannin (10 nM)</td>
<td>45 ± 19</td>
<td>3</td>
</tr>
<tr>
<td>Wortmannin (100 nM)</td>
<td>60 ± 11*</td>
<td>4</td>
</tr>
</tbody>
</table>

\(\times\) Fura 2-labeled MDM were preincubated with buffer, SP-A, or the indicated treatments (thapsigargin, an ER Ca\(^{2+}\)-ATPase inhibitor; wortmannin, an inhibitor of PI3K; U73122, a PLC inhibitor; U73343, a structural analog of U73122 used as a negative control) for 30 min before the addition of APP SP-A (10 \(\mu\)g/ml). The response of MDM was analyzed by Photoscan II.

\(\times\) Significant compared with SP-A alone by Student’s \(t\) test, \(p < 0.05\).

**SP-A activates an InsP\(_3\)-signaling pathway**

To further explore the role of the InsP\(_3\)/Ca\(^{2+}\) pathway in SP-A signaling, we next investigated whether SP-A induced generation of InsP\(_3\) in MDM. The level of InsP\(_3\) in MDM was measured using a commercially available competitive RIA, which determines the InsP\(_3\) content, as well as an assay that measures turnover of inositol phosphates (23, 32). Both assays gave similar results (data not shown); therefore, only the results of the competitive RIA are shown. APP SP-A induced production of InsP\(_3\) in a time- and dose-dependent manner (Fig. 8). A typical time course of InsP\(_3\) formation in response to SP-A is shown in Fig. 8A. Within 15 s of SP-A addition, InsP\(_3\) levels rose significantly above baseline. Maximal InsP\(_3\) levels were reached at 15 s post-SP-A addition. InsP\(_3\) formation returned to basal values by 10 min after stimulation. These kinetics are similar to those observed for the Ca\(^{2+}\) response to SP-A. Additionally, the level of InsP\(_3\) formed was dependent on the concentration of APP SP-A (Fig. 8B). Although the effective range of SP-A in these assays was similar to that of the Ca\(^{2+}\) response, slightly more APP SP-A was needed to induce InsP\(_3\) formation compared with Ca\(^{2+}\) mobilization, a result that may be related to differences in assay sensitivity.

InsP\(_3\) is typically formed through PLC hydrolysis of membrane phospholipids (14). Incubation of MDM with an aminosteroid inhibitor of PLC (U73122), but not its analog (U73343), inhibited the SP-A-induced rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Table I), suggesting that SP-A activates PLC. Thus, these data provide support for SP-A activation of a PLC/InsP\(_3\) pathway leading to the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\). However, wortmannin did not completely inhibit Ca\(^{2+}\) mobilization in response to APP SP-A, suggesting that activation of PI3K plays a role in but may not be essential for Ca\(^{2+}\) mobilization.
PI3K plays a role in SP-A-induced up-regulation of MR expression

The interaction of SP-A with the macrophage results in modification of macrophage functions, including, but not limited to, up-regulation of MR expression (1, 7, 8). To determine whether SP-A signaling through the InsP$_3$/Ca$^{2+}$ pathway results in up-regulation of MR, we used inhibitors against the previously identified signaling molecules activated by SP-A and measured MR expression using flow cytometry. As previously reported (8), APP SP-A or IL-4 significantly increased MR expression on MDM (Table II). The addition of the general PI3K inhibitors wortmannin or Ly294002 before SP-A treatment significantly reduced the SP-A-induced up-regulation of MR in a dose-dependent manner. Wortmannin did not reduce basal MR expression levels (data not shown), nor did it significantly inhibit SP-A binding to MDM at concentrations that inhibited MR up-regulation by SP-A (100 nM) compared with concentrations that had no effect (10 nM; data not shown). Additionally, Ly294002 treatment of MDM had no effect on binding of SP-A to MDM (data not shown), indicating that inhibition of PI3K(s) has no effect on SP-AR expression. Therefore, a PI3K(s) plays an important role in the SP-A-induced signaling pathway that leads to MR up-regulation. The role of PLC-linked Ca$^{2+}$ mobilization in SP-A-induced MR up-regulation is less clear. Inhibition of PLC by U73122 did not significantly affect SP-A induction of MR. Furthermore, while preincubation with the Ca$^{2+}$ inhibitor BAPTA reduced SP-A-induced MR expression, inhibition of calcium mobilization by thapsigargin did not significantly affect SP-A induction of MR. However, it should be noted that 5 μM BAPTA tended ($p = 0.07$) to reduce the basal level of MR surface expression on MDM, suggesting that Ca$^{2+}$ is involved in the normal trafficking of this receptor.

