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Suppression of T-Lymphocyte Activation and Chemotaxis by the Adenylate Cyclase Toxin of *Bordetella pertussis*▽

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The adenylate cyclase toxin (CyaA) released by *Bordetella pertussis* is an essential virulence factor for colonization of the host. This toxin inhibits migration and activation of phagocytes, thereby preventing bacterial killing. In addition, CyaA interferes with the initiation of adaptive immunity by misdirecting dendritic cell differentiation to a suppressive rather than stimulatory phenotype. Here we show that CyaA directly affects adaptive responses by catalyzing cyclic AMP (cAMP) production in peripheral blood lymphocytes. Treatment with CyaA resulted in profound impairment of T-lymphocyte activation and chemotaxis. These effects resulted from inhibition of T-cell antigen receptor and chemokine receptor signaling via a cAMP/protein kinase A (PKA)-dependent pathway. A comparison of the activities of CyaA on T-cell and macrophage activation and migration revealed that the biological effects of the toxin were paralleled by inhibition of the activation of mitogen-activated protein (MAP) kinases, highlighting the conclusion that the ubiquitous and evolutionarily conserved MAP kinase modules are common targets of the PKA-mediated immunosuppressant activities of CyaA and underlining the potential of cAMP-elevating toxins as a means of evasion of immunity by bacterial pathogens.

Bordetella pertussis is a small, nonmotile, gram-negative bacillus which causes whooping cough in humans. *B. pertussis* establishes an infection through the respiratory route and remains generally localized in the upper respiratory tract, causing respiratory disease (9, 30). After aerosol infection, the bacteria adhere to the nasopharyngeal mucosa mainly via the filamentous hemagglutinin protein and fimbriae. They then proliferate and spread to the respiratory tract, where they release a toxin which causes loss of cilia from the bronchial columnar epithelium and cell damage. These microorganisms also produce two cyclic AMP (cAMP)-elevating toxins, pertussis toxin and adenylate cyclase toxin (CyaA), which are essential virulence factors for bacterial colonization of the airways and development of whooping cough (9, 30).

CyaA is composed of a single large polypeptide chain. The N-terminal part of the protein contains the catalytic domain, while the C-terminal part mediates binding of the protein to target cells. CyaA enters eukaryotic cells both in a receptor-independent manner (29, 38) and through high-affinity binding to the $\alpha_M\beta_2$ integrin, CD11b/CD18, which is present on macrophages, neutrophils, dendritic cells, and natural killer cells (19). After membrane translocation, the CyaA catalytic domain remains attached to the cytosolic surface of the plasma membrane, where, following activation by Ca^{2+}/calmodulin, it rapidly converts cellular ATP into cAMP, thus generating large quantities of cAMP (29, 33). CyaA can also form cation-selective pores in cell membranes independent of translocation, thereby perturbing ion homeostasis (7). Moreover, Fiser et al. (16) have recently reported a third activity of CyaA, which involves sustained elevation of intracellular Ca^{2+} levels promoted by membrane translocation of the adelylate cyclase domain and appears to be independent of both the adenylate cyclase activity and the pore-forming activity of the toxin.

The use of *B. pertussis* strains lacking specific virulence factors in the mouse has provided proof that CyaA actively participates in pathogenesis by favoring colonization of the respiratory epithelium and infection of the host (18). Crucial to the successful establishment and spread of the infection is the capacity of this toxin to attenuate the immune defenses of the host at the site of colonization and to delay the development of a systemic immune response. Indeed, CyaA inhibits a variety of innate immune effector functions, including phagocytosis, the oxidative burst, and production of proinflammatory cytokines (7, 33, 44). CyaA has recently been reported to also interfere with initiation of adaptive immune responses by driving monocyte-derived dendritic cell differentiation to a semimature state characterized by increased expression of major histocompatibility complex class II and the costimulatory molecules CD80, CD83, and CD86 (2). This state has been associated with decreased proinflammatory cytokine production and increased expression of the suppressive cytokine interleukin-10 (IL-10), which promotes the expansion of regulatory T cells (40). Furthermore, CyaA promotes macrophage apoptosis, which not only impairs bacterial killing but also prevents antigen presentation (7).

The immunosuppressive activities of CyaA on phagocytes and dendritic cells have been attributed largely to its capacity to increase intracellular cAMP levels (9). cAMP has indeed been demonstrated to act as a potent immunosuppressant by promoting activation of protein kinase A (PKA) (42). Further-

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more, the Rap-specific guanine nucleotide exchange factor EPAC1 has recently been identified as a primary target of cAMP (6). Inhibition of monocyte and macrophage activation by cAMP results from disruption of PKA-dependent intracellular signaling through inhibition of lipopolysaccharide (LPS)-induced extracellular signal-regulated kinase (Erk) and stress-activated protein kinase/Jun N-terminal protein kinase (JNK) activation (12, 46). On the other hand, the inhibitory effect of cAMP on phagocytosis by both alveolar and monocyte-derived macrophages has been ascribed to EPAC1 activation (1, 8).

