Surfactant Releases Internal Calcium Stores in Neutrophils by G Protein–Activated Pathway

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Pulmonary surfactant with surfactant-associated proteins (PS+SAP) decreases pulmonary inflammation by suppressing neutrophil activation. We have observed that PS+SAP inserts channels into artificial membranes, depolarizes neutrophils, and depresses calcium influx and function in stimulated neutrophils. We hypothesize that PS+SAP suppresses neutrophil activation by depletion of internal Ca^{++} stores and that PS+SAP induces depletion through release of Ca^{++} stores and through inhibition of Ca^{++} influx. Our model predicts that PS+SAP releases Ca^{++} stores through insertion of channels, depolarization of neutrophils, and activation of a G protein–dependent pathway. If the model of channel insertion and membrane depolarization is accurate, then gramicidin—a channel protein with properties similar to those of PS+SAP—is expected to mimic these effects. Human neutrophils were monitored for $[Ca^{++}]$ responses after exposure to one of two different PS+SAP preparations, a PS-SAP preparation, gramicidin alone, and gramicidin reconstituted with phospholipid (PLG). $[Ca^{++}]$ responses were reexamined following preexposure to inhibitors of internal Ca^{++} release or the G protein pathway. We observed that (i) 1% PS+SAP—but not PS-SAP—causes transient increase of neutrophil $[Ca^{++}]$ within seconds of exposure; (ii) 1% PLG—but not gramicidin alone—closely mimics the effect of PS+SAP on Ca^{++} response; (iii) PS+SAP and PLG equally depolarize neutrophils; (iv) direct inhibition of internal Ca^{++} stores releases or of G protein activation suppresses Ca^{++} responses to PS+SAP and PLG; and (v) preexposure to either PS+SAP or PLG inhibits Ca^{++} influx following *f*MLP stimulation. We conclude that PS+SAP independently depolarizes neutrophils, releases Ca^{++} from internal stores by a G protein–mediated pathway, and alters subsequent neutrophil response to physiologic stimulants by depleting internal Ca^{++} stores and by inhibiting Ca^{++} influx during subsequent *f*MLP activation. The mimicking of these results by PLG supports the hypothesis that PS+SAP initiates depolarization via channel insertion into neutrophil plasma membrane. Exp Biol Med 229:99–107, 2004

Key words: surfactant; neutrophils; calcium; gramicidin; G proteins

Pulmonary surfactant is a complex mixture of lipids and proteins, and it is widely employed for the treatment of respiratory distress syndrome in preterm infants. Natural pulmonary surfactant contains four apoproteins (SAP-A, SAP-B, SAP-C, and SAP-D) in addition to phospholipids and other neutral lipids (1). For the clinical administration of surfactant to humans, exogenous surfactant derived from bovine or porcine sources retains only the hydrophobic apoproteins, SAP-B and SAP-C (PS+SAP). Most synthetic surfactants lack all surfactant-associated proteins (PS-SAP) (2). By meta-analysis of clinical trials comparing the efficacy of PS+SAP and PS-SAP surfactant compounds, preterm infants treated with PS+SAP require lower mean airway pressures, develop fewer pulmonary air leaks, assume lower oxygen requirements, and experience better survival rates than infants treated with PS-SAP (2, 3).

The release of proteases and production of oxygen radicals by pulmonary neutrophils is believed to be a key injurious mechanism in development both of chronic lung disease following neonatal respiratory distress syndrome and of acute respiratory distress syndrome in older children and adults (4). *In vitro* studies examining the effects of PS+SAP on normal human neutrophils have demonstrated decreased neutrophil adherence, aggregation, chemotaxis, respiratory burst, and elastase production following physi-

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ologic stimulation (5–9). By contrast, exposure to PS-SAP does not significantly affect neutrophil responses. These studies, when viewed in light of the clinical benefits of PS+SAP over PS-SAP, suggest an anti-inflammatory role for the surfactant apoproteins SAP-B and SAP-C.

We have observed that PS+SAP, as well as SAP-B and SAP-C isolated from PS+SAP, insert monovalent, cationic channels into artificial black membranes (10). More recent experiments by our laboratory have demonstrated that PS+SAP, but not PS-SAP, depolarizes neutrophil cell membranes and blocks Ca^{++} influx into neutrophils following physiologic activation (9). We also observed that gramicidin-D, a monovalent, cationic, channel-forming peptide, depolarizes neutrophil cell membranes and blocks Ca^{++} influx following physiologic activation similar to PS+SAP (9).

We hypothesize that PS+SAP suppresses neutrophil activation by depletion of internal Ca^{++} stores and that PS+SAP induces depletion through release of Ca^{++} stores and through inhibition of Ca^{++} influx. Our model predicts that PS+SAP releases Ca^{++} stores through insertion of channels, depolarization of neutrophils, and activation of a G protein–dependent pathway. The proposed model of channel insertion and the observed similarities between PS+SAP and gramicidin regarding cationic channel insertion and neutrophil depolarization suggest that effects of PS+SAP on neutrophils should be closely mimicked by those of gramicidin. To test these predictions regarding PS+SAP and gramicidin, the objectives of the current study are to (i) determine if PS+SAP induces a rise in cytosolic [Ca^{++}] that is dependent on membrane polarization, (ii) demonstrate that PS+SAP depolarizes neutrophils, (iii) test whether cytosolic [Ca^{++}] rise depends on release of internal Ca^{++} stores and activation of G proteins, (iv) ascertain if these effects are mimicked by gramicidin, and (v) test whether PS+SAP and PLG inhibit Ca^{++} influx following *f*MLP stimulation.