Discussion

SP-A is increasingly recognized as a key component of innate immunity in the lung. SP-A plays a critical role in host defense against inhaled pathogens, as demonstrated by the increased susceptibility of the SP-A$^{-/-}$ mouse to infection with a variety of extracellular bacteria (33–35). SP-A has been shown to have direct antimicrobial activity against both bacterial (36) and fungal (37) microorganisms. However, it is also evident that part of the role of SP-A in pulmonary immunity is due to the ability of SP-A to modify alveolar macrophage immunological function (2). Addition of SP-A to macrophages in vitro increases chemotaxis and phagocytosis, decreases NO production, and alters the respiratory burst (reviewed in Ref. 1). Recently, our laboratory has demonstrated that SP-A increases the surface expression and activity of the macrophage MR (8) and that this up-regulation plays a role in the observed increase in *Mycobacterium tuberculosis* phagocytosis by SP-A-treated cells (7).

Table II. Determination of signaling molecules involved in up-regulation of human macrophage MR expression by SP-A$^{a}$

<table>
<thead>
<tr>
<th>Treatment$^{b}$</th>
<th>Percentage of Increase in MFI (Mean ± SEM)$^{c}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP SP-A</td>
<td>102 ± 21*</td>
<td>8</td>
</tr>
<tr>
<td>IL-4</td>
<td>253 ± 96*</td>
<td>10</td>
</tr>
<tr>
<td>Wortmannin (10 nM)</td>
<td>75 ± 19*</td>
<td>2</td>
</tr>
<tr>
<td>Wortmannin (100 nM)</td>
<td>56 ± 31</td>
<td>2</td>
</tr>
<tr>
<td>Ly294002 (9 μM)</td>
<td>22 ± 12</td>
<td>2</td>
</tr>
<tr>
<td>Ly294002 (3 μM)</td>
<td>65 ± 28</td>
<td>3</td>
</tr>
<tr>
<td>Ly294002 (1.5 μM)</td>
<td>77 ± 18*</td>
<td>3</td>
</tr>
<tr>
<td>Ly294002 (0.75 μM)</td>
<td>83 ± 28*</td>
<td>3</td>
</tr>
<tr>
<td>Thapsigargin (10 μM)</td>
<td>95 ± 38*</td>
<td>4</td>
</tr>
<tr>
<td>U73122 (5 μM)</td>
<td>88 ± 30*</td>
<td>3</td>
</tr>
<tr>
<td>U73343 (5 μM)</td>
<td>94 ± 22*</td>
<td>3</td>
</tr>
<tr>
<td>BAPTA (0.5 μM)</td>
<td>61 ± 11</td>
<td>2</td>
</tr>
<tr>
<td>BAPTA (1 μM)</td>
<td>23 ± 20</td>
<td>2</td>
</tr>
<tr>
<td>BAPTA (5 μM)</td>
<td>−28 ± 12</td>
<td>2</td>
</tr>
</tbody>
</table>

$^{a}$ MR MFI measured using flow cytometry.

$^{b}$ MDM were incubated with SP-A proteins (10 μg/ml) for 2 h in all but the IL-4 group. IL-4 added for 20 h was used as the positive control. Inhibitors were added 30 min before APP SP-A treatment.

$^{c}$ Values shown represent the % increase in MFI = (treated MFI-control MFI) × control MFI × 100. Values are corrected for nonspecific binding by subtracting the MFI from the appropriate nonspecific control (IgG1x). Mean ± SEM; n = 6.

* Significant relative to MR expression on untreated MDM by Student’s t test, $p < 0.05$.

FIGURE 8. InsP$_3$ formation in response to SP-A is time and concentration dependent. MDM (1 × 10⁶) were incubated with 20 μg/ml SP-A for the indicated time periods (A) or the indicated concentrations of APP SP-A for 15 s (B). Incubation was stopped, and cells were lifted and lysed by adding ice-cold 20% perchloric acid for 20 min. After centrifugation at 4°C for 15 min at 2000 × g, supernatants were removed and neutralized with ice-cold 1.5 M KOH containing 60 mM HEPES. The resultant KClO₄ was precipitated and InsP$_3$ in supernatant samples determined using a specific protein binding assay. Results are expressed as mean ± SEM of two independent experiments; *, $p < 0.05$. 