The cAMP nucleotide is known to suppress T-lymphocyte activation and chemotaxis. The immunosuppressive effects of cAMP on T cells involve perturbation by PKA and EPAC1 of a number of intracellular pathways which participate in T-cell activation and migration (31, 42). Of paramount importance among these pathways are mitogen-activated protein (MAP) kinase cascades, which can be modulated at multiple steps of their activation by cAMP (15). Here we examined the effects of *B. pertussis* CyaA on T lymphocytes. Our results show that CyaA inhibits T-cell activation and chemotaxis by impairing T-cell antigen receptor (TCR) and chemokine receptor signaling through a cAMP/PKA-dependent pathway. A comparative analysis of the effects of CyaA on T cells and macrophages identified MAP kinase modules as common targets of the PKA-modulating activity of the toxin in the signaling pathways triggered by receptors as diverse as antigen receptors, chemokine receptors, and Toll-like receptors (TLRs).

MATERIALS AND METHODS

Cells, antibodies, reagents, and toxins. Peripheral blood mononuclear cells were purified from buffy coats from anonymous healthy donors (~30 donors, available from authorized blood banks) by density gradient centrifugation (800 × *g* for 20 min at room temperature) on Ficoll-Paque (Amersham Biosciences, Buckinghamshire, United Kingdom), using a Beckman GS-6R tabletop centrifuge (Beckman Coulter SpA, Milan, Italy). Cells were washed twice in phosphate-buffered saline (PBS), resuspended in RPMI 1640 (Invitrogen Ltd., Paisley, United Kingdom) (buffered with sodium bicarbonate to pH 7.2) supplemented with 7.5% fetal calf serum (FCS) (HyClone; Thermofischer Scientific Inc., South Logan, UT), plated in plastic flasks (Sarstedt AG, Numbrecht, Germany), and incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. Nonadherent cells, which consisted principally of peripheral blood lymphocytes (PBL) and >90% of which were T cells ($CD3^+$), were centrifuged at 800 × *g* for 5 min at room temperature using a Beckman GS-6R tabletop centrifuge and resuspended in fresh RPMI 1640 supplemented with 7.5% FCS. Adherent cells, which included principally monocytes ($CD14^+$), were gently rinsed twice with RPMI 1640 to remove residual nonadherent cells, supplemented with fresh RPMI 1640 supplemented with 7.5% FCS, and incubated further as described above for 48 to 72 h. Under these conditions, monocytes differentiated spontaneously to macrophages, as assessed by CD86 surface expression.

Phosphospecific antibodies recognizing the phosphorylated active forms of JNK and Erk1/2 were obtained from Cell Signaling Technology (Beverly, MA). An antibody against the phosphorylated PKA consensus phosphorylation site, R-X-X-pT-X-X/R-R-X-pS-X-X, was purchased from Cell Signaling Technology. Anti-Erk2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorochrome-labeled anti-CD3, anti-CD69, anti-CD25, anti-CD14, and anti-CD86 monoclonal antibodies (MAbs) were obtained from Becton Dickinson Biosciences (Milan, Italy). Unlabeled secondary antibodies were purchased from Cappel (ICN Pharmaceuticals Inc, CA), and peroxidase-labeled antibodies were purchased from Amersham Biosciences Inc. Immunoglobulin G antibodies from OKT3 (anti-CD3; American Type Culture Collection, Manassas, VA) hybridoma supernatants were purified using Mabtrap (Amersham Biosciences, Inc.) and titrated by flow cytometry.

SDF-1α, MIP-1α, forskolin, 8-cyclo-pentenyltheophylline (8-CPT)-cAMP, and *Escherichia coli* lipopolysaccharide (LPS) were purchased from Sigma Aldrich (Milan, Italy), and KT5720 was obtained from Calbiochem (Merck Biosciences GmbH, Schwalbach, Germany).

CyaA and the enzymatically inactive variant CyaA-E5 (resulting from insertion of a Leu-Gln dipeptide between Asp188 and Ile189 in the catalytic core of the enzyme) were expressed in *E. coli* strain BLR (Novagen, La Jolla, CA) and purified to near homogeneity (more than 90% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) by using previously established procedures (25) that were modified as described by Preville et al.(37) in order to eliminate most of the contaminating endotoxin. The specific activity of CyaA, measured as described by Ladant et al. (28), was >500 μmol cAMP/min/mg, whereas CyaA-E5 had no detectable enzymatic activity. In both preparations the endotoxin content, determined using a *Limulus* amoebocyte lysate assay (QCL-1000 kit obtained from Lonza), was <0.5 endotoxin unit/μg protein.