Materials and Methods

Reagents and Chemicals. Phosphatidic acid, phosphatidylglycerol, dipalmitoylphosphatidylcholine, EGTA, dextran, Ficoll, formyl peptide (*f*MLP), *N*-methylglucamine, and Triton X-100 were purchased from Sigma Chemical Company (St. Louis, MO). Fura2-AM, fura2, and gramicidin-D were purchased from Molecular Probes (Eugene, OR). Hanks balanced salt solution with (HBSSw) or without Ca^{++} and Mg^{++} (HBSSw/o) was purchased from Bio-Whitaker (Walkersville, MD), Hypaque 76 from Sanofi Winthrop Pharmaceuticals (New York, NY), and heparin from Elfing-Sinn (Cherry Hill, NJ). 2-APB, SKF-96365, and pertussis toxin were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). PS+SAP preparations, Survanta and Infasurf, were obtained from Ross Products Division, Abbott Labs (Columbus, OH) and Forest Pharmaceuticals (St. Louis, MO), respectively. Survanta and Infasurf contain approximately equal amounts of SP-C, but Survanta contains <10% of the SP-B content of that found in Infasurf (2). PS-SAP preparation, Exosurf, was obtained from Glaxo-Wellcome (Research Triangle Park, NC).

Purification of Neutrophils. Heparinized, venous blood samples were obtained from healthy adult human volunteers and separated by Hypaque-Ficoll step-gradient centrifugation, dextran sedimentation, and hypotonic lysis as previously described (9, 11). Our Institutional Review Board approved collection of blood, and informed consent was waived. Cell preparations typically provided >95% neutrophils confirmed by modified Wright-Giemsa staining, and they were used within 4 hrs of purification.

Reconstitution of Gramicidin in Phospholipid (PLG). Dipalmitoylphosphatidylcholine dissolved in chloroform was combined with phosphatidylglycerol dissolved in methanol and dry phosphatidic acid providing a final phospholipid molar ratio of 7:2:1, respectively. This phospholipid mixture (PL) was employed for experiments examining the effects of PS+SAP-like phospholipids on neutrophil [Ca^{++}] changes (12). For experiments requiring gramicidin reconstituted with PL (PLG), gramicidin-D dissolved in methanol was added in a 1:5 (gramicidin:phospholipid) molar ratio to the phospholipid mixture. Chloroform and methanol were removed under N_2 at 45°C. A 1% emulsion of the PLG complex was prepared with 145 mM NaCl by ultrasonication. To change conformation of gramicidin within the phospholipid mixture from a nonconductive to conductive conformer (13), PLG was heated (60°C, 12 hrs) following initial preparation and reheated (60°C, 1 hr) and cooled (24°C, 30 mins) prior to each experiment. Between experiments, PLG was stored at 4°–8°C. The estimated final [gramicidin] in experiments employing 1% PLG was 10 μM.

Cytosolic Calcium Concentration ([Ca^{++}]) Measurements. Purified neutrophils were suspended in HBSSw/o and exposed to 2 μM fura2-AM—the methyl ester form of fura2, a dual wavelength, fluorescent Ca^{++} probe. After incubation (37°C in the dark, 5% CO_2, 45 mins), cells were sedimented and washed twice in HBSSw/o to remove nonincorporated, extracellular fura2-AM. Neutrophils were resuspended at a concentration of 10×10^6 cells/ml in HBSSw or HBSSw/o depending on the experiment. Subsequently, cells (5×10^6/cuvette) were examined for fluorescence in an LS50B spectrofluorometer (Perkin-Elmer-Cetus, Norwalk, CT) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Maximum (R_{max}) and minimum (R_{min}) fura2 fluorescence ratios were calculated by adding 0.1% Triton and 20 mM EGTA, respectively, to cells in the spectrofluorometer. Neutrophil cytosolic [Ca^{++}] values were then calculated from the equation:

$$[Ca^{++}] = K_d[(R - R_{min})/R_{max} - R)]\beta,$$

where R is the ratio of the 340/380-nm fluorescence, R_{min} is the minimal ratio of 340/380-nm fluorescence, R_{max} is the maximal ratio of 340/380-nm fluorescence, β is the ratio of 380-nm fluorescence under Ca^{++}-free/Ca^{++}-saturated con-

ditions, and K_d is the dissociation constant for Ca^{++} binding to fura2 based on calibration curves from our laboratory (9, 14). Early cytosolic [Ca^{++}] responses in unstimulated neutrophils were examined following addition of either (i) 1% PS+SAP, (ii) 1% PS-SAP, (iii) 1% PL, (iv) 10 μM gramicidin-D, or (v) 1% PLG. Experiments were performed in both calcium-supplemented (HBSSw) and calcium-free buffers. Calcium-free buffer was prepared by adding 30 μM EGTA to HBSSw/o. For experiments examining Na^+ dependence of the PS+SAP response, *N*-methylglucamine (150 mM) was substituted for NaCl in the buffer. Daily pH values of all balanced salt solutions were monitored and maintained at 7.40 ± 0.05.

For experiments examining the effect of neutrophil membrane depolarization on PS+SAP-dependent cytosolic [Ca^{++}] peaking, neutrophils were depolarized through incubation with KCl or gramicidin-D prior to PS+SAP exposure (9). Specifically, neutrophils (5×10^6) were exposed (4 mins, 24°C) to KCl (150 mM) or gramicidin-D (90 μM), and fura2-associated fluorescence changes following addition of 1% PS+SAP were measured as described previously.

For experiments testing the contribution of intracellular Ca^{++} stores to [Ca^{++}] rise, SKF-96365 (10 μM) or 2-APB (30 or 100 μM) was added to neutrophils suspended in HBSS with Ca^{++} 4 mins prior to addition of PS+SAP, PLG, or *f*MLP (15). For those experiments testing coupled G protein activation, neutrophils suspended in HBSS with Ca^{++} (5×10^6 cells/ml) were incubated with or without pertussis toxin (50 ng/ml) (90 mins, darkness, 37°C, 5% CO_2) (16). Neutrophils were subsequently washed twice with HBSS prior to loading with fura2-AM, resuspending in HBSS with Ca^{++}, and exposing to PS+SAP, PLG, or *f*MLP.

To examine sensitivity of Ca^{++} influx to PS+SAP or PLG exposure stimulated by physiologic activation, 5×10^6 neutrophils were exposed to 1% PS+SAP, 1% PLG, or control buffer (HBSSw/o) and washed and resuspended in calcium-free buffer. Following stabilization of baseline fluorescence, 1 μM *f*MLP was added and fluorescence monitored. On achieving the maximal *f*MLP-induced [Ca^{++}] response, 5 mM $CaCl_2$ were added, and fluorescence was monitored for an additional 120 secs. Ca^{++} influx following *f*MLP stimulation was quantitated by the area under the curve following addition of $CaCl_2$ and expressed as nM/min (9). To provide a baseline control in separate experiments, 40 μM EGTA was added following *f*MLP stimulation.

