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Phospholipase A_2 Functions in *Pseudomonas aeruginosa*-Induced Apoptosis

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***Pseudomonas aeruginosa*, a gram-negative, facultative pathogen, causes severe and often even lethal infections in immunocompromised patients, as well as cystic fibrosis patients. We show here that a variety of *P. aeruginosa* strains activate phospholipase A_2 (PLA_2), cultured epithelial cells, and fibroblasts, resulting in increased intracellular and extracellular arachidonic acid release. The use of different PLA_2 inhibitors revealed that *P. aeruginosa*-induced arachidonic acid release is mediated by activation of cytosolic PLA_2 (cPLA2), whereas $iPLA_2$ or $sPLA_2$ do not seem to be involved in the response to *P. aeruginosa*. Likewise, the $cPLA_2$-specific inhibitors MAFP and AACOCF3 prevented apoptosis of cultured epithelial cells upon *P. aeruginosa* infection, whereas inhibitors specific for $iPLA_2$ or $sPLA_2$ were without effect. The physiological significance of these findings is indicated by an inhibition of apoptosis in tracheal epithelial cells upon in vivo infection with *P. aeruginosa*. The data indicate that arachidonic acid generation by activation of $cPLA_2$ during *P. aeruginosa* infection plays an important role in the induction of host cell death.**

Pseudomonas aeruginosa infections are a severe clinical problem, in particular in immunocompromised individuals and in patients with cystic fibrosis (CF) (53). CF patients are highly susceptible to *P. aeruginosa* bacteria, leading to chronic lung infection in the majority of these patients. This lung disease is characterized by persistent pulmonary inflammation that finally results in fibrosis and destruction of lung tissue and respiratory failure. At present, *P. aeruginosa* is the leading cause of mortality in CF patients. Infection of mammalian cells with *P. aeruginosa* results in the release of cytokines (11, 23, 45), internalization of the bacteria (20, 40, 41), and induction of apoptosis of the infected host cells (5, 19–21, 42). We sought here to identify novel mechanisms of *P. aeruginosa*-induced apoptosis. It was previously shown that mice unable to respond with host cell apoptosis to pulmonary infection with *P. aeruginosa* are highly sensitive to the infection, develop sepsis, and die (19). Further, it was demonstrated that *P. aeruginosa*-induced apoptosis is delayed in cells lacking functional cystic fibrosis conductance regulator (5), whose mutations cause cystic fibrosis. Grassmé et al. (19), Jendrossek et al. (25), and Cannon et al. (5) demonstrated an upregulation of the CD95-CD95-ligand system on the surface of infected cells that resulted in apoptosis of *P. aeruginosa*-infected cells by ligation of the endogenous CD95 receptor. Genetic deficiency of CD95 prevented *P. aeruginosa*-induced apoptosis (19, 25), suggesting a central role of the CD95-CD95-ligand system for *P. aeruginosa*-initiated cell death. Further, an involvement of mitochondria (in particular mitochondrial depolarization, synthesis of reactive oxygen intermediates, and release of cytochrome *c*), an activation of Jun N-terminal kinases (JNK), and a stimulation of caspases were demonstrated to be involved in the induction of apoptosis in mammalian cells upon infection with *P. aeruginosa* (19, 25, 26).

In the present study we tested a potential role of phospholipases A_2 (PLA_2) in *P. aeruginosa*-triggered cell death. PLA_2 are lipolytic enzymes that hydrolyze membrane phospholipids and thereby release fatty acids, particularly arachidonic acid from the *sn-2* position of glycerophospholipids (13). PLA_2 plays an important role in signal transduction, in particular by generation of proinflammatory mediators as prostaglandins and leukotrienes and by membrane remodeling. Several subtypes of mammalian PLA_2 have been described that are divided in four main groups according to their function, localization, and calcium dependency. Secretory PLA_2 (low molecular mass enzymes belonging to groups I, II, III, V, and X) are cysteine-rich, secreted proteins that require millimolar concentrations of Ca^{2+} for activity without a preference for a specific fatty acid in the sn-2 position of the phospholipid substrate (13). The second class of PLA_2 includes specific acetylhydrolases such as platelet-activating factor. A third class is composed of Ca^{2+}-independent PLA_2, e.g., $iPLA_2$ isolated from myocardium (55), CHO cells, and macrophages (1, 28). The group IV cytosolic PLA_2 ($cPLA_2$) includes three PLA_2 named α, β, and γ. $cPLA_{2\alpha}$, an 85-kDa protein, requires micromolar Ca^{2+} concentrations for activity and has a preference for arachidonic acid (8). The recently described $cPLA_{2\beta}$, a 110-kDa protein, shows 30% sequence identity with $cPLA_{2\alpha}$ and also depends on Ca^{2+} but is less selective for cleavage at the sn-2 position than $cPLA_{2\alpha}$ (39, 49). $cPLA_{2\gamma}$ with a molecular mass of 61 kDa and 29% sequence identity with $cPLA_{2\alpha}$ is Ca^{2+} independent but distinguishable from $iPLA_2$ by its preference for arachidonic acid at the sn-2 position (3). Previous studies implied PLA_2 in the host response to such diverse pathogens as *Staphylococcus aureus*, *Escherichia coli*, *Aeromonas hydrophila*, *Pasteurella haemolytica*, and *Bacillus anthracis* (6, 9, 16, 18, 24, 44, 54). PLA_2 seem to have a dual function in the infection of mammalian cells by pathogens, since many

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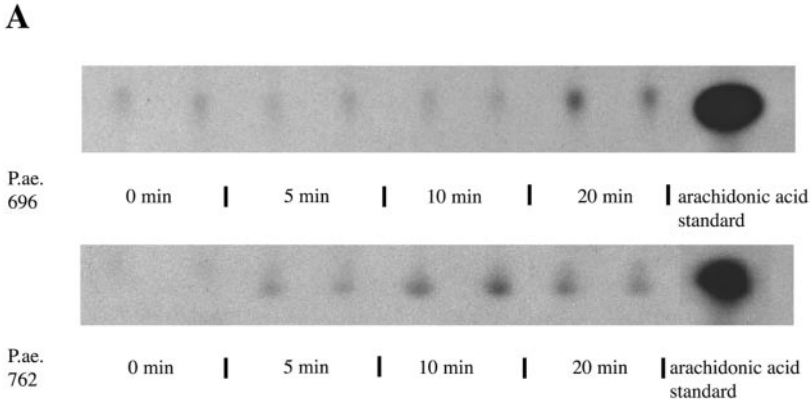

