**Streptococcus pneumoniae** Infection of Host Epithelial Cells via Polymeric Immunoglobulin Receptor Transiently Induces Calcium Release from Intracellular Stores*

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Tauseef M. Asmat†1,2, Vaibhav Agarwal†1,3, Susann Räth§, Jan-Peter Hildebrandt#, and Sven Hammerschmidt‡4

From the ‡Department of Genetics of Microorganisms, Interfaculty Institute for Genetics and Functional Genomics, Ernst Moritz Arndt University of Greifswald, Friedrich-Ludwig-Jahn-Strasse 15a, D-17487 Greifswald and #Animal Physiology and Biochemistry, Zoological Institute, Ernst Moritz Arndt-University, Johann Sebastian Bach-Strasse 11/12, D-17487 Greifswald, Germany

The pneumococcal surface protein C (PspC) is a major adhesin of *Streptococcus pneumoniae* (pneumococci) that interacts in a human-specific manner with the ectodomain of the human polymeric immunoglobulin receptor (pIgR) produced by respiratory epithelial cells. This interaction promotes bacterial colonization and bacterial internalization by initiating host signal transduction cascades. Here, we examined alterations of intracellular calcium ([Ca\(^{2+}\)]\(_i\)) levels in epithelial cells during host cell infections with pneumococci via the PspC-pIgR mechanism. The release of [Ca\(^{2+}\)]\(_i\) from intracellular stores in host cells was significantly increased by wild-type pneumococci but not by PspC-deficient pneumococci. The increase in [Ca\(^{2+}\)]\(_i\) was dependent on phospholipase C as pretreatment of cells with a phospholipase C-specific inhibitor U73122 abolished the increase in [Ca\(^{2+}\)]\(_i\). In addition, we demonstrated the effect of [Ca\(^{2+}\)]\(_i\) on pneumococcal internalization by epithelial cells. Uptake of pneumococci was significantly increased after pretreatment of epithelial cells with the cell-permeable calcium chelator 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid-tetraacetoxymethyl ester or use of EGTA as a extracellular Ca\(^{2+}\)-chelating agent. In contrast, thapsigargin, an inhibitor of endoplasmic reticulum Ca\(^{2+}\)-ATPase, which increases [Ca\(^{2+}\)]\(_i\), in a sustained fashion, significantly reduced pIgR-mediated pneumococcal invasion. Importantly, pneumococcal adherence to pIgR-expressing cells was not altered in the presence of inhibitors as demonstrated by immunofluorescence microscopy. In conclusion, these results demonstrate that pneumococcal infections induce mobilization of [Ca\(^{2+}\)]\(_i\) from intracellular stores. This may constitute a defense response of host cells as the experimental reduction of intracellular calcium levels facilitates pneumococcal internalization by pIgR-expressing cells, whereas elevated calcium levels diminished bacterial internalization by host epithelial cells.

The Gram-positive bacterium *Streptococcus pneumoniae* (pneumococci) is a commensal of the human upper respiratory tract. Depending on the host susceptibility, pneumococci may cause local infections such as otitis media, sinusitis, or life-threatening diseases such as community acquired pneumonia, septicemia, and meningitis (1). Pneumococci colonize the nasopharyngeal epithelium and eventually penetrate the epithelium to reach the vascular compartment. This colonization or transmigration of host tissue barriers is facilitated by a variety of virulence factors or surface-exposed adhesin(s) of pneumococci (2–4). However, various sera or extracellular matrix proteins such as complement factor H, human thrombospondin-1, and vitronectin also facilitate pneumococcal adherence to and invasion into host cells (5–7). Pneumococcal surface protein C (PspC, also designated as CbpA or SpasA) is a multifunctional surface-exposed choline-binding protein and a major virulence factor that plays an important role in invasion and pathogenesis of this versatile pathogen. PspC recognizes directly and in a human-specific manner the ectodomains of the polymeric immunoglobulin receptor (pIgR) (8–11). By adopting the pIgR retrograde transcytosis machinery, pneumococcal binding to pIgR via PspC leads to internalization and transcytosis across epithelial layers (8, 10–12). The pIgR, which is broadly expressed by epithelial cells of the respiratory tract, mediates the transport of polymeric IgA (pIgA) or pIgM across the mucosal epithelial barriers from the basolateral to apical lumen. Studies exploring the mechanism involved in the cellular trafficking of pIgR demonstrated that pIgA binding stimulates rabbit-pIgR transcytosis, because of phospholipase C activation, and increases intracellular calcium levels (13). However, this effect was not observed with human-pIgR (14).