Membrane Potential Measurement. Neutrophils (1×10^6) suspended in 100 μl HBSSw were exposed (24°C, 4 mins) to either 1% PLG, 1% PS+SAP, or HBSSw (control). Cells were washed and resuspended in HBSSw. Potentiometric fluorescent dye $diOC_5(3)$ (225 nM) in HBSSw was incubated (25°C, 4 mins) in the dark before baseline fluorescence was recorded at excitation and emission wavelengths of 460 and 507 nm (9). Neutrophils were added, creating a 20-fold dilution, and on restabilization of the fluorescence signal, 150 mM KCl were added to fully depolarize neutrophils and quench fluorescence. Quenching was followed until a final plateau was observed. Change in membrane potential was measured as a ratio of the change in fluorescence units (9).

Statistical Analysis. Data were analyzed by paired sample Student's *t* tests. *P* values less than 0.05 were considered significant. All data were expressed as mean ± SEM. *SPSS 7.5 for Windows* software employing the Marquardt-Levenberg algorithm was employed to determine the logarithmic regression of [Ca^{++}] peaking on PS+SAP or PLG concentration. Where dose relationships were sought, correlation coefficients (R^2) were determined by the same software. Replicate experiments used neutrophils from different human donors.

Results

PS+SAP Induces a Transient Neutrophil [Ca^{++}] Response. Exposure of neutrophils to 1% PS+SAP induced transient peaking of cytosolic [Ca^{++}] (Fig. 1). Within 60 secs of PS+SAP addition (closed circles), [Ca^{++}] peaked and was followed by prompt return toward baseline over the next 120 secs. The observation of this internal [Ca^{++}] response in the absence of extracellular Ca^{++} strongly suggests that intracellular Ca^{++} stores provide the source for cytosolic [Ca^{++}] peaking.

In contrast to the effect of PS+SAP, exposure to 1% PS-SAP (open circles) induced minimal change in neutrophil [Ca^{++}] (Fig. 1). Likewise, exposure of neutrophils to 1% PL (composed of phospholipids more closely resembling the phospholipid content of PS+SAP than those of PS-SAP) failed to increase neutrophil [Ca^{++}] (closed triangles). The negligible Ca^{++} response to PS-SAP or PL indicates that SAP (SAP-B and/or -C) is necessary for the observed neutrophil Ca^{++} response.

To provide further support for a causal relationship between PS+SAP and the Ca^{++} response by neutrophils, the dose effect of [PS+SAP] on magnitude of intracellular neutrophil Ca^{++} response was examined. At concentration as low as 0.2%, PS+SAP induced release of cytosolic Ca^{++} in Ca^{++}-free buffer (inset, Fig. 1). Release was not observed at lower concentrations, and concentrations greater than 1% could not be examined because of excessive optical interference. However, between 0.1% and 1% concentrations, dose dependency was observed by a logarithmic response of neutrophil [Ca^{++}] to [PS+SAP] (magnitude of Ca^{++} peak = 53.07 + 20.64 [ln{PS+SAP}], $R^2 = 0.93$).

[Ca^{++}] Response Depends on Membrane Polarization and Extracellular Na^+. We hypothesize that the effect of PS+SAP on neutrophil [Ca^{++}] is mediated by insertion of monovalent cationic channels into plasma membrane and that resulting dissipation of transmembrane, monovalent cation (i.e., Na^+ and K^+) gradients causes membrane depolarization via collapse of electrochemical potential gradients. To test for the dependency of Ca^{++}

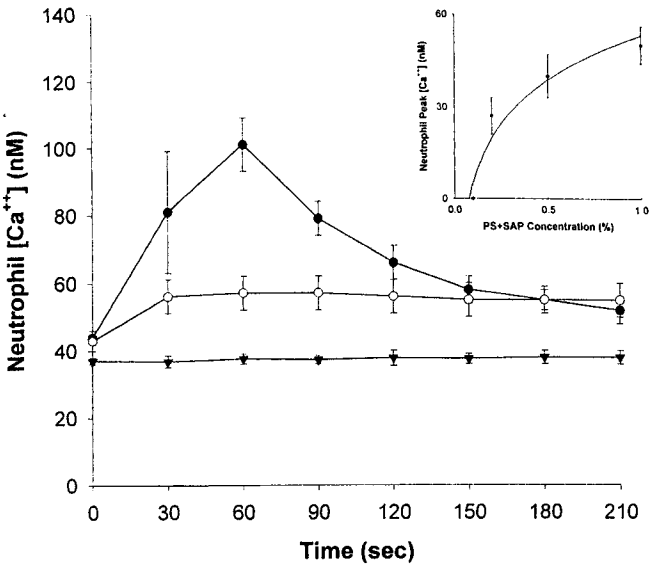

Figure 1. Effect of PS+SAP Don neutrophil [Ca^{++}] response. Neutrophils suspended in Ca^{++}-free buffer were exposed to 1% PS+SAP (closed circles), 1% PS-SAP (open circles), or 1% purified phospholipids (closed triangles) at time 0. [Ca^{++}] response was monitored over time. Data are expressed as mean ± SEM values (n = 4). The inset graph depicts peak Ca^{++} responses observed following exposure to PS+SAP at concentrations ≤1%. Peak Ca^{++} response is logarithmically related to PS+SAP concentration by the equation provided in text.

response on preexisting membrane polarization, we prevented PS+SAP-induced membrane depolarization by depolarizing neutrophils prior to PS+SAP exposure. Two methods were employed to induce membrane depolarization (Fig. 2A). The first substituted extracellular 150 mM KCl (open circles) for extracellular NaCl (closed circles), and the second employed 90 μM gramicidin (closed triangles).

Collectively, both approaches demonstrated that depolarization of neutrophils prior to PS+SAP exposure blocks Ca^{++} response to PS+SAP (Fig. 2A). Gramicidin achieved this inhibition of PS+SAP-induced Ca^{++} peaking while inducing significantly higher baseline cytosolic [Ca^{++}] (control [closed circles]: 50 ± 2 nM; KCl [open circles]: 52 ± 3 nM; gramicidin [closed triangles]: 76 ± 13 nM; $P < 0.05$, gramicidin vs. control or KCl). The cause of higher baseline cytosolic [Ca^{++}] is explored in subsequent experiments.