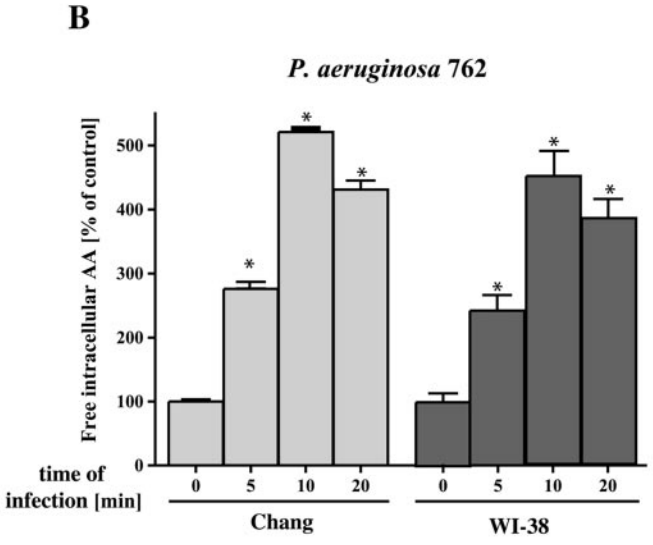

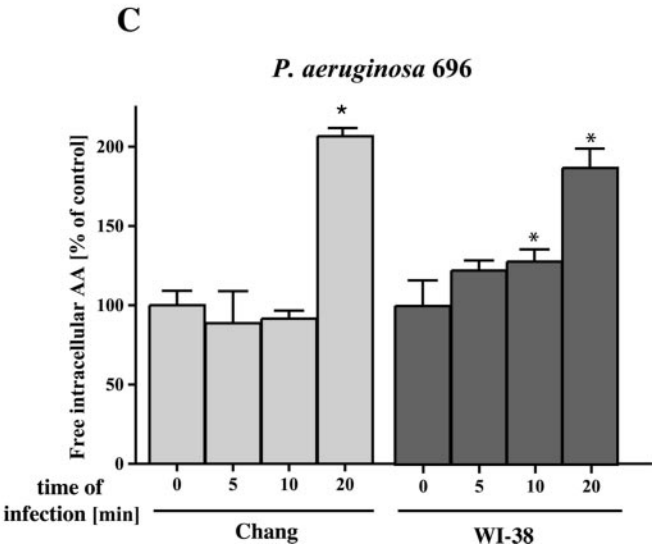

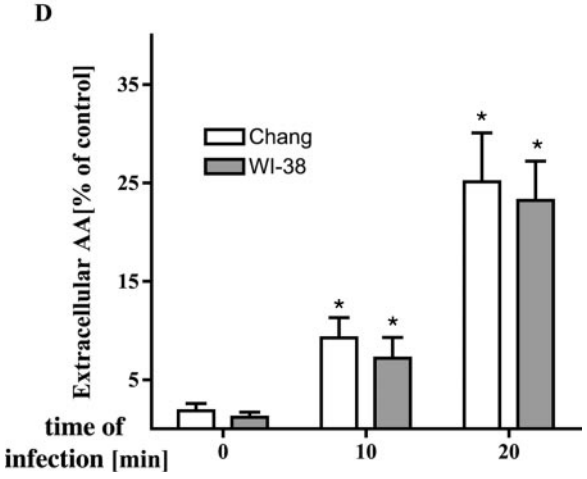

FIG. 1. *P. aeruginosa* induces intra- and extracellular arachidonic acid release. (A) *P. aeruginosa* (P.ae.) 762 or 696 infection induces rapid intracellular arachidonic acid (AA) release as shown by extraction of free fatty acids, followed by TLC separation of [^{3}H]arachidonic acid-labeled Chang conjunctiva epithelial cells. Cells were infected 5, 10, and 20 min with the bacteria or left uninfected and then harvested, and intracellular arachidonic acid was quantified as described above. *P. aeruginosa* induced a rapid increase in intracellular arachidonic acid release with a maximum at 10 min for *P. aeruginosa* strain 762 and at 20 min for *P. aeruginosa* strain 696. The data were determined in duplicate and are representative for three independent experiments. (B and C) Quantification of intracellular arachidonic acid release was performed by removing the arachidonic acid spots from the TLC plates, followed by liquid scintillation counting. The data refer to the percentages of untreated control and represent means ± the standard deviations (SD) of three independent experiments. (D) Release of extracellular arachidonic acid release 0, 10, and 20 min after *P. aeruginosa* 762 infection of Chang epithelial cells and WI-38 fibroblasts. Cells were labeled with [^{3}H]arachidonic acid overnight and infected, and the radioactivity released into the supernatant was quantified by liquid scintillation counting. The data are normalized to total incorporated arachidonic acid and represent means ± the SD of at least four independent experiments. ❋, Significant differences compared to the control ($P < 0.05$, *t* test for unpaired samples).

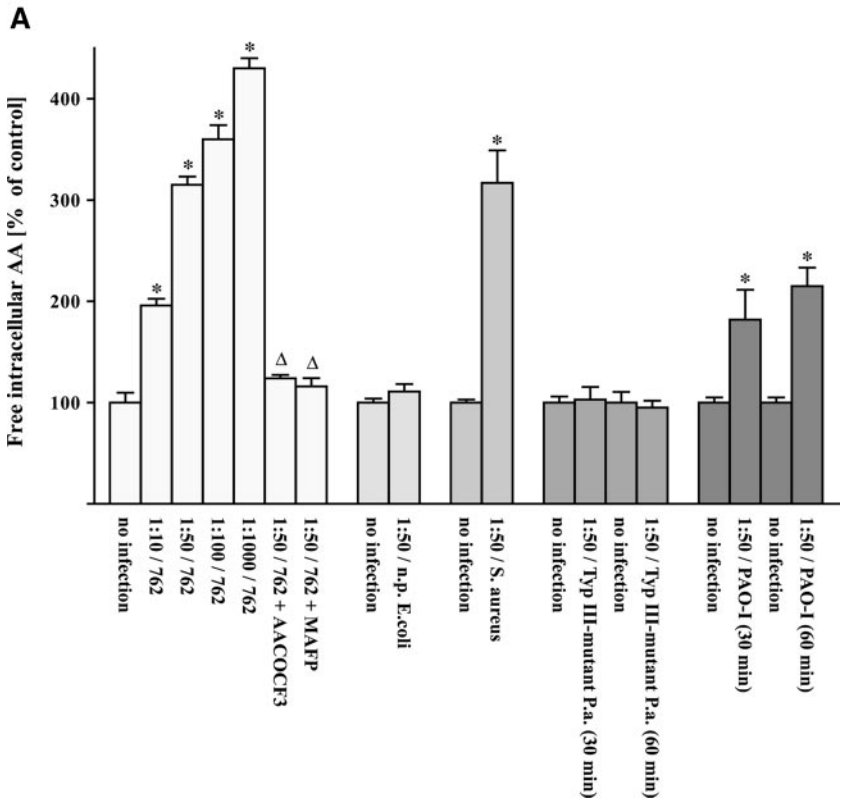

FIG. 2. Infection of human cells with different *P. aeruginosa* strains results in arachidonic release prevented by $cPLA_2$ inhibitors. Chang cells were prelabeled with [^{3}H]arachidonic acid and infected with *P. aeruginosa* 762 for 20 min, *P. aeruginosa* 769 or ATCC 27853 for 45 min, and *P. aeruginosa* 696 or PAO-1 or a type III-deficient *P. aeruginosa* mutant for 60 min, respectively. In addition, the cells were infected with a nonpathogenic *E. coli* strain for 120 min or *S. aureus* strain 8325 for 60 min. Cells were infected at the indicated MOIs, and the release of intracellular (A and B) and extracellular (C) arachidonic acid was determined. The effect of the $cPLA_2$ inhibitors MAFP or AACOCF3 was determined for each *P. aeruginosa* strain at an MOI of one cell to 50 bacteria. Displayed are the means ± the SD of each of three independent experiments. ✻, Significant differences between infected samples and noninfected controls and significant differences between infected cells treated with inhibitor or left untreated, respectively, are labeled with a delta ($P < 0.05$, *t* test for unpaired samples).