Cell activation and lysis and immunoblotting. Cells were plated at a concentration of 5×10^6 cells/ml in plastic flasks in RPMI 1640 supplemented with 7.5% FCS and 2 mM $CaCl_2$ (required for CyaA entry into the cells [39]) and containing 45 nM CyaA or CyaA-E5 or 100 μM 8-CPT-cAMP, and they were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 2 h (cultures containing CyaA or CyaA-E5) or 30 min (cultures containing 8-CPT-cAMP) before activation. When required, cells were preincubated in RPMI 1640 containing 7.5% FCS as described above with 56 nM KT5720 for 1 h before addition of CyaA or 8-CPT-cAMP. Activation of PBL and macrophages with 1.2 nM (10 ng/ml) SDF-1α or activation of macrophages with 3.2 nM (25 ng/ml) MIP-1α was carried out at 37°C in RPMI 1640 containing 1% bovine serum albumin (BSA) for 1 min in 1.5-ml microcentrifuge vials. Activation by TCR/CD3 cross-linking was performed by incubating PBL with saturating concentrations of anti-CD3 MAb (as assessed by flow cytometry) and 50 μg ml^{-1} secondary antibodies (goat anti-mouse immunoglobulin) in RPMI 1640 for 5 min at 37°C as previously described (5). LPS activation of macrophages was accomplished by incubation for 10 min at 37°C with 10 μg ml^{-1} LPS in RPMI 1640 containing 7.5% FCS in 24-well plates. None of the stimulation conditions described above (SDF-1α, MIP-1α, anti-CD3 MAb, or LPS) affected cell viability, as assessed by trypan blue exclusion (data not shown).

Cells were recovered by centrifugation at 16,000 × *g* for 30 s at 4°C using an Eppendorf 5415R microcentrifuge (Eppendorf srl, Milan, Italy), washed twice in PBS, and lysed in 1% (vol/vol) Triton X-100 in 20 mM Tris-HCl (pH 8), 150 mM NaCl (in the presence of 0.2 mg/ml sodium orthovanadate, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 10 mM phenylmethylsulfonyl fluoride). To normalize for variations in protein content among samples (particularly for macrophages, where CyaA treatment resulted in ~20% cell lysis [see Results]), equal amounts of proteins from each sample (measured using a kit obtained from Pierce, Rockford, IL) were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.45-μm nitrocellulose filters (Whatman GmbH, Dassel, Germany). Prestained molecular mass markers (Invitrogen) were included in each gel.

Immunoblotting was carried out using primary antibodies and peroxidase-labeled secondary antibodies according to the manufacturers' instructions and a chemiluminescence detection kit (Pierce). Blots were scanned using a laser densitometer (Duoscan T2500; Agfa, Milan, Italy) and quantified using the ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

T-cell activation and proliferation assays and measurement of cytokines and cAMP. For analysis of CD69/CD25 expression, cytokine production, and proliferation, cells (1×10^6 cells/sample) were plated in 96-well plates in RPMI 1640 supplemented with 7.5% FCS and 2 mM $CaCl_2$ and containing CyaA or CyaA-E5 (0.14 to 45 nM) or 8-CPT-cAMP (100 μM) and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 2 h (cultures containing CyaA or CyaA-E5) or 30 min (cultures containing 8-CPT-cAMP) before activation. Cells were activated by CD3 cross-linking on secondary antibody-coated plates as described previously (5) and were processed 16 to 48 h after activation. Flow cytometric analysis of CD69 and CD25 surface expression was carried out with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using fluorochrome-labeled anti-CD69 and anti-CD25 MAbs and gating on live cells, as assessed by forward and side scatter characteristics. For proliferation assays [^{3}H]thymidine (1 mCi) was added to each microtiter well (96-well plates) for the last 18 h of culture. After the cells were harvested with an automatic harvester (Micromate 196; Canberra Packard, Meriden, CT), proliferation was determined by measuring the [^{3}H]thymidine (Amersham, Buckinghamshire, United Kingdom) incorporation with a β-counter (Matrix 9600; Canberra Packard, Meriden, CT). Cytokines in the cell supernatants were measured using a human cytokine enzyme-linked immunosorbent assay kit obtained from Biosource Europe SA (Nivelles, Belgium) and a Bio-Rad model 680 microplate plate reader (Bio-Rad Laboratories Srl, Milan, Italy).

Intracellular cAMP was quantitated with an enzyme-linked immunoassay kit (Biotrak EIA; Amersham Biosciences) used according to the manufacturer's instructions. For these experiments, cells (1×10^6 cells plated in 96-well plates in 200 μl RPMI 1640 containing 7.5% FCS) were treated with CyaA or CyaA-E5

as described above for 10 min to 24 h in a humidified atmosphere with 5% CO_2. Alternatively, cells were incubated with forskolin (25 μM) for 30 min in the same conditions. At the end of the treatment, cells were washed twice in PBS and lysed in the lysis reagent included in the kit.