The addition of macrophage functions, including, but not limited to, up-regulation of MR expression (1, 7, 8). To determine whether SP-A signaling through the InsP$_3$/Ca$^{2+}$ pathway results in up-regulation of MR, we used inhibitors against the previously identified signaling molecules activated by SP-A and measured MR expression using flow cytometry. As previously reported (8), APP SP-A or IL-4 significantly increased MR expression on MDM (Table II). The addition of the general PI3K inhibitors wortmannin or Ly294002 before SP-A treatment significantly reduced the SP-A-induced up-regulation of MR in a dose-dependent manner. Wortmannin did not reduce basal MR expression levels (data not shown), nor did it significantly inhibit SP-A binding to MDM at concentrations that inhibited MR up-regulation by SP-A (100 nM) compared with concentrations that had no effect (10 nM; data not shown). Additionally, Ly294002 treatment of MDM had no effect on binding of SP-A to MDM (data not shown), indicating that inhibition of PI3K(s) has no effect on SP-AR expression. Therefore, a PI3K(s) plays an important role in the SP-A-induced signaling pathway that leads to MR up-regulation. The role of PLC-linked Ca$^{2+}$ mobilization in SP-A-induced MR up-regulation is less clear. Inhibition of PLC by U73122 did not significantly affect SP-A induction of MR. Furthermore, while preincubation with the Ca$^{2+}$ inhibitor BAPTA reduced SP-A-induced MR expression, inhibition of calcium mobilization by thapsigargin did not significantly affect SP-A induction of MR. However, it should be noted that 5 μM BAPTA tended ($p = 0.07$) to reduce the basal level of MR surface expression on MDM, suggesting that Ca$^{2+}$ is involved in the normal trafficking of this receptor.

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SP-A is increasingly recognized as a key component of innate immunity in the lung. SP-A plays a critical role in host defense against inhaled pathogens, as demonstrated by the increased susceptibility of the SP-A$^{-/-}$ mouse to infection with a variety of extracellular bacteria (33–35). SP-A has been shown to have direct antimicrobial activity against both bacterial (36) and fungal (37) microorganisms. However, it is also evident that part of the role of SP-A in pulmonary immunity is due to the ability of SP-A to modify alveolar macrophage immunological function (2). Addition of SP-A to macrophages in vitro increases chemotaxis and phagocytosis, decreases NO production, and alters the respiratory burst (reviewed in Ref. 1). Recently, our laboratory has demonstrated that SP-A increases the surface expression and activity of the macrophage MR (8) and that this up-regulation plays a role in the observed increase in *Mycobacterium tuberculosis* phagocytosis by SP-A-treated cells (7).

SP-A has been shown to associate with macrophages in a saturable and specific manner, consistent with receptor-mediated binding (38). Although several SP-A binding proteins have been identified on rat macrophages, myeloid cell lines, and in purified protein systems (10), the receptor(s) responsible for the effects of SP-A on MR expression and activity have not yet been determined. Interactions between SP-A and its macrophage receptor(s) activate signaling pathway(s) that ultimately effect the reported alterations...
in macrophage functions (12, 39–43). Our laboratory is particularly interested in the identification of signaling pathways leading to up-regulation of the MR. The results presented here indicate that at least one signal transduction pathway activated in human macrophages by SP-A involves Ca\(^{2+}\) signaling and that PI3K contributes to the SP-A-induced increase in MR expression.

Ca\(^{2+}\) is an important second messenger in phagocytic cells and has been linked to many cellular processes, including vesicle trafficking (reviewed in Ref. 44). [Ca\(^{2+}\)]\(_{cyt}\) in resting cells is low; upon stimulation, [Ca\(^{2+}\)]\(_{cyt}\) increases 5- to 10-fold, activating Ca\(^{2+}\)-sensitive processes. Our data demonstrate that SP-A significantly increases [Ca\(^{2+}\)]\(_{cyt}\) within seconds of SP-A addition to cultured MDM. The immediate increase in [Ca\(^{2+}\)]\(_{cyt}\) in response to APP SP-A is similar to that reported by Ohmer-Schro¨ck et al. (12) using rat alveolar macrophages. However, there may be species differences in threshold responsiveness to SP-A: we found that 10 \(\mu\)g/ml APP SP-A induced mobilization of calcium in 100% of MDM. In contrast, Ohmer-Schro¨ck et al. (12) reported 30 \(\mu\)g/ml APP SP-A stimulated Ca\(^{2+}\) mobilization in 36% of rat alveolar macrophages, and 120 \(\mu\)g/ml APP SP-A achieved mobilization in 69% of these cells (12).