To test whether hypothesized channels inserted by PS+SAP specifically conduct small monovalent cations, 150 mM *N*-methylglucamine was substituted for extracellular NaCl. In the absence of extracellular Na^+, PS+SAP would not be expected to depolarize membrane by conducting Na^+ across plasma membrane. By these experiments, *N*-methylglucamine (open circles) fully inhibited PS+SAP-induced Ca^{++} peaking without significantly raising baseline cytosolic [Ca^{++}] (Fig. 2B).

Gramicidin Mimics PS+SAP Effects on Neutrophil [Ca^{++}] as PLG. Recalling that gramicidin blocks PS+SAP-induced Ca^{++} peaking (Fig. 2A) and that this inhibition is achieved by insertion of monovalent cationic

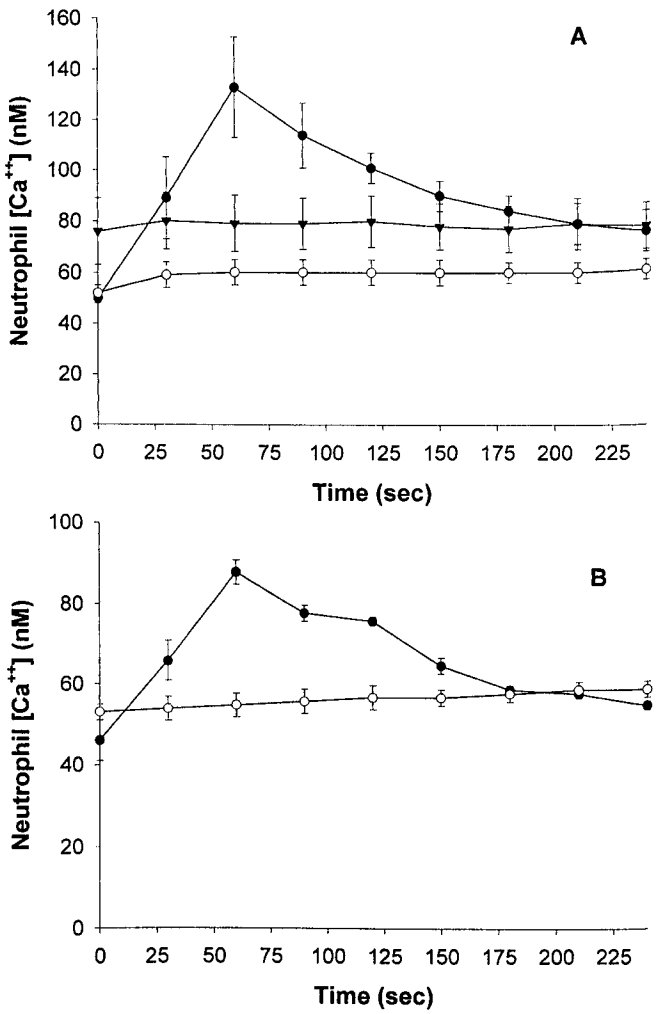

Figure 2. Inhibition of PS+SAP-induced [Ca^{++}] response by neutrophil depolarization or extracellular Na^+ removal. (A) Prior to exposure of neutrophils to 1% PS+SAP at time 0, neutrophils were suspended in either Ca^{++}-free KCl buffer (open circles) or Ca^{++}-free NaCl buffer containing 90 μM gramicidin (closed triangles) to depolarize neutrophils or in Ca^{++}-free NaCl buffer (control, closed circles). [Ca^{++}] response was monitored over time. Data are expressed as mean ± SEM values (n = 4). (B) Prior to exposure of neutrophils to 1% PS+SAP at time 0, neutrophils were suspended in either Ca^{++}-free NaCl buffer (control, closed circles) or Ca^{++}-free buffer substituting *N*-methylglucamine for NaCl (open circles). [Ca^{++}] response was monitored over time. Data are expressed as mean ± SEM values (n = 4).

channels into neutrophil membrane, we reasoned that substitution of gramicidin for PS+SAP should mimic results observed with PS+SAP. Experiments were repeated in both the presence (Fig. 3A) and absence (Fig. 3B) of extracellular Ca^{++} and while employing a second PS+SAP preparation (Infasurf) for comparison with original observations (Survanta). Unexpectedly, 10 μM gramicidin did not cause the predicted Ca^{++} peaking within the time frame of earlier experiments (Fig. 3A and B). Although gramicidin (open circles) induced a gradual rise in cytosolic [Ca^{++}] observable after 250 secs (data not shown), the rise did not mimic the Ca^{++} peaking caused by PS+SAP (open and closed triangles). The maximal [Ca^{++}] (approximately 80

nM) associated with gramicidin exposure did not occur for greater than 400 secs, and this gradual rise in cytosolic [Ca^{++}] likely explains the elevated baseline [Ca^{++}] observed in Figure 2A.

The failure of gramicidin to mimic the effects of PS+SAP prompted us to reconstitute gramicidin with phospholipids. Gramicidin has hydrophobic properties similar to those of SAP-B and SAP-C. Combination of SAP-B and SAP-C with phospholipids promotes spreading of PS+SAP in the lung. Similarly, reconstitution of gramicidin with phospholipids was expected to promote better contact between gramicidin and neutrophil membranes through improved diffusion in the aqueous extracellular environment.

We observed that reconstitution of gramicidin as a phospholipid mixture mimicked the effect of PS+SAP on neutrophil [Ca^{++}] in both magnitude and kinetics. The 1% PLG (closed circles, Fig. 3A and B) caused Ca^{++} peaking within 25 secs followed by return to baseline levels similar to the patterns of both PS+SAP preparations. Also like the PS+SAP preparation, Survanta, PLG induced Ca^{++} response in a logarithmic, dose-dependent manner (magnitude of Ca^{++} peak = 34.07 + 8.98 [ln{PLG}]; $R^2 = 0.88$) (data not shown).