Chemotaxis assays. For chemotaxis assays, cells (5×10^5 cells/sample) were plated in 24-well plates in RPMI 1640 supplemented with 1% BSA and 2 mM $CaCl_2$ and containing CyaA or CyaA-E5 (0.14 to 45 nM) or 8-CPT-cAMP (100 μM) and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 2 h (cultures containing CyaA or CyaA-E5) or 30 min (cultures containing 8-CPT-cAMP) before activation. Chemotaxis assays were carried out using 24-well Transwell chambers with 5-μm-pore-size polycarbonate membranes (Corning Life Sciences B.V., Schiphol-Rijk, The Netherlands) essentially as described previously (36). Filters were soaked overnight at 4°C in a sterile solution of 0.2% BSA in Hanks' balanced salt solution without Ca^{2+}/Mg^{2+}. The chemotaxis medium (500 μl RPMI 1640 containing 1% BSA) with or without the selected chemokine was placed in the lower chamber, and cells were placed in the upper chamber. After 2 h of incubation at 37°C in humidified air with 5% CO_2, the upper chamber was emptied, the filters were removed, and the contents of the lower chamber were recovered. After two washes in PBS, the cells in the lower chamber were counted by flow cytometry, with gating on live cells, as assessed by forward and side scatter characteristics. The migration index was calculated by determining the ratio of migrated cells in chemokine-treated cells to migrated cells in untreated cells. The concentrations of chemokines used in these experiments are in the middle range of the dose-response curve previously determined both for T cells and for macrophages (36, 41; unpublished data). Notwithstanding some variability among donors, the average migration indexes in the experimental conditions used for the chemotaxis assays were ~10 and ~8 for T cells and macrophages stimulated with SDF-1α, respectively, and ~4 for macrophages stimulated with MIP-1α.

Transfection and confocal microscopy. Jurkat cells (1×10^6 cells) were transfected by electroporation with 20 μg each of two pcDNA3 (Invitrogen srl) plasmids, one carrying the catalytic subunit of PKA fused to yellow fluorescent protein (YFP) (C-YFP) and one carrying a MyrPalm-tagged version of the regulatory subunit fused to cyan fluorescent protein (CFP) (RII-CFP). In MyrPalm-tagged RII-CFP, a myristoylated and palmitoylated peptide (MGCIKSKRKDNLNDD) from the Lyn kinase (48) was fused to the N terminus of the RII-CFP chimera (11). After transfection, T cells were plated at a density of 5×10^5 cells/ml on fibronectin-coated glass coverslips in RPMI 1640 containing 10% FCS and incubated at 37°C in a humidified atmosphere with 5% CO_2. At 24 to 48 h after transfection, cells were transferred to a balanced salt solution (135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4$, 20 mM HEPES, 1.8 mM $CaCl_2$, 5.4 mM glucose; pH 7.4) in a microscope-adapted microincubator equipped with a temperature controller (HTC, Italy) at 37°C and constant 5% CO_2 pressure. CyaA or CyaA-E5 (final concentration, 1 nM) was added after 5 min of imaging to stabilize the baseline, and then images were obtained every 10 s. After 25 min of imaging, 25 μM forskolin was added. The integration time was 100 ms or less. At each time point, the intracellular cAMP level was estimated by measuring the ratio of the background-subtracted cyan emission image (480 nm) to the yellow emission image (545 nm) upon excitation at 430 nm (CFP/YFP ratio) (34). Images were acquired using an oil immersion ×60 (zoom ×1.6) PlanApo 1.40 NA objective with a wide-field Olympus IX81 microscope equipped with a dichroic mirror. Images were processed with WCIF ImageJ v1.37. cAMP variations were represented by using pseudocolors (Rainbow2 ImageJ LUT), where red indicates high cAMP levels (high CFP/YFP ratio) and green indicates low cAMP levels (low CFP/YFP ratio).

Statistical analyses. Mean values and standard deviations were calculated and Student's *t* test (unpaired) was performed using Microsoft Excel. A *P* value of <0.05 was considered statistically significant.

RESULTS

CyaA increases the cAMP level and activates PKA in T lymphocytes. The $\alpha_M\beta_2$ integrin (CD11b/CD18), which is expressed on all myeloid cells but not on T lymphocytes, is a high-affinity CyaA receptor (19). However, CyaA has been shown to intoxicate cells lacking this receptor through a mechanism involving receptor-independent binding to the cell surface and subsequent translocation of the catalytic domain through the plasma membrane (29). Treatment with CyaA of human monocyte-depleted PBL, consisting of >90% T cells, resulted in increases in the levels of intracellular cAMP to levels comparable to those elicited by forskolin, a potent activator of endogenous adenylate cyclase (Fig. 1A), suggesting that CyaA can enter T cells in a CD11b/CD18-independent manner. The increase, which reached a plateau at 10 min and was sustained for up to 24 h (Fig. 1B and data not shown), was not observed when T cells were exposed to a genetically inactivated variant of CyaA, CyaA-E5 (Fig. 1A), which is devoid of adenylate cyclase activity due to a dipeptide insertion in the ATP binding site (28). No effect of CyaA or CyaA-E5 on T-cell viability was observed with the concentrations and treatment times used, as assessed by trypan blue exclusion ($n = 2$) and by flow cytometric analysis of forward and side scatter characteristics ($n > 3$) (data not shown), consistent with the requirement for CD11b/CD18 binding for the pore-forming activity of the toxin (19).