Calcium acts as a secondary messenger in eukaryotic signal transduction cascades and plays an essential role in a wide variety of cellular processes, including gene expression (15, 16), vesicular trafficking (17), cytokoskeletal rearrangements, apoptosis (18), growth and proliferation, and cytokine secretion as well (19). Calcium signaling has been implicated in various steps of bacterial infections of eukaryotic host cells, including *Listeria*.

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† Both authors contributed equally to this work.

‡ Recipient of a fellowship from the German Academic Exchange Commission and German Academic Exchange Service.

§ Recipient of a fellowship from the German Academic Exchange Service. Present address: Dept. of Laboratory Medicine, Medical Protein Chemistry, Malmö University Hospital, Lund University, S-205 02 Malmö, Sweden.

# To whom correspondence should be addressed. Tel.: 49-3834-864161; Fax: 49-3834-86-4172; E-mail: sven.hammerschmidt@uni-greifswald.de.

5 The abbreviations used are: pIgR, polymeric immunoglobulin receptor; h, human; AM, acetoxymethyl ester; BAPTA, 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid; 2-APB, 2-aminobiphenyl borate; PLC, phospholipase C; MDCK, Madin-Darby canine kidney cells; InsP\(_3\), inositol 1,4,5-triphosphate; PFA, paraformaldehyde.
monocytogenes (20, 21), Campylobacter jejuni (22), or Salmonella typhimurium (23, 24). Bacterial toxins may induce an increase in the cytosolic concentration of free calcium ions in host cells (25) or bacteria may induce, independently of toxins, calcium responses that play a role in cytoskeleton rearrangements that facilitate cell adherence or even internalization of pathogenic bacteria into host cells.

Recently, we demonstrated that pneumococcal invasion of host epithelial cells via the PspC-hplgR interaction requires the small GTPase member Cdc42, phosphatidylinositol 3-kinase (PI3K), and Akt activity (26). In addition, PspC-hplgR-mediated invasion of pneumococci requires a coordinated signaling of Src protein-tyrosine kinase, focal adhesion kinase, ERK1/2, and JNK (27). Several of these cellular signaling cascades are directly or indirectly dependent upon transient or sustained elevations in intracellular calcium (28). However, whether pneumococcal infections of host epithelial cells via the PspC-hplgR mechanism require intracellular calcium mobilization has not been addressed yet. Here, we assessed the role of calcium during PspC-hplgR-mediated internalization of pneumococci. We demonstrated that pneumococcal infection of host epithelial cells induces release of calcium from intracellular stores in a phospholipase C-dependent manner. In contrast, experimental lowering of intracellular calcium levels facilitates pneumococcal uptake by these cells.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Culture Conditions, and Recombinant PspC Proteins—** S. pneumoniae (NCTC10319; serotype 35A) were cultured in Todd-Hewitt broth (Oxoid, Basingstoke, UK) supplemented with 0.5% yeast extract (THY) to mid-log phase or grown on blood agar plates (Oxoid). Constructions of the pspC mutant and anti-PspC antibodies used in this study have been described previously (6, 8). Expression and purification of His6-tagged PspC proteins PspC-SH12 (amino acid residues 38–482 of PspC3.3) and PspC-SH3 (amino acid residues 38–159 of PspC2.1) employed in this study have also been described previously (6, 11).