The presence of extracellular Ca^{++} had limited demonstrable effect on results (Fig. 3A vs. 3B). Although Ca^{++} peaks induced by PS+SAP in the absence of extracellular Ca^{++} were higher (Infasurf [open triangles]: 182 ± 10 nM vs. 144 ± 17 nM, $P < 0.05$; Survanta [closed triangles]: 131 ± 10 nM vs. 90 ± 14 nM, $P < 0.05$; Fig. 3A and B), peaks were narrower and Ca^{++} runoffs quicker. Presence of extracellular Ca^{++} had no demonstrable effect on PLG results (PLG [closed circles]: 104 ± 13 nM vs. 112 ± 15 nM, NS]. Among PS+SAP preparations, 1% Infasurf induced consistently larger Ca^{++} responses than 1% Survanta in both the presence and the absence of extracellular Ca^{++} as quantified by either Ca^{++} peak data (see previous discussion) or by area under the curve data (extracellular Ca^{++} present: 141 ± 23 nM/min vs. 32 ± 4 nM/min, $P < 0.01$ Infasurf vs. Survanta; extracellular Ca^{++} absent: 159 ± 20 nM/min vs. 117 ± 13 nM/min, $P < 0.05$).

PS+SAP and PLG Independently Depolarize Neutrophils. To demonstrate that PLG depolarizes neutrophils as demonstrated in the past for PS+SAP (9), the magnitude of depolarization induced by PLG was compared to that induced by PS+SAP preparation, Survanta. Neutrophils were exposed (4 min 25°C) to PS+SAP, PLG, or control buffer; washed; and then resuspended in buffer before diluting with buffer containing potentiometric dye, $diOC_5(3)$ (225 nM final) in the spectrofluorometer. On stabilization of fluorescence, depolarization of neutrophils was completed with 150 mM KCl, and the change in fluorescent signal following prior PS+SAP or PLG exposure was expressed relative to fluorescent change observed under control conditions. These experiments demonstrated that prior exposure of neutrophils to 1% PS+SAP or 1% PLG each independently decreased further

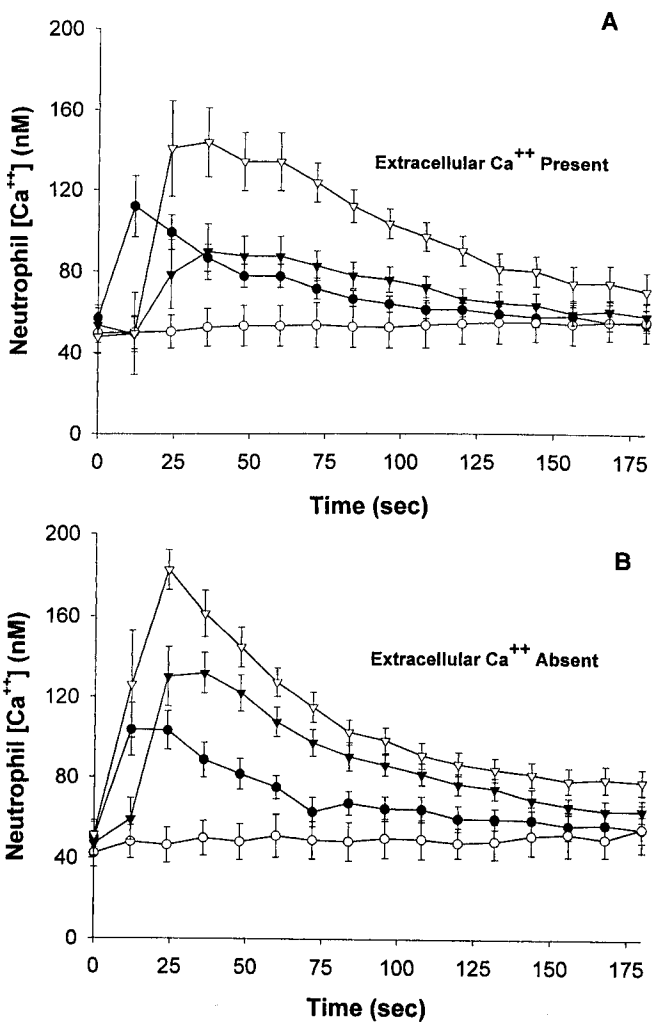

Figure 3. Effect of PS+SAP, PLG, or gramicidin on neutrophil [Ca^{++}] response. Neutrophils suspended in Ca^{++}-supplemented (A) or Ca^{++}-free (B) buffer were exposed to either of two 1% PS+SAP preparations (closed triangles [Survanta] or open triangles [Infasurf]), 1% PLG (closed circles), or 10 μM gramicidin (open circles) at time 0. [Ca^{++}] response was monitored over time. Data are expressed as mean ± SEM values (n = 5–8).

membrane depolarization by 150 mM KCl 36 ± 7% and 31 ± 6%, respectively. These results confirmed preexisting, partial membrane depolarization of approximately 25 mV (9) by 1% PS+SAP and 1% PLG without significant difference between the two agents.

Inhibition of Internal Ca^{++} Store Release Eliminates Ca^{++} Responses Induced by PS+SAP and PLG. Prior experiments conducted in the absence of extracellular Ca^{++} strongly suggested that release of internal Ca^{++} storage sites provides the source of Ca^{++} responses. To distinguish between release of internal Ca^{++} stores and altered intracellular Ca^{++} buffering or extracellular Ca^{++} influx, direct inhibitors of Ca^{++} release from endoplasmic reticulum storage sites were employed. Experiments were conducted in the presence of extracellular Ca^{++} to avoid possible masking caused by precedent depletion of internal Ca^{++} stores. 2-APB inhibits Ca^{++} release by blocking G