The increase in the concentration of intracellular cAMP induced by CyaA was imaged in living cells using a fluorescence resonance energy transfer (FRET)-based assay which measures PKA activation. Jurkat T cells were cotransfected with two constructs, one encoding the catalytic subunit of PKA fused to YFP and one encoding the regulatory PKA subunit fused to CFP. As shown in Fig. 1C (upper panel), untreated cells displayed high FRET, indicating close proximity of the regulatory (CFP) and catalytic (YFP) subunits. Treatment with CyaA resulted in a decrease in FRET that was clearly detectable after 15 min and still maximal at the last time point analyzed, due to the cAMP-dependent release of the catalytic subunit from the regulatory subunit. Conversely, no change in FRET was detected when Jurkat cells were treated with the adenylate cyclase-defective CyaA-E5 mutant (Fig. 1C, lower panel). As a further indication of PKA activation, we used an antibody specific for the phosphorylated PKA consensus sequence, R-X-X-pT-X-X/R-R-X-pS-X-X. In agreement with the homeostatic production of low levels of cAMP by eukaryotic cells, PKA displayed a basal level of activity, as revealed by the presence of a number of phosphorylated protein substrates in untreated cells. The increase in the intracellular cAMP level following treatment with CyaA resulted in an increase in PKA activity, as shown by the qualitative and quantitative changes in the phosphoprotein pattern, similar to the results obtained with the nonhydrolyzable cAMP analogue 8-CPT-cAMP (Fig. 1D, bands indicated by an asterisk). Hence, CyaA catalyzes the long-lasting production of large amounts of cAMP and triggers sustained PKA activation in T cells.

CyaA inhibits T-cell activation through its cAMP-elevating activity. T-cell activation is initiated by TCR engagement by a cognate antigen. PKA acts as a potent inhibitor of T-cell activation by interfering with multiple steps of the TCR signaling cascade (42). The increase in the cAMP level promoted by CyaA suggests that this toxin may affect T-cell responses initiated by the TCR. As shown in Fig. 2A (top panel), T-cell proliferation was inhibited in a dose-dependent manner by CyaA. In agreement with the inhibition of T-cell proliferation, production of IL-2, which is expressed by activated T cells and drives their proliferation by establishing an autocrine loop together with IL-2R, was found to be potently inhibited by CyaA (Fig. 2A, bottom panel).

The effect of CyaA on surface activation markers in peripheral T cells stimulated with an agonistic anti-CD3 MAb was also assessed. Expression of the early activation marker, CD69,