**Chemicals and Reagents—**Digitonin, EGTA, and latex beads (3 μm) were obtained from Sigma. Indo-1/AM was obtained from Invitrogen; 1,2-bis-(o-aminophenoxy)-ethane-N,N,N’,N’'-tetraacetic acid, tetracetoxyethyl ester (BAPTA-AM) and thapsigargin were obtained from Calbiochem. Trypsin (without EDTA) was purchased from PAN-Biotech (Aidenbach, Germany). U73122 and U73343 were obtained from Axxora (Loërrach, Germany). All other chemicals were obtained from Roth (Karlsruhe, Germany).

**Cell Culture, Infection Experiments, and Inhibitor Studies—** Madin-Darby canine kidney (ATCC CCL-34) epithelial cells stably transfected with the hplgR cDNA in pcB6 (MDCK-hplgR) and plgR-expressing human epithelial cell line Calu-3 (ATCC HTB-55) were cultured in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin G (100 IU ml⁻¹), and streptomycin (100 μg ml⁻¹) (all from PAA Laboratories, Colbe, Germany) at 37 °C under 5% CO₂. The medium for Calu-3 cells was further supplemented with 1 mM sodium pyruvate and 0.1 mM nonessential amino acids (PAA).

Epithelial cells were seeded on glass coverslips (diameter 12 mm) or directly in wells of a 24-well plate (Cellstar, Greiner, Germany). 5 × 10⁶ cells per well were cultured to form cell monolayers with ~2 × 10⁶ cells per well. Prior to the infection with pneumococci, cells were washed three times with Dulbecco’s modified Eagle’s medium containing HEPES (DMEM-HEPES, PAA Laboratories, Colbe, Germany) supplemented with 1.0% FBS. Host cells were infected as described using a multiplicity of infection of 50 bacteria per host cell. Infections were carried out for 3 h at 37 °C and 5% CO₂. To remove unbound bacteria from the supernatant of the infection experiment, host cells were thoroughly washed with DMEM-HEPES. The infection dose (colony-forming units) per infection and cell culture well was controlled by serial plating of pneumococci on blood agar plates. Pharmacological inhibitors used here to study the role of calcium on pneumococcal invasion were solved in dimethyl sulfoxide (DMSO). Host cells were preincubated at 37 °C for 5–10 min with U73122 and U73343 or for 30 min with the other inhibitors prior to host cell infection, and the infection assays were performed in the presence of the inhibitors. To evaluate the effect of DMSO, control experiments were performed in which the host cells were incubated with DMSO alone and infected as indicated. Our results revealed no effect of DMSO on bacterial and host cell viability and pneumococcal adherence as determined by immunofluorescence microscopy (data not shown). To quantify the total number of epithelial cell-associated bacteria (attached and invasive bacteria), unbound bacteria were removed, and saponin-treated cells (1% w/v in DMEM) were plated on blood agar plates. After overnight incubation at 37 °C, 5% CO₂, the colony-forming units per well were determined. The antibiotic protection assays was conducted to quantify the total number of internalized and recovered pneumococci after infection experiments as described recently (7).

**Measurement of Intracellular Calcium—** Cultured epithelial cells were trypsinized and suspended in prewarmed (37 °C) HEPES-buffered saline (132.5 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5.95 mM HEPES (free acid), 9.05 mM HEPES (sodium salt), 6 mM glucose, and 1.3 mM CaCl₂, pH 7.4) (29). The cells were loaded with 5 μM indo-1/AM in HEPES-buffered saline for 30 min in the dark at 37 °C with gentle shaking. Following incubation, indo-1/AM-loaded cells were washed and further incubated for 30 min at 37 °C (recovery period) in the dark with gentle shaking. Thereafter, the cells were washed three times by centrifugation (2 min at 600 × g) with HEPES-buffered saline and finally resuspended in 3 ml of HEPES-buffered saline in the methacrylate cuvette (Sarstedt, Nümbrecht, Germany) of the spectrofluorimeter (Fluoro-Max-3, HORIBA Jobin Yvon, Bensheim, Germany) as described earlier (25). In all experiments, the contents of the cuvette were stirred and held at a constant temperature (37 °C) during the measurements. Calcium measurements were performed as single wavelength experiments. Excitation and emission wavelengths were set at 338 and 400 nm, respectively, with slit widths of 2 nm. Fluorescence data were recorded in intervals of 2 s. For calibration of the indo-1/AM signals, cells were lysed by addition of 50 μM digitonin and reading of the maximum fluorescence followed by chelation of free Ca²⁺ by addi-
tions of EGTA (5 mM, pH 8.8) and reading of the minimum fluorescence. Calcium concentrations were calculated using the equation for single wavelength measurements and a binding constant ($K_d$) between indo-1 and $Ca^{2+}$ of 250 nM as reported previously (30).