bacterial toxins seem to kill mammalian cells via PLA_2 (4, 12, 16, 18, 27), whereas expression of PLA_2 was shown to protect mice from acute infections at least with *S. aureus*, *P. aeruginosa*, and *E. coli* (15, 33). Thus, the exact function of PLA_2 in infectious processes requires definition.

In the present study, we investigated the role of PLA_2 for the in vitro and in vivo infection of cultured epithelial cells or fibroblasts, respectively, as well as tracheal epithelial cells with *P. aeruginosa*. We demonstrate an activation of PLA_2 by a variety of *P. aeruginosa* strains and reveal by the use of a panel of PLA_2 inhibitors a significant role of $cPLA_2$ for the induction of host cell apoptosis by *P. aeruginosa* both in vitro and in vivo.

MATERIALS AND METHODS

Materials and cell culture. The human conjunctiva epithelial cell line Chang (ATCC CCL 20.2) was cultured in RPMI 1640 (Gibco-BRL/Life Technologies) supplemented with 5% fetal calf serum (FCS) at 37°C as monolayers in tissue culture flasks in 5% CO_2 atmosphere. The human lung fibroblast cell line WI-38 was maintained in minimal essential medium supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acids, and 1% penicillin-streptomycin. Infections were performed in RPMI 1640 supplemented with 10 mM HEPES (pH 7.2) to avoid interactions of serum proteins with the bacteria. Prior to infections, FCS and antibiotics were omitted from the culture media. The PLA_2 inhibitors MAFP, BEL, 12-Episcalaradial, and AACOCF3 were purchased from Biomol.

Bacterial strains. Three clinical isolates and two laboratory strains of *P. aeruginosa* were used. The isolate 762 was originally obtained from an urinary tract infection, strain 696 was isolated from the sputum of a hospitalized patient, and strain 769 was from a patient with urosepsis (18). The laboratory strains used were *P. aeruginosa* ATCC 27853 and PAO-1. Furthermore, we infected cells with a previously described (26) *P. aeruginosa* strain deficient for the type III secretion system (*P. aeruginosa* PAK *pcrD*::Smr, kindly provided by J. Heesemann), *S. aureus* strain ATCC 8325, and a nonpathogenic *E. coli* isolate.

Infection experiments. Bacteria originating from glycerol stock cultures were plated overnight on tryptic soy agar plates at 37°C, resuspended in tryptic soy broth (TSB) at an optical density at 550 nm of 0.25, shaken at 120 rpm for 1 h at 37°C, and harvested during logarithmic growth phase by pelleting and resuspension in fresh TSB. Prior to infection, cells were washed twice in RPMI 1640 (Chang cells) or minimal essential medium (WI-38 cells) and maintained in the same medium during infection. Infection was performed by inoculating subconfluent cell layers at a host cell/bacterium ratio of 1:1,000, 1:100, 1:50, or 1:10. Synchronous infection conditions and an enhanced bacterium-host cell interaction were achieved by a 2-min centrifugation ($35 \times g$) of the bacteria onto the cells. The end of the centrifugation step was defined as the starting point of all infections.

Arachidonic acid release assays. Chang cells (0.4×10^5 per well) or WI-38 cells (0.3×10^5 per well) were labeled for 18 to 20 h with 0.05 μCi of [^{3}H]arachidonic acid [5,6,8,9,11,12,14,15-^{3}H(N)] (0.1 mCi/ml stock; New England Nuclear)/ml. Prior to infection, cells ware washed three times with a buffer containing 132 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, and 0.8 mM $MgSO_4$ (H/S) supplemented with 1% bovine serum albumin and 10 mM glucose and incubated in the same buffer during infection. Infection was performed as described above at the indicated multiplicity of infection (MOI).