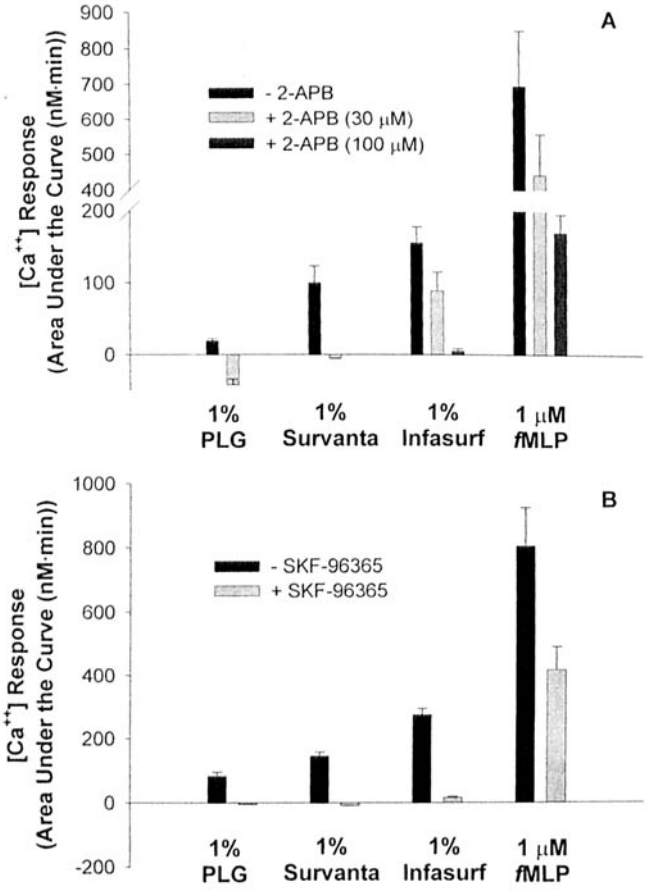

Figure 4. Effect of Ca^{++} store release inhibitors on PLG-, PS+SAP-, or *f*MLP-induced Ca^{++} responses. Two Ca^{++} store release inhibitors were employed: 2-APB at 30 or 100 μM (A) or SKF-96365 at 10 μM (B). Inhibitor or equal volume of solvent was added to neutrophils suspended in Ca^{++}-supplemented buffer 4 mins prior to addition of 1% PLG, 1% PS+SAP (Survanta or Infasurf), or 1 μM *f*MLP. [Ca^{++}] responses were monitored, and data are presented as areas under the Ca^{++} response curve for the first 240 secs of exposure ($n = 4$–6).

protein–sensitive IP3 receptors, and SKF-96365 inhibits both store-operated and receptor-mediated Ca^{++} release. The influences of inhibitors on the PS+SAP-, PLG-, and *f*MLP-induced Ca^{++} responses were compared. In the presence of 2-APB at 30- or 100-μM or SKF-96365 at 10-μM concentrations, both inhibitors nearly eliminated neutrophil Ca^{++} responses to PLG ($P < 0.01$), Survanta ($P < 0.01$), and Infasurf ($P < 0.01$ except for 30 μM 2-APB [$P < 0.05$]; Fig. 4A and B). Though presented data come from experiments that were conducted in the presence of extracellular Ca^{++}, results in the absence of extracellular Ca^{++} were nearly identical (data not shown).

Inhibition of G Protein Activation Limits Ca^{++} Response Induced by PS+SAP and PLG. Inhibition of internal Ca^{++} release from storage sites influenced by G protein–coupled IP3 receptors suggested that upstream inhibition of G protein activation also would limit Ca^{++} response to PS+SAP and PLG. Accordingly, Ca^{++} responses to PLG, Survanta, Infasurf, and *f*MLP were compared with and without prior exposure to pertussis toxin. Preexposure to pertussis toxin reduced observed Ca^{++} responses in neutrophils by 95%, 74%, 48%, and 27% by PLG ($P < 0.01$), Survanta ($P < 0.01$), Infasurf ($P < 0.05$), and *f*MLP, respectively (Fig. 5). At the relatively low *f*MLP dose employed, the limited effect of pertussis toxin on Ca^{++} response to *f*MLP was expected.

PS+SAP and PLG Limit Ca^{++} Influx by Subsequent fMLP Activation. Previously reported experiments from our laboratory demonstrated that preexposure of neutrophils to PS+SAP decreases activated Ca^{++} influx by subsequent *f*MLP exposure (9). To compare the preexposure effects of PLG with those of PS+SAP on subsequent activation of neutrophils, we examined the effect of PLG on *f*MLP-activated Ca^{++} influx. Neutrophils exposed to 1% PS+SAP, 1% PLG, or control buffer were washed, suspended in Ca^{++}-free buffer, and then stimulated with 1 μM *f*MLP. After neutrophil [Ca^{++}] peaked from *f*MLP-induced release of internal Ca^{++} stores, 10 mM $CaCl_2$ were added to the extracellular space. Influx of calcium was measured over 2 mins, and net Ca^{++} responses were compared by calculating areas under the curves of falling neutrophil [Ca^{++}] (9). *f*MLP-activated Ca^{++} influx was reduced significantly relative to control by pretreatment of neutrophils with either PS+SAP or PLG (control [*f*MLP only]: 204 ± 22 nM/min; PS+SAP: 95 ± 31 nM/min; PLG: 131 ± 31 nM/min; $P < 0.01$, PS+SAP or PLG vs. control).

Discussion

Commercially available natural surfactant (PS+SAP) significantly improves survival from adult respiratory distress syndrome (17, 18). In neonatal respiratory distress syndrome, PS+SAP promotes better short- and long-term outcomes than PS-SAP (3). These clinical investigations demonstrating therapeutic superiority of PS+SAP over PS-SAP suggest that the presence of surfactant-associated apoproteins SAP-B and SAP-C contributes to the improved clinical outcomes.