To study the effects of pneumococcal infection on intracellular calcium levels in epithelial cells, live or paraformaldehyde (PFA)-fixed pneumococci at a multiplicity of infection of 50 bacteria per host cells were used. The bacteria were added to the cuvette 5 min after starting the recording of intracellular calcium concentration. Latex beads were used as control.

**Fluorescence Staining and Microscopy**—For immunofluorescence microscopy, the infected host cells were fixed on the glass coverslips (diameter, 12 mm) using 3% paraformaldehyde. Immunofluorescence staining of pneumococci attached to the host cells was carried out as described recently (26). Briefly, extracellular pneumococci were stained using a polyclonal anti-pneumococcal antiserum and secondary goat anti-rabbit IgG coupled to Alexa Fluor 488 (green) (Invitrogen). The stained samples were viewed with a confocal laser scanning microscope (Zeiss LSM510Meta). LSM510 software was used for image acquisition. Bars in the images represent 10 μm.

**Statistical Analysis**—All data are reported as mean ± S.D. unless otherwise noted. For cytosolic calcium measurements, significant differences were detected by analyzing the means at each time point. Results were statistically analyzed using the paired two-tailed Student’s $t$ test, and a value of $p < 0.05$ was accepted as indicating significance.

**RESULTS**

**Cross-talk between hpIgR-expressing Respiratory Epithelial Cells and Pneumococci Generates Changes in [Ca$^{2+}$],**—The level of cytosolic free $Ca^{2+}$ measured in noninfected plgR expressing Calu-3 and MDCK-hpIgR epithelial cells was ~200 nM. The presence of pneumococci significantly increased $[Ca^{2+}]$, in Calu-3 and MDCK-hpIgR cells. The levels increased 15 min after addition to ~400–500 nM $[Ca^{2+}]$, when using live bacteria and to ~300 nM $[Ca^{2+}]$, when using PFA-fixed pneumococci (Fig. 1, A and B). These data demonstrated that live and fixed pneumococci had similar effects on calcium release from intracellular stores. To verify that these changes in $[Ca^{2+}]$, were specifically mediated by the cross-talk between the eukaryotic cells and pneumococci, latex beads were used instead of bacteria. However, there were no changes in calcium levels upon addition of these inert particles to the experimental cells (Fig. 1C). These data demonstrate that the increases in $[Ca^{2+}]$, levels were induced by pneumococci and not just by contact of eukaryotic cells with inert particles.