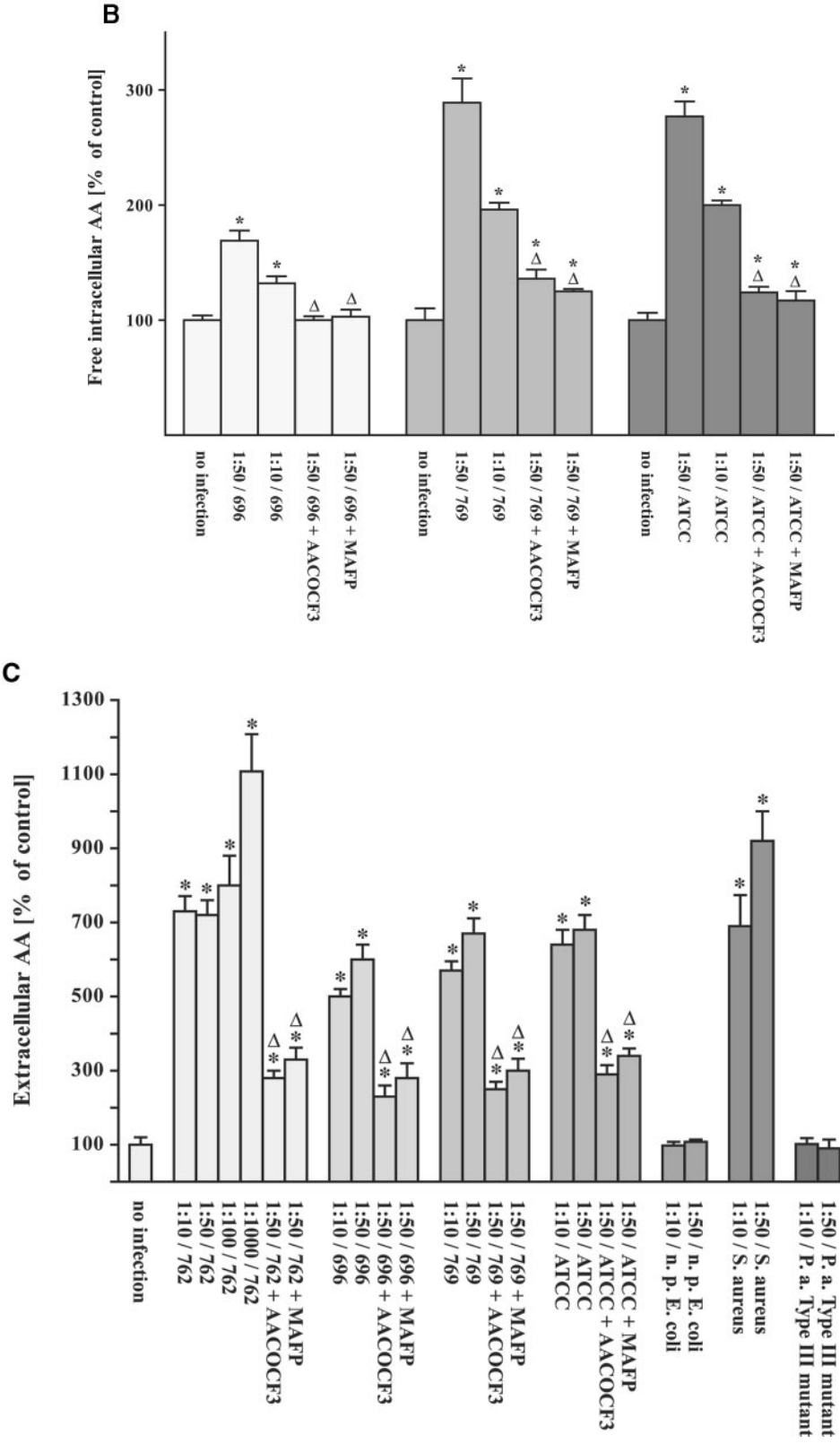

FIG. 2—*Continued.*

Cells were preincubated with the PLA_2 inhibitors MAFP, 12-episcalaradial, and bromoenol lactone (BEL) at the indicated concentrations for 15 min prior to infection. The slow-acting inhibitor AACOCF3 was added to the cells 3 h prior to infection.

Extracellular arachidonic acid release. To determine the release of arachidonic acid into the extracellular space, supernatants were collected after the indicated time of infection and centrifuged at 2,000 rpm for 5 min in a microcentrifuge to remove any cells that may have detached. The supernatants were quantified by liquid scintillation counting. The remaining cell-bound radioactivity was determined by adding 0.5 ml of 0.1% Triton X-100 in H_2O to the cells, and lysed cells were removed from the plates and subjected to liquid scintillation counting. Extracellular arachidonic acid release was calculated as the percentage of radioactivity released into the supernatant of the total radioactivity (the latter being the sum of released and cell-bound radioactivity). Since only free arachidonic acid is released into the supernatant, the measurement of radioactivity in the supernatant is an accurate measurement for the formation of extracellular arachidonic acid.

Intracellular arachidonic acid release. Chang cells were seeded at a density of 3×10^5 cells/well in six-well plates. Labeling with [^{3}H]arachidonic acid, pretreatment with PLA_2 inhibitors, and infection were performed as described above. Infections were terminated by removal of the supernatants, washing, and the addition of 1 ml of methanol–15% acetic acid to each well. Cells were scraped off the plates, and wells were washed with 0.5 ml of methanol. Chloroform (0.75 ml) was added, and samples were extracted by incubation for 30 min at room temperature. Next, 0.75 ml each of chloroform and H_2O were added, and the phases were separated by low-speed centrifugation for 10 min. The lower phase containing the fatty acids was removed, dried, solubilized in 30 μl of petrol ether-diethyl ether (96:1), spotted onto a silica gel 60 plate, and separated by thin-layer chromatography (TLC) in a solvent system consisting of ethyl acetate–glacial acetic acid–2,2,4-trimethyl pentane–H_2O (45:10:25:50, by volume). An arachidonic acid standard was run on each plate to confirm migration of free arachidonic acid. After separation, TLC plates were air dried and a Biomax MS film was exposed at −80°C for several days. To quantify free arachidonic acid, spots representing arachidonic acid were scraped off the plates and subjected to liquid scintillation counting. The TLC analysis permits separation of free arachidonic acid from arachidonic acid incorporated into lipids and, therefore, the analysis of free intracellular arachidonic acid.

Detection and quantification of apoptosis. Apoptosis was quantified by fluorescein isothiocyanate (FITC)-annexin V staining and confirmed by trypan blue staining. To this end, cells were infected as described above, treated with trypsin, washed twice in 10 mM HEPES (pH 7.4), 140 mM $NaCl_2$, and 5 mM $CaCl_2$ and resuspended in the same buffer supplemented with FITC-annexin V (dilution 1:50; Roche Biochemicals, Mannheim, Germany). After incubation for 15 min cells were subjected to fluorescence-activated cell sorter (FACS) analysis using a FACSCalibur flow cytometer using the CELLQuest software (Becton Dickinson, Mountain View, Calif.).

In vivo infections. To perform infection of tracheal epithelial cells in vivo, C57BL/6 wild-type mice were anesthetized by intraperitoneal injection of avertin, the trachea was carefully isolated, and the distal part was catheterized and ligated immediately proximal of the catheter. We then injected MAFP, AACOCF3, or buffer into the proximal part of the trachea. After an incubation period of 15 min for MAFP, we injected 10^8 CFU of *P. aeruginosa* 762 into the proximal trachea. Mice were killed after 45 min, and the tracheas were rapidly excised and fixed for 30 min in 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.2). Epithelial cells were isolated by disintegration of the trachea by scratching with a 27-gauge needle and a 5-min digestion in trypsin. Cells were washed and stained with FITC-annexin V to detect apoptosis. Apoptosis of epithelial cells in the trachea was also determined by TUNEL staining. To this end, the trachea was excised and fixed in 4% PFA in PBS (pH 7.2) for 36 h. The tissues were embedded in paraffin, and sections were deparaffinized and treated for 5 min in 0.1 M citrate (pH 6.0) with 350-W microwave irradiation for 5 min. The sections were then stained for 30 min at 37°C with FITC-coupled dUTP in the presence of terminal deoxynucleotidyl transferase. The samples were washed, incubated at 70°C for 10 min to reduce unspecific binding, and stained with alkaline phosphatase-coupled anti-FITC-antibodies. The slides were finally developed using Fast Red Tablets (Roche) as a substrate resulting in the formation of a red dye.

RESULTS

***P. aeruginosa* induces extra- and intracellular arachidonic acid release upon infection of epithelial cells.** To gain insight into the interactions of *P. aeruginosa* with epithelial cells, we investigated the role of PLA_2 upon bacterial infection. To this end, we measured *P. aeruginosa*-induced PLA_2 activity by quantification of the extracellular and intracellular arachidonic acid release from two different cell lines, i.e., Chang conjunctiva epithelial cells and WI-38, a lung fibroblast cell line. Intracellular arachidonic acid release was determined by prelabeling cells with [^{3}H]arachidonic acid, extraction of fatty acids, and subsequent separation by TLC. Extracellular arachidonic acid release was determined by labeling cells with [^{3}H]arachidonic acid and quantification of released radioactivity in the supernatant by liquid scintillation counting. The latter data were related to the total radioactivity recovered from the samples. The results revealed a marked increase of free, intra- and extracellular arachidonic acid after bacterial infection with the *P. aeruginosa* strains 696, 762, 769, ATCC 27853, and PAO-1 (Fig. 1 and Fig. 2).