Neutrophils are the primary cellular mediators of acute inflammation, and they are largely responsible for the lung damage seen in respiratory distress syndromes. During development of adult respiratory distress syndrome, neutrophils migrate into lung parenchyma. On release of proteases and oxygen radicals, neutrophils injure the respiratory epithelium and inactivate native pulmonary surfactant (19). PS+SAP treatment improves clinical outcomes by suppression of neutrophil functions. Suwabe *et al.* demonstrated that Surfacten, a PS+SAP preparation, inhibits adherence and superoxide production by human neutrophils (5). Ahuja *et al.* reported that native porcine PS+SAP inhibits human neutrophil superoxide production induced by either *f*MLP or phorbol myristate acetate (6). PS-SAP does not cause inhibition. Tegtmeyer *et al.* observed that elastase release by activated human neutrophils is decreased by PS+SAP preparations, while PS-SAP exhibits only modest effects (8). We recently reported that PS+SAP decreases neutrophil adherence and aggregation

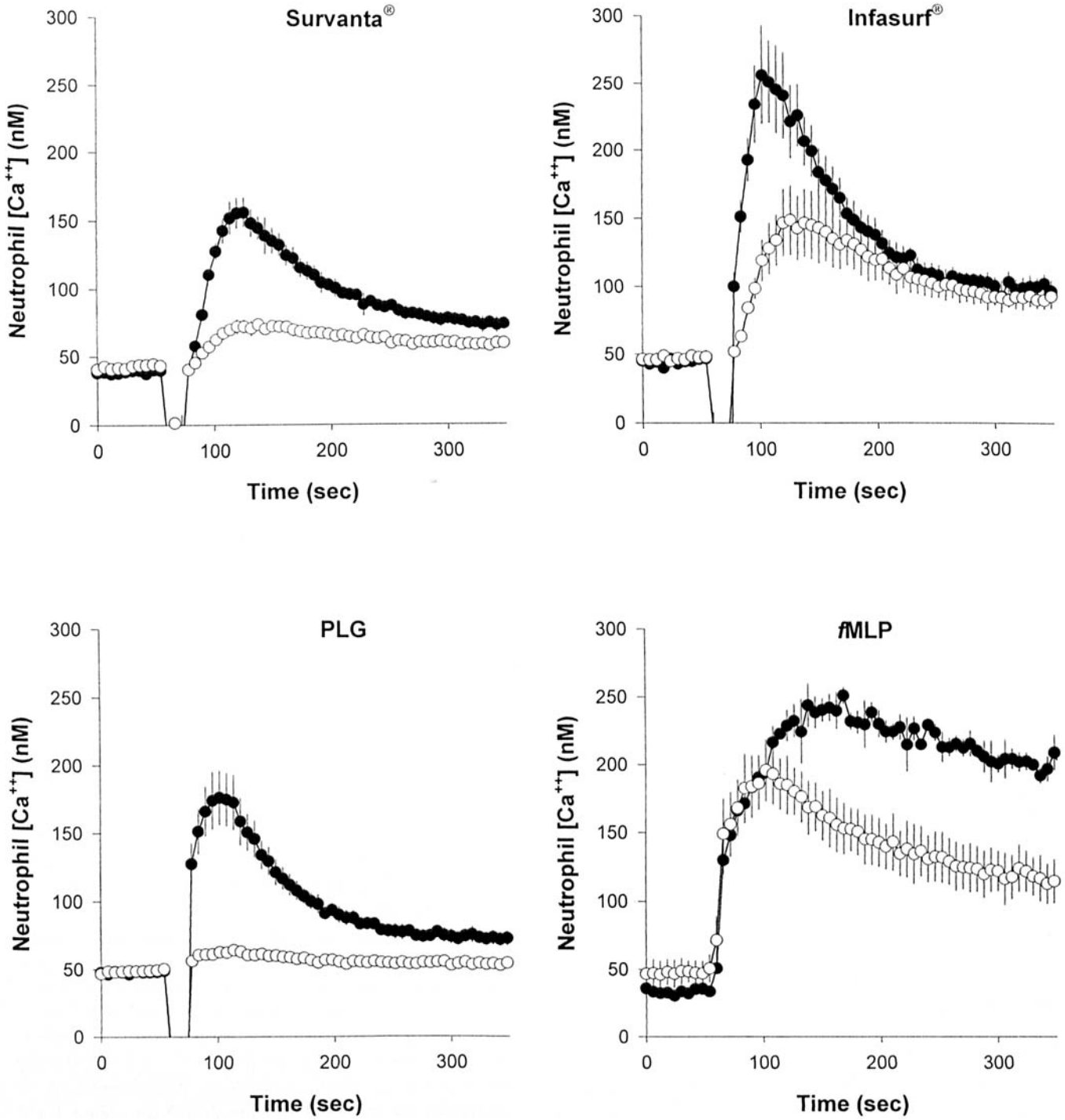

Figure 5. Effect of G protein activation inhibitor on PLG-, PS+SAP-, or *f*MLP-induced Ca^{++} responses. Neutrophils suspended in Ca^{++}-supplemented buffer were exposed to pertussis toxin (50 ng/ml) (open circles) or solvent (closed circles) as described in text before washing, loading with fura2, and exposing to 1% PLG, 1% PS+SAP (Survanta or Infasurf), or 1 μM *f*MLP. $[Ca^{++}]$ responses were monitored over time, and data are expressed as mean ± SEM values ($n = 4$).

following physiologic stimulation, while PS-SAP does not (9). The pathway by which PS+SAP suppresses neutrophil activation is the focus of our present experiments.

A common link among most neutrophil functions is change in cytosolic Ca^{++} activity. An increase in cytosolic $[Ca^{++}]$ is the earliest measurable event following neutrophil activation, and it serves to initiate or enhance response (20, 21). Therefore, we proposed during our earliest investigations that PS+SAP inhibits neutrophil function via direct modulation of neutrophil $[Ca^{++}]$. We subsequently established that PS+SAP does inhibit both activation of Ca^{++} influx and activation of neutrophil function stimulated by *f*MLP and other physiologic activators (9). We also observed that PS+SAP depolarizes neutrophils. However, it

remained unknown how depolarization by PS+SAP was linked to neutrophil [Ca^{++}] and subsequent inhibition of *f*MLP activation. The primary goal of the present experiments was to investigate whether depolarization of neutrophils by PS+SAP or gramicidin inhibits subsequent *f*MLP activation through depletion of internal Ca^{++} stores. We hypothesized that depletion occurs through both release of internal Ca^{++} stores and inhibition of Ca^{++} influx. Internal store release would lead to depletion through leakage of Ca^{++} to the extracellular space, and decreased Ca^{++} influx would reduce replenishment of depleted Ca^{++} stores.