**Pneumococcus-mediated Changes in [Ca$^{2+}$], Are Dependent upon PspC-hpIgR**—We have recently shown that pneumococcal adherence to and internalization by host epithelial cells via the PspC-hpIgR activate several signal transduction pathways in epithelial cells (26, 27). To investigate whether the observed changes in $[Ca^{2+}]$, (Fig. 1, A and B) were mediated via a direct interaction between the bacterial adhesin PspC and the host cellular polymeric immunoglobulin receptor, live and paraformaldehyde-preserved PspC-deficient *S. pneumoniae*, respectively, were employed in calcium mobilization assays using indo-1-loaded epithelial cells. Addition of PspC-deficient pneumococci did not induce any increases in the level of $[Ca^{2+}]$, as compared with the resting state of experimental cells, irrespective of using live or PFA-fixed bacteria (Fig. 2, A and B). Moreover, pretreatment of wild-type pneumococci with anti-PspC antibodies prevented changes in $[Ca^{2+}]$, (Fig. 2C). To corroborate the role of PspC-hpIgR interaction on increases in $[Ca^{2+}]$, two different PspC protein fragments were employed in calcium assays with eukaryotic cells. The first set of experiments was performed using PspC3.3 subtype derivative SH12, consisting of the N-terminal part of PspC (residues 38–482), including the hexameric peptides within the repeat domains (termed R1 or R2) that recognize human-specific amino acids in ectodomains D3 and D4 of plgR (8). In another set of experiments, the C-terminally truncated PspC derivative PspC-SH3 (residues 38–159), which lacks plgR-binding domains, was used. Addition of PspC-SH12 protein (3 μg) induced an increase in $[Ca^{2+}]$, to similar levels observed in experiments using pneumococci producing PspC, although the addition of PspC-SH3 had no effect (Fig. 2D). These results indicate that the surface-exposed PspC protein containing the plgR interaction domain is critical for the observed bacterial effects on $[Ca^{2+}]$. To demonstrate that the cellular plgR is essential for pneumococci and PspC-mediated changes of $[Ca^{2+}]$, we performed control calcium...
assays using wild-type MDCK cells that do not express the pIgR (Fig. 2E). In these cells neither pneumococci nor the PspC-SH12 protein induced any changes in the levels of $[\text{Ca}^{2+}]_i$ as compared with MDCK cells expressing hpIgR (Figs. 1B and 2D).

### Pneumococcal PspC Interaction with the Cellular hpIgR Induces Ca$^{2+}$ Release from Intracellular Stores

In most epithelial cells, the endoplasmic reticulum has inositol 1,4,5-triphosphate (InsP$_3$) receptors that trigger Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores upon activation (31). To elucidate the mechanisms underlying the observed changes in $[\text{Ca}^{2+}]_i$ in human epithelial cells in response to pneumococci or purified PspC protein, we used a cell-permeable antagonist of InsP$_3$ receptor, 2-aminobiphenyl borate (2-APB) (32–34). Epithelial cells were pretreated with 50 μM 2-APB, and the levels of cytosolic calcium were assayed before and after the bacteria or the purified PspC-SH12 fragments were added to the host cell suspension. The results showed that 2-APB pretreatment of cells significantly decreased the effect of pneumococci (Fig. 3, A and B) or PspC-SH12 protein (Fig. 3C) on $[\text{Ca}^{2+}]_i$ in both Calu-3 and MDCK-hpIgR cells. This indicates that the observed changes in cytosolic calcium levels in epithelial cells in response to pneumococci or PspC protein were due to calcium release from intracellular stores mediated by activation of the InsP$_3$ receptor.

### Activation of Phospholipase C (PLC) Is Required for PspC-hpIgR-mediated Ca$^{2+}$ Release

To assess the contribution of PLC to pneumococcus-induced calcium signals, Calu-3 and MDCK-hpIgR cells were pretreated with U73122, a specific inhibitor of phospholipase C (35, 36). U73122 inhibits the gen
**Ca^{2+} Flux during Pneumococcal Infection**

**FIGURE 4.** PspC-mediated increases in intracellular calcium levels in Calu-3 and MDCK-hpIgR cells depend on activation of phospholipase C. Effect of the PLC inhibitor U73122 (10 \(\mu\)M) or its inactive analog U73343 on [Ca^{2+}], in Calu-3 and MDCK-hpIgR upon addition of live *S. pneumoniae* (A), PFA-fixed *S. pneumoniae* (B), PspC protein SH12 (C), or after activation of purinergic receptors with ATP (100 \(\mu\)M) (D). Traces represent means ± S.D. (S.D. at very full minute for clarity) of \(n = 4\) experiments using different cell preparations.