The release of arachidonic acid occurred very rapidly after infection with *P. aeruginosa* (Fig. 1) and was dependent on the MOI used in the infection experiments (Fig. 2). However, even at the lowest MOI of 10 bacteria/cell, we were still able to detect some release of arachidonic acid (Fig. 2). A type III mutant of *P. aeruginosa* did not induce a release of intra- or extracellular arachidonic acid (Fig. 2A).

The release of arachidonic acid was not restricted to *P. aeruginosa* and was also observed upon infection with a different pathogen, i.e., *S. aureus* (Fig. 2A). In contrast, nonpathogenic *E. coli* were unable to trigger a significant release of arachidonic acid (Fig. 2A).

Arachidonic acid release induced by *P. aeruginosa* infection is diminished by $cPLA_2$ inhibitors but not by $sPLA_2$ or $iPLA_2$ inhibitors. Next, we aimed to further characterize the PLA_2 subclass involved in *P. aeruginosa*-induced arachidonic acid release. To this end, we investigated the influence of various PLA_2 inhibitors on arachidonic acid release into the supernatant and on cell-bound arachidonic acid release. Two inhibitors (MAFP, AACOCF3) specific for both $cPLA_2$ and $iPLA_2$ were used, as well as the $sPLA_2$-specific inhibitor 12-Episcalaradial and the $iPLA_2$-specific inhibitor BEL. The results reveal that MAFP and AACOCF3 significantly reduced intra- and extracellular arachidonic acid release upon infection with *P. aeruginosa* strains 696, 762, 769, and ATCC 27853 (Fig. 2). A dose-response analysis revealed that doses of 1 μM MAFP or 2 μM AACOCF3, respectively, were already sufficient to reduce arachidonic acid release (Fig. 3A and B). In contrast, neither the $iPLA_2$ inhibitor BEL nor the $sPLA_2$ inhibitor 12-episcalaradial affected *P. aeruginosa*-induced arachidonic acid release upon infection of WI-38 or Chang cells (Fig. 3C and D).

Since MAFP specifically blocks $cPLA_2$ and $iPLA_2$, whereas BEL specifically targets $iPLA_2$ and 12-Episcalaradial inhibits type II $sPLA_2$, the data suggest that $cPLA_2$ is activated by *P. aeruginosa*.

The $cPLA_2$ inhibitors MAFP and AACOCF3 reduce *P. aeruginosa*-induced apoptosis. To gain insight into the functional significance of arachidonic acid release, we investigated the role of PLA_2 for the induction of apoptosis during *P. aeruginosa* infection. *P. aeruginosa*-induced apoptosis of epithelial cells has been previously demonstrated (5, 19, 20) and seems to be critical for the defense of the host against acute infections with these bacteria (19). First signs of apoptosis as

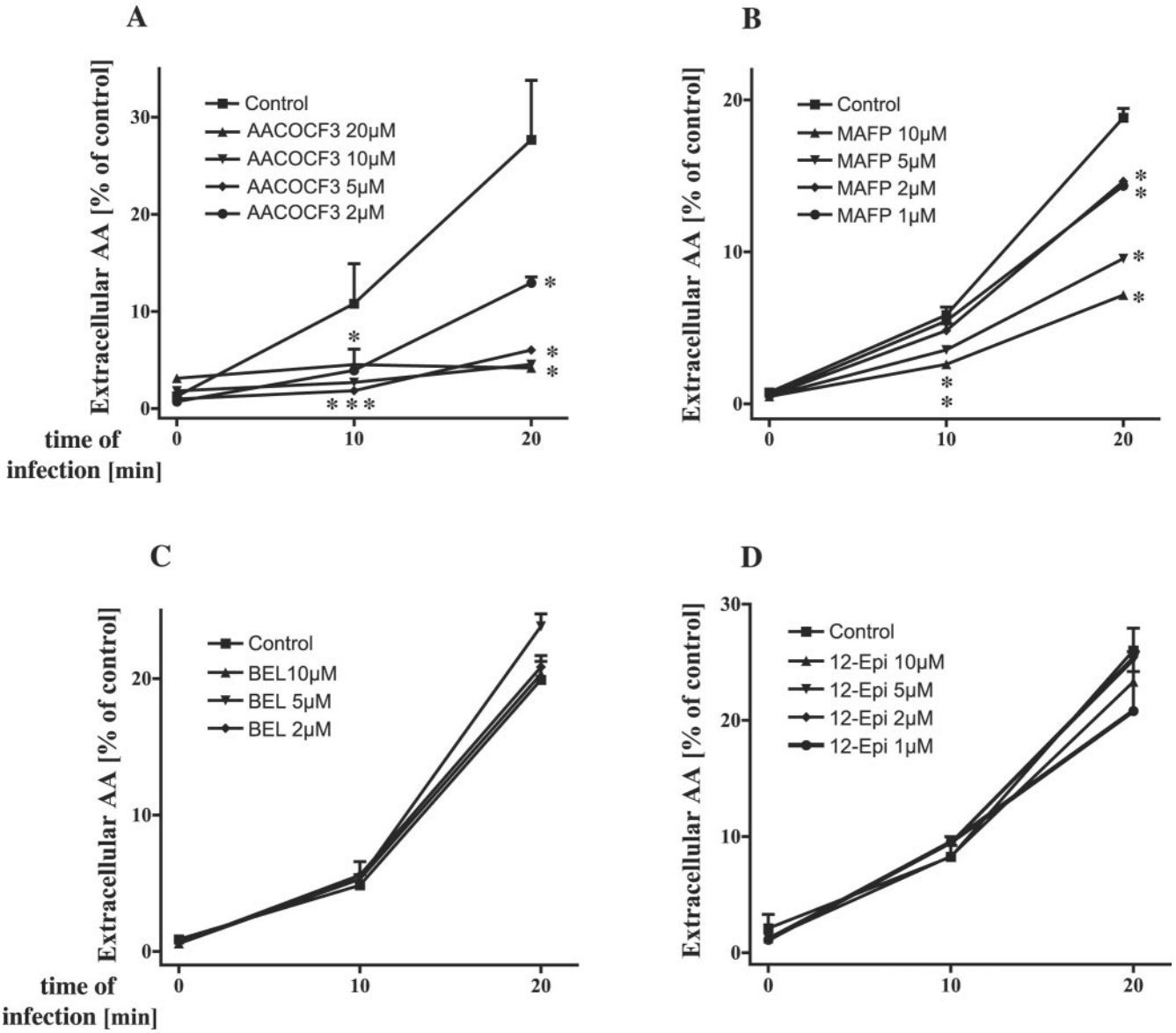

FIG. 3. Influence of various PLA$_2$ inhibitors on *P. aeruginosa*-induced arachidonic acid release. Preincubation of WI-38 lung fibroblasts with cPLA$_2$ inhibitors AACOCF3 (A) and MAFP (B) prevents the release of extracellular arachidonic acid in a dose-dependent way, whereas the iPLA$_2$ inhibitor BEL (C) or sPLA$_2$ inhibitor 12-Episcalaradial (D) was without effect. The data are means ± the SD of duplicate samples and representative of three experiments. Significant differences between inhibitor-treated and -untreated values are indicated by asterisks ($P < 0.05$, *t* test for unpaired samples).