The present experiments established that two different preparations of PS+SAP induce transient peaking of cytosolic Ca^{++} via the release of internal Ca^{++} stores (Figs. 1 and 3). By contrast, neither PS-SAP nor purified PL induced transient Ca^{++} peaking. Though possible contribution of extracellular Ca^{++} to the observed responses could not be excluded, the similarity of responses in the presence or absence of extracellular Ca^{++} suggested that the extracellular contribution is minimal (Fig. 3A and B). Evidence for a causal relationship between PS+SAP exposure and internal Ca^{++} release was demonstrated by (i) consistent stimulus-response timing between presentation of PS+SAP and peaking of cytosolic [Ca^{++}], (ii) a dose-response relationship between [PS+SAP] and magnitude of [Ca^{++}] peaking, (iii) elimination of response by specific internal Ca^{++} release inhibitors (2-APB and SKF-96365), and (iv) lack of an observed response in the absence of SAP. Differences in the magnitudes of Ca^{++} responses between the two 1% PS+SAP preparations (Fig. 3A and B) is likely related to significantly higher SAP concentrations in Infasurf and/or the relative lack of SAP-B in Survanta (22). The 1% concentrations of Survanta (0.25 mg/ml) and Infasurf (0.35 mg/ml) employed in these experiments are well within the physiologic range of surfactant concentrations observed in alveolar lining layers (5–10 mg/ml) (23).

Duplication of PS+SAP results by the exposure of neutrophils to PLG—a well-characterized cationic channel protein packaged in phospholipids resembling PS—supports the argument that cationic channel insertion is the initiating event for Ca^{++} store release. Gramicidin is a small hydrophobic peptide that has been extensively studied for its ability to form monovalent cationic channels in cell membranes. It exists naturally in two basic conformations—a double helix and a β-helical dimer (14, 24). The latter conformation is the one believed responsible for forming channels in cell membranes (14, 25). Surfactant-associated apoproteins SAP-B and SAP-C are hydrophobic lipopolypeptides containing extended α-helical regions within their secondary structures (1). Amphipathic, α-helical structures within the peptide subunits resemble other monomeric proteins recognized for insertion in membrane bilayers and for combination with other subunits forming channel oligomers (10, 26).

Insertion of monovalent cationic channels by PS+SAP and PLG would be expected to depolarize neutrophils in the K^+-predominant intracellular and Na^+-predominant extracellular environments. Consistent with this expectation, current experiments also established that PS+SAP (and PLG) depolarizes neutrophils and that prior polarization of neutrophils is necessary for the Ca^{++} peaking. This was substantiated by the observed membrane depolarization following PS+SAP or PLG exposure and by the dependence of Ca^{++} response on preexisting neutrophil polarization (Fig. 2). Substitution of *N*-methylglucamine for NaCl also demonstrated dependency of Ca^{++} peaking on extracellular Na^+ presence. This Na^+ dependency is best explained by (i) the demonstrated Na^+ conductances of SAP and gramicidin channels inserted in plasma membrane (10) and (ii) elimination of available monovalent cations for channel conduction following removal of extracellular Na^+.

To explain the potentiometric sensitivity of the PS+SAP-induced Ca^{++} response, we speculated that internal Ca^{++} release is related to membrane potential through an unidentified intermediate event that couples membrane depolarization with internal Ca^{++} release. Others have observed that pore-forming toxins release internal Ca^{++} stores in neutrophils by membrane depolarization that is accompanied by G protein activation (16). 2-APB inhibits G protein–activated Ca^{++} store release in neutrophils, and our observation of 2-APB inhibition (Fig. 4A) supports the possibility of G protein–activated Ca^{++} release by PS+SAP (27). In pursuit of this possibility, sensitivity to pertussis toxin was explored. It is well documented that pertussis toxin inhibits G protein–activated Ca^{++} release in neutrophils (16). We found that pertussis toxin significantly inhibits PLG-induced and PS+SAP-induced Ca^{++} release (Fig. 5). Closer review of the time courses for PS+SAP-induced releases revealed that much of the pertussis toxin–dependent inhibition occurred shortly after PS+SAP exposure, thereby delaying the observed Ca^{++} peaks approximately 60 secs and reducing magnitudes. The observed delay in Ca^{++} peaks suggests that at least two pathways—one that is dependent and one that is independent of pertussis toxin–sensitive G protein activation—contribute to Ca^{++} release by PS+SAP.

Finally, we observed that preexposure to either PS+SAP or PLG inhibits activated Ca^{++} influx following *f*MLP exposure. Prior observations that preexposure of neutrophils to PS+SAP inhibits *f*MLP-stimulated Ca^{++} influx suggested to us that reduced Ca^{++} influx was sufficient to explain the reduced fMLP response (9). However, this explanation ignored the possible contribution of Ca^{++} store release and the observed sensitivities of *f*MLP-induced Ca^{++} response to 2-APB and SKF-96365 (Fig. 4A and B). Hence, depletion of internal Ca^{++} stores by both PS+SAP-induced Ca^{++} store release and inhibited Ca^{++} influx would be more consistent with our collective observations.

In summary, our experiments demonstrate that both PS+SAP and PLG preparations independently depolarize

neutrophils, release Ca^{++} from internal stores by a G protein–activated pathway, and inhibit Ca^{++} influx to recognized physiologic stimulants. Our model proposes that both PLG and PS+SAP insert monovalent cationic channels into neutrophil membranes causing cell depolarization and G protein–mediated release of intracellular Ca^{++} stores. Release of Ca^{++} stores, combined with reduced Ca^{++} influx, depletes Ca^{++} stores and blunts responsiveness to physiologic activators. This PS+SAP-initiated cascade of events modulates the response of human neutrophils during inflammation and thereby improves clinical outcomes in patients with respiratory distress syndromes. Although the PS+SAP preparation currently employed does not contain all four of the major surfactant apoproteins, the excluded SAP-A and SAP-D play important roles in local lung defense by enhancing chemotaxis, opsonizing bacteria, enhancing macrophage function, and stimulating antibody production (28, 29). Therefore, in addition to reducing surface tension, it would appear that native pulmonary surfactant provides both pro- and anti-inflammatory activities. We speculate that in the course of pulmonary disease, a balance is sought between pro- and anti-inflammatory activities to both counter invading microorganisms (pro-inflammatory) and modulate host inflammation (anti-inflammatory).

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