Ca^{2+} Flux during Pneumococcal Infection

Pneumococci were added to epithelial cells pretreated with U73122 or U73343 (10 \(\mu\)mol each) and loaded with indo-1/AM, respectively. In the presence of U73122, pneumococcus-induced calcium release from intracellular stores was blocked, although preincubation of cells with U73343 had no inhibitory effect (Fig. 4, A and B). Similarly, U73122 blocked the increase in [Ca^{2+}], mediated by incubation of cells with the PspC protein fragment PspC-SH12, whereas the inactive analog U73343 did not (Fig. 4C). The specificity of PLC inhibition by U73122 in Calu-3 and MDCK-hpIgR cells was tested by activation of InsP3 generation and calcium release from intracellular stores by using ATP, an agonist of endogenous purinergic receptors that are known to be coupled to PLC in these cells. Addition of ATP (100 \(\mu\)mol) to indo-1-loaded Calu-3 and MDCK-hpIgR cells mediated a transient release of calcium ions from the endoplasmic reticulum (Fig. 4D). This response was abolished by pretreatment of cells with U73122, but not by pretreatment with U73343. Taken together, these results indicated that activation of phospholipase C plays an essential role in PspC-mediated increases in cytoplasmic calcium in human epithelial cells.

**Cytosolic Ca^{2+} Regulates plgR-mediated Internalization of Pneumococci by Host Cells—Thapsigargin is a plasma membrane-permeable inhibitor of sarco-/endoplasmic reticulum Ca^{2+} ATPase (37–39). Different types of epithelial cells respond to addition of thapsigargin with increases in cytosolic calcium due to uncompensated leakage of calcium ions from intracellular stores and activation of capacitative calcium influx through the plasma membrane. In MDCK-hpIgR and Calu-3 cells, addition of thapsigargin (250 nM) caused a sustained increase in cytosolic calcium (Fig. 5A).** To test whether such an increase in [Ca^{2+}], affects the rate of internalization of pneumococci by these epithelial cells, Calu-3 and MDCK-hpIgR cells were pretreated for 30 min with thapsigargin prior to pneumococcal infection. The results revealed that pneumococcal internalization by Calu-3 and MDCK-hpIgR cells was significantly reduced in a dose-dependent manner by pretreatment of cells with thapsigargin (Fig. 5B).

To address whether attenuation of intracellular calcium signaling interferes with pneumococcal endocytosis and may result in higher rates of pneumococcal internalization by host cells, BAPTA-AM as a cell-permeable intracellular calcium chelator (40), cell-permeable antagonist 2-APB, or EGTA as agents for chelation of extracellular calcium ions were employed in assays to suppress increases in [Ca^{2+}], upon contact of cells with pneumococci. Treatment of indo-1/AM-loaded epithelial cells with BAPTA-AM (10 \(\mu\)M) during the recovery phase of epithelial cells resulted in a reduction in basal calcium levels as compared with the control (Fig. 6A). In BAPTA-AM-pretreated cells, the rate of pneumococcal internalization was significantly increased, and a dose-dependent effect was measured (Fig. 6B). Similar results were obtained when cells were pretreated with 2-APB. In the presence of 2-APB, a significant increase in the number of internalized pneumococci was observed (Fig. 6C).

The chelation of extracellular calcium by 3 mM EGTA caused a drop in basal levels of free cytosolic calcium and resulted in a shift in the calcium balance across the plasma membrane of both Calu-3 and MDCK-hpIgR cells (Fig. 7A). To investigate the effects of extracellular calcium buffering on internalization of pneumococci, epithelial cells were treated with EGTA 30 min prior to the infection with pneumococci. The antibiotic protection assay indicated that chelation of extracellular calcium by EGTA resulted in a significant increase in pneumococcal uptake by plgR-expressing epithelial cells (Fig. 7B). These results deciphered the key role of intracellular calcium concentrations on pneumococcal internalization by epithelial cells.