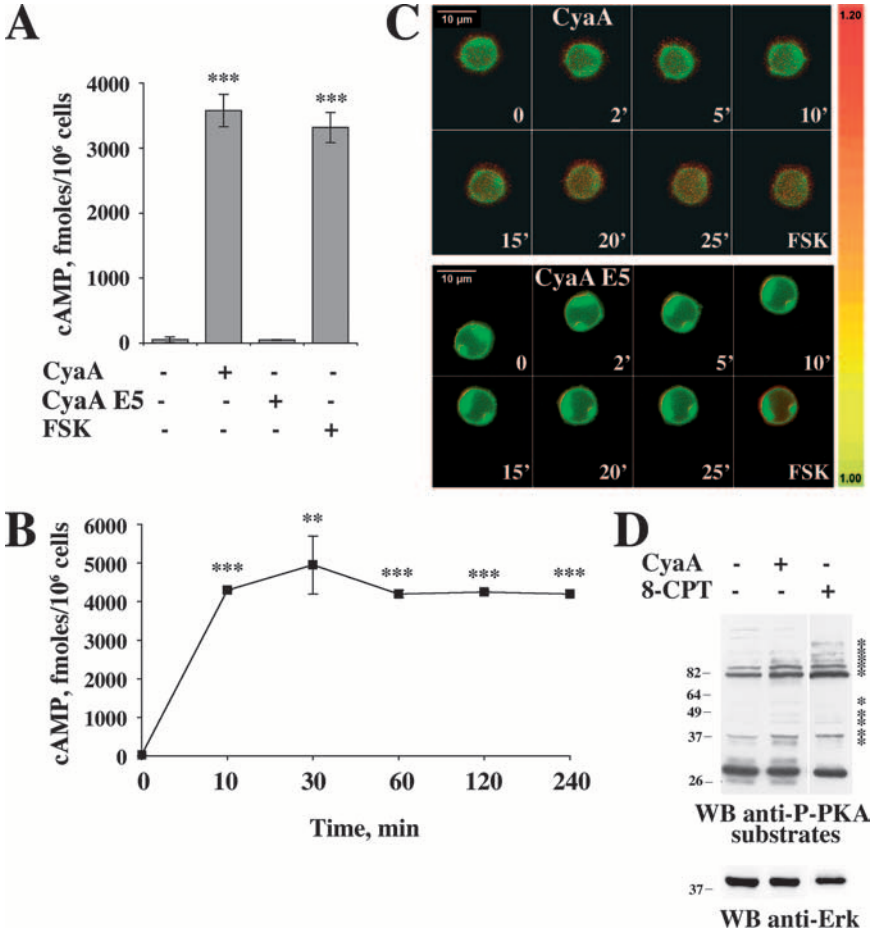

FIG. 1. cAMP production and PKA activation by CyaA in PBL. (A) Quantification of cAMP in lysates of PBL treated with 45 nM CyaA or CyaA-E5 for 2 h or with 20 μM forskolin (FSK) for 30 min ($n \geq 3$). (B) Quantification of cAMP in lysates of PBL treated for different times with 45 nM CyaA. The results for duplicate samples from a representative experiment are shown ($n \geq 2$). The error bars indicate standard deviations. Three asterisks, $P \leq 0.001$; two asterisks, $P \leq 0.01$. (C) FRET imaging of cAMP production in Jurkat cells transiently transfected with two constructs, one carrying the catalytic subunit of PKA fused to YFP (C-YFP) and one carrying a MyrPalm-tagged version of the regulatory subunit fused to CFP (RII-CFP). The transfected cells were treated with CyaA or CyaA-E5 (1 nM) for the indicated times. After 30 min of imaging, 25 μM forskolin was added. cAMP concentrations are indicated by false colors (the concentrations increase from green to red). Representative cells are shown. (D) Immunoblot analysis of the phosphorylation state of PKA substrates in postnuclear supernatants of PBL treated with 45 nM CyaA for 2 h ($n > 3$) or with 100 μM 8-CPT-cAMP for 30 min ($n = 2$), using an antibody which recognizes a phosphorylated PKA consensus sequence (see Materials and Methods). Filters were stripped and reprobed with control anti-Erk antibody. The asterisks indicate proteins whose phosphorylation by PKA is either induced or enhanced by CyaA or 8-CPT-cAMP. The results of a representative experiment are shown. The positions of molecular mass markers are indicated on the left.

as well as of CD25, the high-affinity subunit of the IL-2R, was potently inhibited by CyaA but not by the adenylate cyclase-defective CyaA-E5 mutant (Fig. 2B). CyaA was at least as effective as the nonhydrolyzable cAMP analogue 8-CPT-cAMP in inhibiting CD69/CD25 expression. Hence, CyaA potently suppresses T-cell activation through its cAMP-elevating activity.

CyaA inhibits TCR-dependent MAP kinase activation via a cAMP/PKA-dependent pathway. Among the effector pathways triggered following TCR engagement, of paramount importance to expression of CD69/CD25, as well as to expression of a number of other genes implicated in the process of T-cell activation, is the MAP kinase cascade (13). The activation of MAP kinases is negatively controlled by cAMP through both PKA-dependent and PKA-independent mechanisms (15). The effect of CyaA on activation of the MAP kinase cascade by the TCR was assessed by measuring phosphorylation of Erk1/2, the terminal components of the cascade, as readout. As shown in Fig. 3, CyaA potently suppressed TCR-dependent Erk activation in peripheral T cells.

To assess the contribution of the adenylate cyclase activity of CyaA, we measured TCR-dependent Erk activation in cells treated with the adenylate cyclase-defective CyaA-E5 mutant. In contrast to CyaA, which inhibited Erk activation by >90%, CyaA-E5 inhibited Erk activation by ~15% (Fig. 3), indicating that the suppressive activity of the toxin is primarily dependent on its capacity to catalyze the production of cAMP. Conversely, TCR-dependent Erk activation was effectively blocked by 8-CPT-cAMP (Fig. 3B). To understand whether the effects of the increase in cAMP due to CyaA are mediated by PKA, T cells were activated using a TCR agonist in the presence of a combination of CyaA and the pharmacological PKA inhibitor