The effects of SP-A on [Ca\(^{2+}\)]\(_{cyt}\) were observed in our system at concentrations as low as 1 \(\mu\)g/ml. Although the precise SP-A concentration in pulmonary surfactant is not known, the concentration of SP-A in rat lung hypophase has been estimated at 300 \(\mu\)g/ml to 1.8 mg/ml (reviewed in Ref. 1) based on SP-A levels in lavage fluid. However, this does not account for the possibility that microenvironments could exist where the local concentrations of SP-A are much higher or lower (1). It is important to note that SP-A activity may also be affected by its association with lipid, as SP-A has been shown to be concentrated in tubular myelin in the human lung (45).

In our studies, after the maximum [Ca\(^{2+}\)]\(_{cyt}\) was reached, the [Ca\(^{2+}\)]\(_{cyt}\) declined over the following 10 min until reaching a steady-state level slightly above baseline. Interestingly, when these macrophages were stimulated with a second dose of SP-A (lesser or equal concentration) within 10 min of the first, no second rise in [Ca\(^{2+}\)]\(_{cyt}\) was detected. When 2- or 4-fold greater APP SP-A was used as the secondary stimulus following an initial SP-A treatment, the [Ca\(^{2+}\)]\(_{cyt}\) was increased but never achieved the level reached in response to the first stimulus. However, SP-A-treated MDM were still able to fully respond to the second stimulus PAF. This suggests that the inhibition is not due to depletion of available Ca\(^{2+}\) but that SP-A facilitates an agonist-specific refractory period, which is consistent with the hypothesis that SP-A induces homologous desensitization of its receptor(s) on the macrophage surface.

Desensitization facilitates decreased responsiveness of a cell to successive exposure of the same extracellular stimulus over time. We envision that the functional state of the SP-AR(s) is under continuous regulation in response to changes in SP-A levels in the lung, such as has been reported in bacterial pneumonia (46). Additionally, the degree of SP-AR desensitization is likely influenced by the differentiation and/or activation state of the cell. In the alveoli, alveolar macrophages are in continuous contact with surfactant containing SP-A and ingest abundant amounts of this material (47); therefore, it is possible that resident alveolar macrophages are in a constant state of desensitization to SP-A. In other words, maintenance of the alveolar macrophage phenotype may require constant (tonic) ligand and desensitization of SP-AR(s).

In contrast, monocytes or interstitial macrophages that migrate into the lung would be sensitive to the effects of SP-A upon first encounter. In this model, SP-A would serve as a local tissue factor that contributes to the induction and maintenance of the unique biological properties of alveolar macrophages compared with macrophages in other tissue compartments. These include increased surface expression of receptors of innate immunity such as the MR (9), greater phagocytosis of both nonopsonized and opsonized particles (48), decreased production of inflammatory cytokines (48), a reduced oxidative burst in response to stimuli (6, 49), and decreased expression of costimulatory molecules (50)—an anti-inflammatory phenotype consistent with a state referred to as “alternative activation” (51). Homologous receptor desensitization is dependent on agonist occupancy of the receptor; consequently, under conditions in which SP-A concentration is significantly decreased, the alveolar macrophages would become resensitized to SP-A. This would explain why alveolar macrophages are capable of responding to SP-A in vitro (7, 12).

In the current study, the threshold level of SP-A needed to induce a Ca\(^{2+}\) response and the magnitude of this response were both dependent on the source of SP-A used. Although human APP, native human and rat, and SP-A\(^{op}\) demonstrate shared functional characteristics, differences in the magnitude of select biological responses related to SP-A type have been observed previously (52, 53). Our laboratory has reported previously that APP SP-A was more effective than either native human or SP-A\(^{op}\) in up-regulating MR receptor expression and enhancing the phagocytosis of M. tuberculosis by MDM (7, 8).