**Pneumococcal Adherence to Host Cells Is Not Affected by Ca^{2+}-chelating Inhibitors—**To exclude potential artifacts in the internalization assays by bacteria that adhere to but are not internalized by host epithelial cells, confocal laser scanning microscopy and enumeration of attached bacteria by plating were performed to quantify adherence of pneumococci to both types of host epithelial cells and the effects of inhibitors as well.
No significant differences were observed for pneumococcal adherence to inhibitor-treated cells as compared with untreated epithelial cells (Fig. 8, A and B). To detect any potentially deleterious effects of inhibitors on pneumococcal growth, bacteria were cultured in the presence of inhibitors. There were no significant differences in growth behavior of pneumococci as compared with bacteria grown without inhibitors (data not shown). However, after uptake by host epithelial cells, intracellular pneumococci are killed time-dependently as shown by the antibiotic protection assay. Pneumococcal invasion in the absence of thapsigargin was set to 100%. Pneumococcal uptake by Calu-3 and MDCK-hplgR cells was significantly attenuated by pretreatment of epithelial cells with thapsigargin. Data represent means $\pm$ S.D. ($n = 3$). Means of experiments in the presence of thapsigargin were significantly different from those in the untreated controls ($*, p < 0.001$).

DISCUSSION

*S. pneumoniae* is a highly versatile Gram-positive bacterium that asymptptomatically colonizes the surface of the nasopharynx in humans. Depending on the host susceptibilities, it may cause potentially life-threatening diseases such as septicemia, pneumonia, or meningitis (1). PspC has been identified as a major virulence factor that recognizes directly and in a human-specific manner the ectodomain of the polymeric immunoglobulin receptor (pIgR) (8–11) thereby playing an important role in adherence and invasion of this versatile pathogen. Although a significant knowledge base has been generated delineating the mechanism of pneumococcal pathogenesis along with induced host cell signal cascades, the consequence of this host-pathogen interaction on host cells as well as on pathogen functions is still not fully understood. We have demonstrated previously that pIgR-mediated pneumococcal uptake by host epithelial cells requires the small GTPase Cdc42, phosphatidylinositol 3-kinase (PI3K) and Akt activity (26), as well as activation of Src PTKs, focal adhesion kinase, ERK1/2, and JNK (27). Many of these cellular signaling cascades are directly or indirectly dependent upon transient or sustained elevations in the intracellular calcium concentration (28). However, whether pneumococcal infection of host epithelial cells via PspC-hpIgR interaction requires calcium mobilization has not yet been addressed. In this study, we have assessed the role of calcium during pneumococcal infection of host cells expressing hpIgR.

Calcium is an important intra- and extracellular divalent cation that plays an essential role in wide variety of processes ranging from forming structural elements in bone matrix to intracellular signaling. The modulation of calcium signals is a very important mechanism by which pathogenic bacteria influence host cells. Bacterial toxins such as listeriolysin O, the pore-forming toxin from *L. monocytogenes*, has been identified as a *sine qua non* of calcium mobilization mediating intracellular calcium oscillations (20, 41–43). Moreover, bacteria such as *C. jejuni* and *S. typhimurium* induce, independently of toxins, calcium responses that play a role in cytoskeleton rearrangements thus facilitating their cell association or even internalization.
Increased significantly the release of \([Ca^{2+}]\), from intracellular stores in host cells (Fig. 2). Similarly, PspC protein PspC-SH12 but not the pIgR nonbinding protein PspC-SH3 induced an increase in \([Ca^{2+}]\), to similar levels observed when using bacteria (Fig. 2D). Importantly, these effects on \([Ca^{2+}]\), were not observed during stimulation of wild-type and thus non-hpIgR-expressing MDCK cells when using wild-type pneumococci or the PspC-SH12 protein (Fig. 2E). Taken together, these results demonstrate that the interaction of PspC with hpIgR is critical for the observed intracellular calcium mobilization.