determined by breakdown of phosphatidylserine asymmetry in the cell membrane were detected within 15 to 30 min after infection with *P. aeruginosa* 762. MAFP and AACOCF3 dose dependently inhibited *P. aeruginosa*-induced apoptosis (Fig. 4A and B), whereas pretreatment with 12-Episcalaradial (sPLA$_2$ inhibitor) (Fig. 4C) was without effect on apoptosis induction. Inhibition of apoptosis by MAFP and AACOCF3, respectively, was not restricted to *P. aeruginosa* strain 762 and also observed for the *P. aeruginosa* strains 696, 769, ATCC 27853, and PAO-1 (Fig. 4D), as well as the *S. aureus* strain ATCC 8325 (not shown).

To test the in vivo role of PLA$_2$ activation for *P. aeruginosa*-induced apoptosis, we used an in vivo infection model. To this end, we applied the PLA$_2$ inhibitor MAFP into the trachea in vivo, followed by local infection with *P. aeruginosa* strains 762 or ATCC 27853. The results reveal an induction of apoptosis in respiratory epithelial cells in vivo upon infection with *P. aeruginosa*, which was prevented by inhibition of cPLA$_2$ (Fig. 5A and B).

DISCUSSION

In the present study we investigated the role of PLA$_2$ in *P. aeruginosa* infection and its significance in *P. aeruginosa*-induced epithelial cell death. We show that several *P. aeruginosa* strains, i.e., the clinical isolates *P. aeruginosa* 762, 696, and 769,

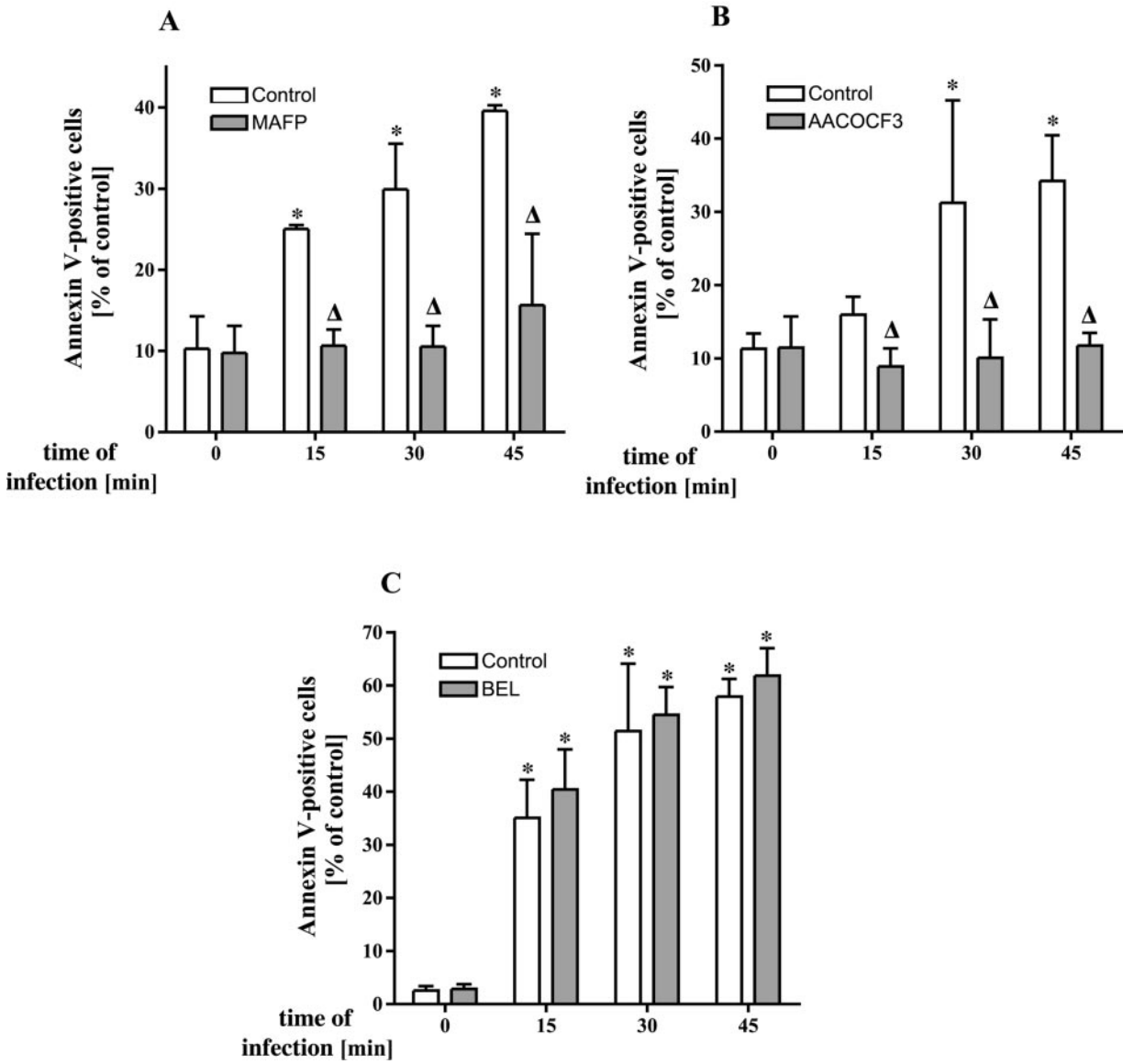

FIG. 4. $cPLA_2$ and $iPLA_2$ inhibitors prevent *P. aeruginosa*-induced apoptosis. *P. aeruingosa* strain 762-induced apoptosis is abrogated by preincubation of Chang epithelial cells with the $cPLA_2$ inhibitors MAFP (A) or AACOCF3 (B) as determined by FITC-annexin staining, but not with the $sPLA_2$-specific inhibitor 12-Episcalaradial (C). Cells were infected with *P. aeruginosa* 762 for the indicated times, stained with FITC-labeled annexin V, and subjected to flow cytometry analysis. (D) Inhibition of PLA_2 with MAFP or AACOCF3 also prevents apoptosis triggered by other *P. aeruginosa* strains 696, 769, ATCC 27853, and PAO-1. Cells were infected at an MOI of 1:100 with the strains 696 and PAO-1 for 6 h, with 769 and ATCC 27853 for 90 min. Apoptosis was quantified by FITC-annexin V binding and FACS analysis. Displayed are means ± the SD of two to four independent experiments. Significant differences between infected samples and noninfected controls are indicated by asterisks, and significant differences between infected cells treated with inhibitor or left untreated are indicated by a delta ($P < 0.05$, *t* test for unpaired samples).