One notable difference among the SP-As used is the degree of higher-order molecular organization (54); our results suggest that these differences are related to the ability of SP-A to induce Ca\(^{2+}\) mobilization. APP SP-A, which can form complexes larger than the native SP-A octadecamer (52, 55), demonstrated the greatest amount of Ca\(^{2+}\) mobilization. In contrast, SP-A\(^{op}\), which oligomerizes to a lesser extent than native SP-A due to a lack of proline hydroxylation, demonstrated the lowest amount of Ca\(^{2+}\) mobilization. Additionally, mutants that lacked the collagen-like domain and/or the N-terminal region, both of which are believed to be essential for forming higher order multimers (3), were capable of stimulating only a slight Ca\(^{2+}\) response. These data suggest that higher order oligomers are essential for maximally effective receptor engagement, which may involve recognition of a multitiulin epitope, and/or receptor clustering by ligation of multiple CRD. It should also be noted that, in addition to differences in multimerization, human APP, native human and rat, and SP-A\(^{op}\) also demonstrate differences in glycosylation patterns (3), which may play a role in the current results.

To further investigate the role of individual structural components of SP-A on Ca\(^{2+}\) mobilization, we used SP-A\(^{op}\) variants lacking part or all of the collagen-like domain or one or both attached carbohydrates. Wild-type SP-A\(^{op}\) contains two sugars, one at Asn\(^1\) in the N-terminal segment and one at Asn\(^187\) in the CRD (3). Removal of one or both sugars significantly decreased the SP-A-induced Ca\(^{2+}\) burst, which is consistent with studies showing that these carbohydrate moieties are important for some of its biological effects (7, 56). When both sugars remained intact but the collagen-like domain was partially or completely removed, the SP-A-induced calcium response was also diminished. This is supported by work by Chronoes et al. (10), which showed that collagen V inhibits SP-A binding to macrophages, as well as work by Vandivier et al. (57), which showed that C1q collagen tails inhibit macrophage uptake of SP-A-coated erythrocytes. It is not yet clear whether the collagen-like domain participates in direct protein-protein interactions that result in a calcium flux or whether it acts primarily as scaffolding that amplifies the binding activities of other domains of the molecule. None of the mutations completely eliminated the Ca\(^{2+}\) response, and inhibition of the Ca\(^{2+}\) response by these mutations was not additive because the removal of both sugars and the collagen-like domain or of the N-terminal and collagen-like domains did not further diminish the Ca\(^{2+}\) response. It
is unclear whether the small remaining Ca\(^{2+}\) response is physiologically relevant. Viewed as a whole, our studies demonstrate that both the protein-associated carbohydrates and collagen-like domain of SP-A play a role in SP-A-induced Ca\(^{2+}\) mobilization.

The possibility exists that there is more than one receptor for SP-A on macrophages, each one of which induces a different signal pathway and biological effect. Several proteins have been identified as putative receptors for SP-A, not all of which are present on human macrophages (38, 58). One SP-A-binding protein reported to be on U-937 cells and rat alveolar macrophages is the 210-kDa protein SP-R210. Our assays failed to reveal a role for this receptor in the Ca\(^{2+}\) response or up-regulation of the MR because Abs against SP-R210 failed to inhibit SP-A-induced increases in Ca\(^{2+}\), or MR expression (data not shown). U937 cells also required 10 times more APP SP-A for the induction of calcium than did human MDM, suggesting that the SP-AR(s)s on U937 cells that signals through Ca\(^{2+}\) is expressed at an extremely low level compared with that on primary human macrophages, has a lower affinity for SP-A, or lacks expression of a coreceptor (39, 40, 59).