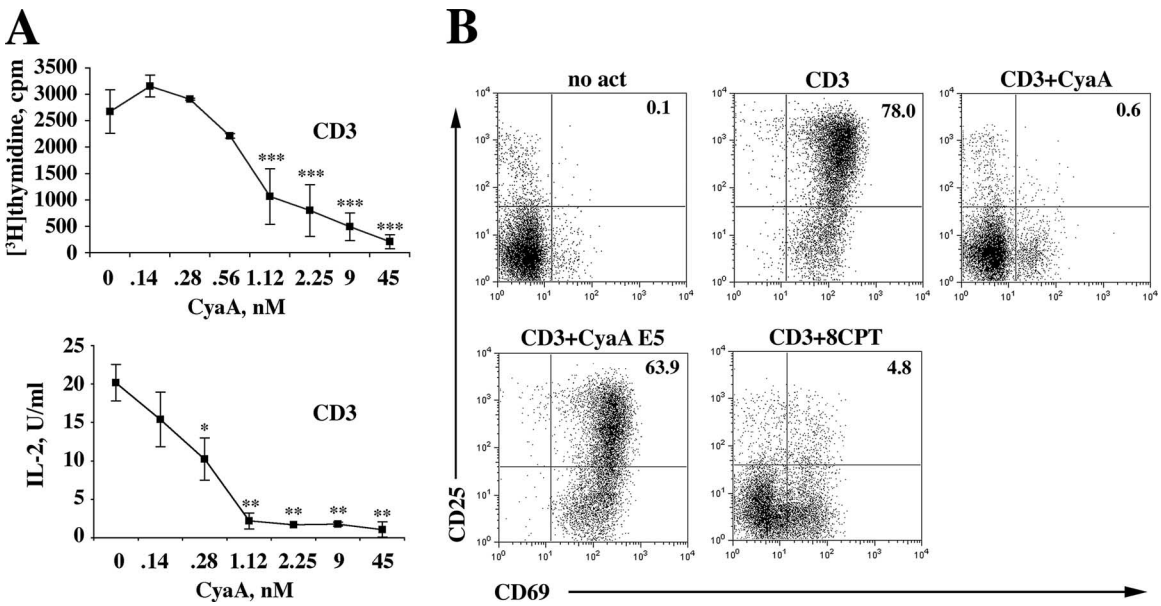

FIG. 2. Suppression of T-cell proliferation, IL-2 production, and CD69/CD25 expression by CyaA. (A) (Top panel) [^{3}H]thymidine uptake by PBL. PBL were stimulated for 48 h by CD3 cross-linking in the presence or absence of the indicated concentrations of CyaA. The results are expressed as mean values for [^{3}H]thymidine uptake obtained with triplicate samples of PBL from four independent donors. (Bottom panel) Quantification of IL-2 in the supernatants of duplicate samples of PBL from two independent donors activated for 30 h by CD3 cross-linking in the presence or absence of the indicated concentrations of CyaA. The error bars indicate standard deviations. Three asterisks, $P \leq 0.001$; two asterisks, $P \leq 0.01$; one asterisk, $P \leq 0.05$. (B) Flow cytometric analysis of CD69 and CD25 expression on PBL activated for 24 h by CD3 cross-linking in the presence of 45 nM CyaA or CyaA-E5 or 100 μM 8-CPT-cAMP (8CPT). The percentages of CD25$^+$/CD69$^+$cells are indicated in the upper right quadrants. Representative results obtained for PBL from three independent donors are shown. no act, no activation.

KT5720. The inhibitor was used at a low concentration (56 nM) which selectively blocked PKA without affecting other serine/threonine kinases (26). The inhibitory effects of CyaA were partially reversed by KT5720, indicating that the inhibition of TCR-dependent Erk phosphorylation by CyaA is mediated, at least to a significant extent, by PKA (Fig. 3B). Collectively, these data show that CyaA inhibits T-cell activation and proliferation by blocking TCR signaling through increasing cAMP and PKA activation.

CyaA inhibits T-cell chemotaxis through its cAMP-elevating activity by interfering with chemokine receptor signaling. Initiation of adaptive immune responses requires migration of activated dendritic cells to regional lymph nodes, where they encounter antigen-specific T cells, which continuously recirculate between the lymphatic system and the bloodstream. This process is finely regulated by a complex chemokine network (45). Chemokine receptors are seven-span membrane proteins coupled to heterotrimeric Gi proteins which, when activated, suppress homeostatic cAMP production by adenylate cyclases (47). This suggests that by increasing the levels of intracellular cAMP, CyaA may inhibit chemotaxis and thereby prevent T-cell activation. The effect of CyaA on T-cell (CD3$^+$) migration triggered by the chemokine SDF-1α/CXCL12, which interacts with the CXCR4 receptor, was assessed. As shown in Fig. 4A, CyaA inhibited CXCR4-dependent T-cell chemotaxis in a dose-dependent manner. The adenylate cyclase-defective CyaA-E5 mutant did not affect this process. Furthermore, similar to the results for CyaA, treatment of T cells with 8-CPT-cAMP resulted in inhibition of CXCR4-dependent chemotaxis (Fig. 4A), supporting the notion that this biological activity of the toxin is related to its capacity to increase intracellular cAMP levels.

By inhibiting cAMP production and PKA activity, chemokine receptors activate MAP kinases, which act as central regulators of the dynamics of the actin cytoskeleton and, as such, control cell motility (22, 47). Treatment of monocyte-depleted peripheral lymphocytes with CyaA resulted in suppression of Erk1/2 activation by CXCR4 (Fig. 4B). A reduction in CXCR4-dependent Erk1/2 activation was also observed following treatment with 8-CPT-cAMP, while, conversely, CyaA-E5 did not affect this process (Fig. 4B and C). Furthermore, CXCR4-dependent Erk phosphorylation in CyaA-treated cells was rescued to a large extent by the PKA inhibitor KT5720 (Fig. 4C). Hence, CyaA inhibits T-lymphocyte chemotaxis through a cAMP/PKA-dependent pathway.

The inhibitory effects of CyaA on macrophage activation and chemotaxis involve perturbation of TLR4 and chemokine receptor signaling to MAP kinases via cAMP/PKA. While CyaA has been demonstrated to inhibit production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), and, conversely, to enhance production of the immunosuppressive cytokine IL-10 by macrophages (2), the underlying mechanisms are only beginning to be addressed. MAP kinases play pivotal roles in signaling by receptors controlling macrophage activation, motility, and effector functions (27). The ubiquity of these cascades suggests that the inhibitory activity of CyaA for MAP kinase activation in T cells may also be operational in macrophages. On the other hand, in contrast to T lymphocytes, macrophages express