as well as the laboratory strains *P. aeruginosa* ATCC 27853 and PAO-1, activate $cPLA_2$, release arachidonic acid, and employ this pathway to induce apoptosis of host cells. The use of various subtype-specific inhibitors indicates an activation of $cPLA_2$ by *P. aeruginosa*-mediating the release of arachidonic acid. Our data further show that $cPLA_2$ activation plays a significant role in *P. aeruginosa* apoptosis in vitro as well as in vivo, since $cPLA_2$ inhibitors reduce *P. aeruginosa*-induced cell death. A *P. aeruginosa* mutant that lacked the type III secretion system failed to stimulate PLA_2 and to induce apoptosis. This is consistent with previous findings that demonstrated the failure of a *P. aeruginosa* type III mutant to induce significant apoptosis of epithelial cells (26).

At present the signals mediating the activation of $cPLA_2$ upon infection with *P. aeruginosa* are unknown. We have previously shown that *P. aeruginosa* triggers an activation of the acid sphingomyelinase and the formation of ceramide (19). Ceramide forms membrane platforms that serve to reorganize receptor molecules involved in internalization of the bacteria, as well as the induction of apoptosis (19). However, it is possible that ceramide does not only triggers the reorganization of receptor molecules in ceramide-enriched membrane domains

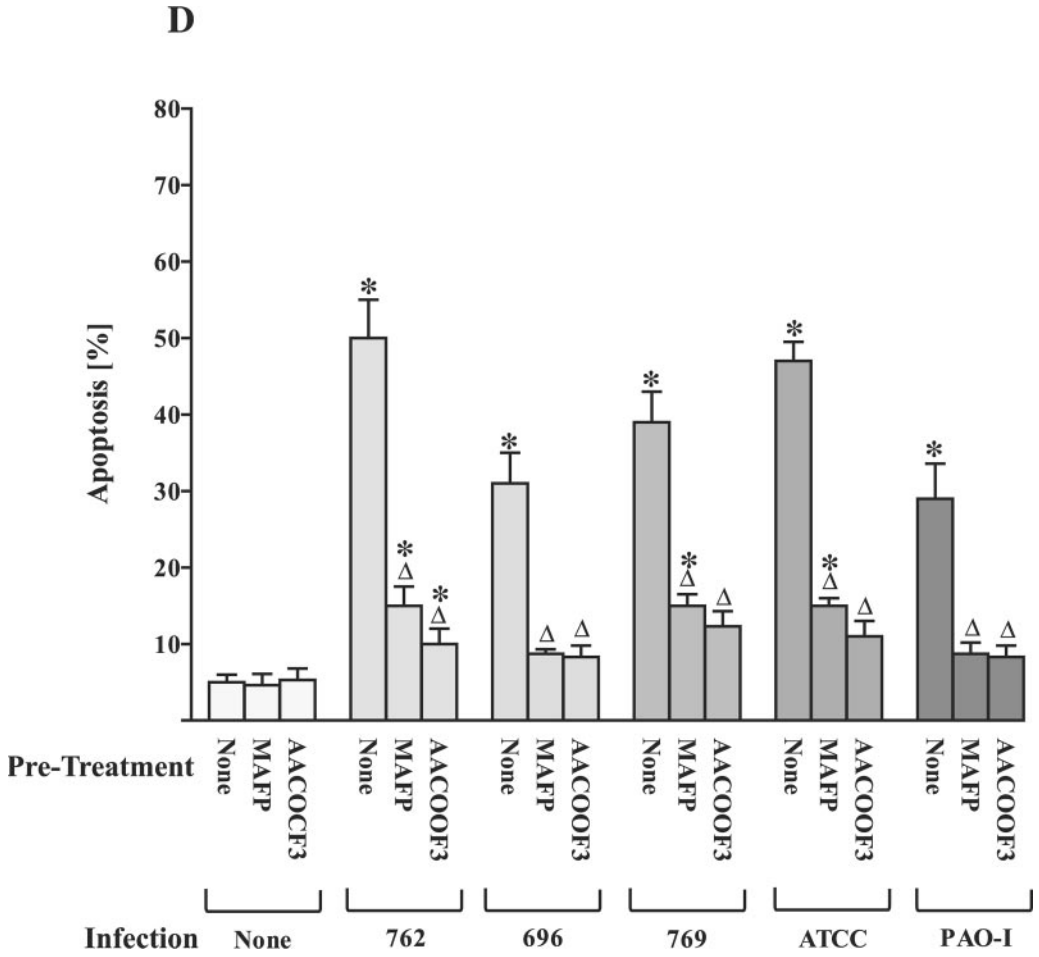

FIG. 4—*Continued.*

upon infection but also mediates a stimulation of PLA$_2$ (22, 31). In this context it is interesting that ceramide has been shown to bind to the CaLB domain of PLA$_2$ and facilitates membrane docking of and lipid hydrolysis by PLA$_2$ (22, 31). Thus, ceramide might be involved in the spatial reorganization of PLA$_2$ required for the function of this enzyme. In addition, a recent study demonstrated an involvement of ceramide-kinase in the activation of PLA$_2$ by ceramide (36, 37). Since ceramide-1-phosphate was shown to be a direct activator of PLA$_2$ (36, 37), the metabolism of ceramide to ceramide-1-phosphate after infection with *P. aeruginosa* may also contribute to PLA$_2$ activation. Further, we have previously shown that infection of human and murine epithelial cells and fibroblasts with *P. aeruginosa* results in the induction of apoptosis by upregulation of the endogenous CD95 receptor/CD95 ligand system (5, 19). The critical role of the CD95/CD95 ligand system for induction of apoptosis was demonstrated by the use of CD95- and CD95-ligand-deficient cells. These cells were resistant to *P. aeruginosa*-induced cell death (19, 25). Recent studies from Cannon et al. confirmed the CD95-dependent induction of apoptosis in mammalian cells by *P. aeruginosa* (5). CD95 might also mediate the activation of PLA$_2$ upon infection with *P. aeruginosa*, although the activation of PLA$_2$ by CD95 and the role of this enzyme for CD95-triggered death seem to be controversial. For instance, studies of Ulisse et al. (51) and Cifone et al. (7) demonstrated an activation of PLA$_2$ by CD95 in Sertoli and HuT78 cells that was mediated by a signaling pathway involving extracellular regulated kinases. Activation of PLA$_2$ activity was required, at least in part, for the induction of CD95-induced cell death in these cells. On the other hand, it was shown by Luschen et al. (32), Enari et al. (17), de Valck et al. (14), and Atsumi et al. (2) that an activation of PLA$_2$ is not required for CD95-triggered apoptosis and cPLA$_2$ seems to be even cleaved by proteolysis after CD95 stimulation.