The calcium mobilized into the cytoplasm in response to stimulation originates from either the extracellular space or the ER (14). One standard approach to examine whether extracellular Ca\(^{2+}\) plays a role in induced rises in [Ca\(^{2+}\)]\(_{\text{cyt}}\) is to chelate extracellular Ca\(^{2+}\) using EGTA. However, this approach cannot be used in our system to study the contribution of extracellular Ca\(^{2+}\) to signaling by SP-A, as we have recently shown that binding of SP-A to its receptor(s) on primary human macrophages is Ca\(^{2+}\)-dependent (6). We have shown that depletion of ER Ca\(^{2+}\) stores by preincubation with thapsigargin completely blocks the SP-A-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise, indicating that the initial calcium response is mobilized from intracellular stores. We also found that SP-A induces a rise in InsP\(_3\), an important molecule involved in release of Ca\(^{2+}\) from ER stores (14). Based on these observations, we have concluded that SP-A-induced Ca\(^{2+}\) mobilization at least initially originates from intracellular stores. The level of [Ca\(^{2+}\)]\(_{\text{cyt}}\) above baseline seen as late as 20 min after SP-A addition may be due to an influx of extracellular Ca\(^{2+}\) through capacitative calcium entry, which is controlled by the efflux of calcium from the ER. Recent evidence supports InsP\(_3\),R involvement in the initiation of this entry process (60). Therefore, we propose that the SP-A-induced rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) is composed of two phases: an initial spike in [Ca\(^{2+}\)]\(_{\text{cyt}}\) through InsP\(_3\)-induced release of Ca\(^{2+}\) from specific intracellular stores, followed by a plateau phase (essential for replenishment of the intracellular stores) that is sustained by Ca\(^{2+}\) influx from the extracellular medium.

Our data further demonstrate that SP-A induction of Ca\(^{2+}\) mobilization is mediated through activation of PLC. PLC not only catalyzes the formation of InsP\(_3\) from phosphatidylinositol 4,5-bisphosphate but also controls cellular phosphatidylinositol 4,5-bisphosphate concentrations, thereby intersecting with other signal transduction pathways such as that of PI3K. Pretreatment with wortmannin before stimulation with SP-A resulted in a markedly decreased calcium response, suggesting that PI3K is an upstream signaling molecule involved in Ca\(^{2+}\) mobilization by SP-A. Taken together, these studies demonstrate that SP-A activates a Ca\(^{2+}\)/PLC/InsP\(_3\) signal transduction pathway. We next investigated whether signaling through this pathway by SP-A results in up-regulation of MR expression. This hypothesis is supported by inhibition of MR up-regulation by the calcium chelator BAPTA. However, BAPTA may also chelate local concentrations of calcium or other cations needed at the membrane for vesicular fusion, thereby inhibiting processes in addition to calcium release that are necessary for MR up-regulation. The role of SP-A signaling through PLC in MR up-regulation is less clear because inhibitors of PLC did not significantly reduce the effect of SP-A on MR expression. However, inhibitors of PLC decreased but did not completely abolish the calcium response to SP-A; the residual low-level calcium response may have been sufficient to induce MR up-regulation in these cells. Alternatively, redundancy in signaling is common in immunological systems, and inhibition of PLC may therefore cause a compensatory increase in the activity of other contributing enzymes or pathways, which may also account for our results.

Using the inhibitors wortmannin and Ly294002, we have shown that PI3K is involved in both SP-A-induced mobilization of Ca\(^{2+}\) and in SP-A up-regulation of MR. PI3K, as with calcium, is involved in intracellular trafficking and acts as a regulator of multiple aspects of membrane trafficking (61–63). A PI3K may be the common molecule involved in two separate signaling cascades—one resulting in MR up-regulation and the other involving the Ca\(^{2+}\)/PLC/InsP\(_3\) pathway; or, more likely, a different member of the PI3K family is involved in each event. Interestingly, the lipid products of PI3K have been found to directly activate certain protein kinase C (PKC) isoforms. If PKC plays a role in MR up-regulation, it would provide an additional explanation for our data using BAPTA because treatment of cells with BAPTA-AM leads to a translocation and inactivation of PKC (64).

In conclusion, SP-A binding to human macrophages activates a signal transduction pathway(s) that results in increased levels of [Ca\(^{2+}\)]\(_{\text{cyt}}\). The magnitude of this increase is dependent on 1) the type of SP-A used and 2) the sugars and collagen-like domain of the protein. Our experiments make the novel observation that macrophages become refractory to SP-A after initial exposure, suggesting that the receptor(s) for SP-A undergoes homologous desensitization. This finding may be particularly important for setting the anti-inflammatory “tone” of macrophages within the lung. Our data indicate that SP-A triggers the increase in calcium by inducing PLC activation, leading to the hydrolysis of membrane phospholipids and yielding InsP\(_3\). We have shown that activation of PI3K(s) plays a role in SP-A up-regulation of MR and may act as a component of the PLC signal pathway. These studies provide further insight into the signaling pathways activated in human macrophages by SP-A which play direct roles in SP-A modulation of macrophage biology.

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Disclosures
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References
Schlesinger, L. S. 1993. Macrophage phagocytosis of virulent but not attenuated

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