Various studies investigating the mechanism involved in the intracellular pathway of plgR revealed that plgA binding stimulates rabbit-plgR transcytosis, because of phospholipase C activation and increases in intracellular calcium levels (13). However, whether similar processes are also implicated in pneumococcal PspC-hlpgR-mediated adherence and uptake mechanism has not been addressed. Here, we show that the PLC signaling pathway plays a pivotal role in pneumococcal PspC-mediated increase in cytoplasmic calcium. Pretreatment of cells with U71322, an inhibitor of PLC, blocked release of calcium from the intracellular stores of host cells in response to the pneumococcal stimuli or presence of the PspC-SH12 protein fragment (Fig. 4). In contrast, treatment of cells with U73343, an inactive analog of U71322, did not show this blocking effect. Moreover, inhibition of release from \(Ca^{2+}\) stores by Ins(1,4,5)P3 receptors and influx of \(Ca^{2+}\) through store-operated calcium channels by 2-APB treatment significantly reduced the increase in \([Ca^{2+}]\), levels in both Calu-3 and MDCK-hlpGR cells exposed to pneumococci or PspC-SH12 protein fragment (Fig. 3). Our results point toward a negative correlation between the intracellular calcium level and pnu-
moccocal internalization by host cells (Figs. 5–7). Similar to our findings, endocytosis of uropathogenic *Escherichia coli* is enhanced by calcium-chelating chemicals, although release of calcium from intracellular stores blocks uropathogenic *E. coli* internalization by human bladder epithelial cells (44). Contrary to this notion, it has been previously suggested that increased intracellular free Ca\(^{2+}\) concentrations in host cells may be associated with pathogenicity of a number of intracellular bacterial pathogens. Examples for such relationships are *Chlamydia trachomatis* elementary bodies that mobilize Ca\(^{2+}\) upon internalization into lysosomal compartments within host cells (45) or *C. jejuni* that, upon interaction with a membrane receptor protein, induces activation of a number of signal transduction events and the release of Ca\(^{2+}\) from intracellular stores thereby aiding the necessary cytoskeletal rearrangements occurring during bacterial entry (22). Moreover, increased intracellular Ca\(^{2+}\) concentrations seem to be associated with the *S. typhimurium* microfilament-dependent invasion of cultured eukaryotic cells (23, 46). Similarly, purified invasion protein InlB of *L. monocytogenes* was observed to stimulate PLC\(\gamma\) resulting in the release of intracellular Ca\(^{2+}\) via the InsP\(_3\) pathway (21). Whether these examples provide proof for a role of calcium elevations in eukaryotic host cells in facilitating the bacterial invasion process or subsequent steps in the infection process remains open.

The results of our experiments, however, seem to indicate that elevations in [Ca\(^{2+}\)]\(i\) may be part of a protective response of host cells upon contact with bacteria or individual bacterial products. On the contrary, pneumococci are extracellular pathogens and unable to persist inside of eukaryotic cells (Fig. 8).
Therefore, the induction of elevated $[Ca^{2+}]_i$ may also be beneficial for pneumococci as internalization by host cells is prevented. This is indicated by the fact that pretreatment of epithelial cells with a cell-permeable calcium chelator BAPTA-AM (Fig. 6), which blocks the elevation in $[Ca^{2+}]_i$, due to mobilization of $Ca^{2+}$ from the endoplasmic reticulum (40, 47), or the use of EGTA as an extracellular $Ca^{2+}$-chelating agent (Fig. 7), which suppresses elevations in $[Ca^{2+}]_i$, due to dissipation of calcium influx-mediating concentration gradients (22, 47), increased significantly pneumococcal uptake of bacteria by epithelial cells in a dose-dependent manner (Fig. 7). In contrast, pretreatment of cells with thapsigargin, which inhibits the Ca$^{2+}$ ATPase in the endoplasmic reticulum membrane, significantly reduced pIgR-mediated pneumococcal internalization (Fig. 5). These results indicate that decreases in $[Ca^{2+}]_i$ or suppression of physiologically induced calcium elevations appear to be supportive for efficient bacterial internalization of pneumococci, although elevated cytosolic calcium concentrations may prevent bacterial internalization.

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