Next, it is also possible that *P. aeruginosa* triggers an activation of PLA$_2$ independent of the acid sphingomyelinase, ceramide, and the CD95 receptor/CD95 ligand system. In this respect, it is interesting that activation of PLA$_2$ by *P. aeruginosa* required a functional type III secretion system. Although the exact nature of proapoptotic *P. aeruginosa* factors that are delivered into infected cells and mediate apoptosis is unknown, it might be possible that bacterial factors directly or indirectly activate PLA$_2$.

Finally, *P. aeruginosa* expresses enzymes that display PLA$_2$ activity and the observed PLA$_2$ activity could be caused by an activation of a bacterial PLA$_2$ (43, 44). In particular, it was shown that *P. aeruginosa* exotoxin U exhibits a PLA$_2$ activity that is activated by still unknown mammalian cell factors (38,

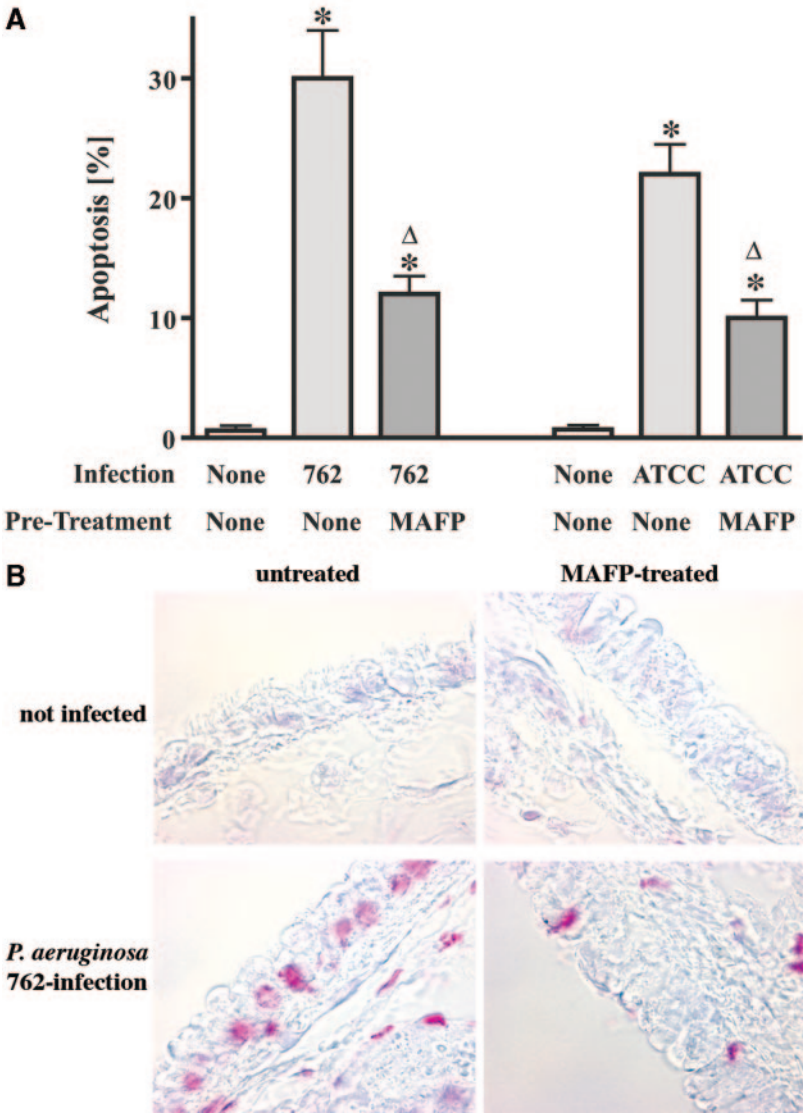

FIG. 5. Inhibition of PLA_2 in vivo prevents apoptosis of tracheal epithelial cells after *P. aeruginosa* infection. The PLA_2 inhibitor MAFP was locally applied into the trachea in vivo. The trachea was infected with *P. aeruginosa* strain 762 or ATCC 27853, respectively, 15 min after application of the inhibitor. Apoptosis was determined by TUNEL and quantified by counting TUNEL-positive cells. Apoptosis was confirmed by staining with FITC-annexin V after disintegration of the trachea. (A and B) Shown are the means ± the SD of three independent experiments (A) and a typical result of the TUNEL staining (B). Asterisks indicate significant differences between control and infected tracheae, and significant differences between infected samples treated with MAFP or left untreated are indicated by a delta ($P < 0.05$, *t* test for unpaired samples).

49). Moreover, the PLA_2 activity of *P. aeruginosa* exotoxin U was inhibited by the $cPLA_2$ inhibitor MAFP and, accordingly, MAFP also prevented exotoxin U-induced cell death (38). However, we used doses of MAFP of 10 μM, while inhibition of *P. aeruginosa* exotoxin U by MAFP required concentrations of the inhibitor up to 1.35 mM and prolonged incubation of the enzyme with the inhibitor up to 16 h (38). Therefore, it seems to be unlikely that the observed effects of MAFP are due to an inhibition of the bacterial protein, and our data strongly suggest that a cellular PLA_2 is activated by *P. aeruginosa* to mediate the release of arachidonic acid.

P. aeruginosa-induced PLA_2 might be involved in apoptosis by the activation of several proapoptotic pathways. It was demonstrated that arachidonic acid, but not products of the lipoxygenase and the cyclooxygenase pathway, induces a depolarization of mitochondria, a typical event of mitochondrial alterations during apoptosis (29, 30, 34, 35, 46, 50, 52). Furthermore, it was suggested that PLA_2 induces the formation of proapoptotic reactive oxygen intermediates and an activation of sphingomyelinases with a concomitant release of ceramide and induction of apoptosis (10, 56). Finally, a recent study demonstrated a change of the nuclear architecture by PLA_2 in the course of apoptosis (47). However, the molecular details of arachidonic acid-induced apoptosis are still unknown.

The present data suggest PLA_2 and arachidonic acid as novel components of the death machinery in the host cell and the pulmonary host response upon *P. aeruginosa* infection. Our studies show that *P. aeruginosa* infection activates $cPLA_2$, re-

sulting in arachidonic acid release. Pharmacological inhibition of PLA$_2$ prevents *P. aeruginosa*-induced apoptosis, suggesting an important role of PLA$_2$ in the biological response of the lung to infections with *P. aeruginosa*.

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