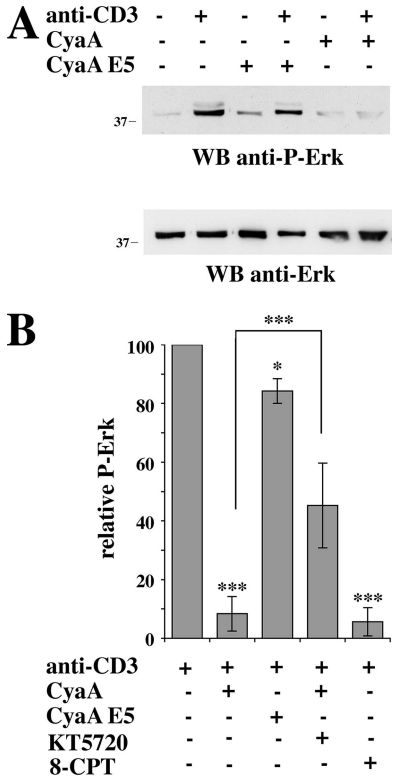

FIG. 3. Suppression of TCR-dependent MAP kinase activation by CyaA. (A) Immunoblot analysis, using a phosphospecific antibody, of Erk1/2 phosphorylation in postnuclear supernatants from PBL activated for 5 min by CD3 cross-linking (anti-CD3) in the presence of 45 nM CyaA or CyaA-E5. Filters were stripped and reprobed with control anti-Erk antibody. The results of a representative experiment are shown. The positions of molecular mass markers are indicated on the left. (B) Quantification by laser densitometry of the relative levels of Erk1/2 phosphorylation (the Erk1/2 phosphorylation in control cells was defined as 100%) in PBL activated for 5 min by CD3 cross-linking in the presence of 45 nM CyaA or CyaA-E5 or 100 μM 8-CPT-cAMP (8-CPT). Where indicated, PBL were pretreated for 1 h with 56 nM KT5720 before addition of CyaA ($n \geq 3$). The error bars indicate standard deviations. Three asterisks, $P \leq 0.001$; one asterisk, $P \leq 0.05$. Neither CyaA, CyaA-E5, nor 8-CPT-cAMP affected to a significant extent the basal level of Erk1/2 phosphorylation. Furthermore, KT5720 did not affect to a significant extent either basal or CD3-dependent Erk1/2 phosphorylation (data not shown).

the high-affinity CyaA receptor CD11b/CD18 (19), which is required for the Ca^{2+}-elevating activity of CyaA in these cells and as such may introduce an additional level of complexity to the effects of the toxin on intracellular signaling.

As expected, treatment of macrophages with CyaA, but not treatment with the adenylate cyclase-defective CyaA-E5 mutant, resulted in the production of large amounts of cAMP comparable to the amounts induced by the endogenous adenylate cyclase activator forskolin (Fig. 5A). The increase in the amount of cAMP was paralleled by an increase in PKA activity, as assessed by immunoblot analysis of CyaA-treated macrophages using an antibody which recognizes phosphorylated PKA substrates (Fig. 5B, bands indicated by an asterisk). Under these conditions, treatment with CyaA and CyaA-E5 resulted in modest reductions in cell viability (lysis of 19.5% ± 0.5% and 9.5% ± 0.5% of the cells, respectively), as assessed by trypan blue exclusion ($n = 2$), in agreement with the reduced lytic activity of recombinant CyaA on macrophages compared to CyaA purified from *B. pertussis* culture supernatants (D. Ladant, unpublished data).

As previously reported (7), CyaA suppressed LPS-dependent TNF-α expression, albeit only at the highest dose used (Fig. 5C). It is noteworthy that TNF-α production by LPS-activated macrophages was inhibited only by high concentrations of CyaA. The sensitivity of TLR4 to cAMP may, however, be underestimated, as LPS has been reported to potently activate PDE4 in macrophages (23), thereby promoting cAMP degradation. Strong inhibition of LPS-dependent TNF-α expression was also observed following treatment of macrophages with 8-CPT-cAMP, while this effect was not elicited by CyaA-E5 (Fig. 5C), even though this mutant has both the pore-forming (lytic) and Ca^{2+}-elevating activities of the toxin. These findings suggest that of the three activities of CyaA with macrophages (44), the adenylate cyclase activity may be primarily responsible for the alterations in the cytokine expression pattern observed following treatment of these cells with CyaA (7).

Both MAP kinases and stress-activated kinases have been implicated in TLR4 signaling (20). The effect of CyaA on LPS-dependent activation of Erk and JNK was assessed. CyaA potently suppressed activation of both Erk and JNK in macrophages treated with LPS (Fig. 5D and 5F). Little effect on Erk or JNK activation was observed when macrophages were activated in the presence of the adenylate cyclase-defective CyaA-E5 mutant (Fig. 5E and 5F). The PKA inhibitor KT5720 largely reversed the inhibitory effect of CyaA on Erk activation (89.5% ± 6.8% of LPS-dependent Erk phosphorylation). Collectively, these data show that CyaA inhibits LPS-dependent macrophage activation and TLR4 coupling to MAP kinases through cAMP production and PKA activation.

The inhibitory effect of CyaA on polymorphonuclear cell migration determined using the migration-under-agarose technique, where fresh serum is used as a chemotactic agent, was described many years ago (17). The effects of CyaA on macrophage chemotaxis toward a chemokine gradient and the underlying mechanisms have, however, not been investigated so far. Since chemokine receptors inhibit endogenous adenylate cyclases through activation of heterotrimeric Gi proteins, thereby reducing homeostatic cAMP production, CyaA might be expected to antagonize chemotactic signaling through its cAMP-elevating activity. As shown in Fig. 6A, CyaA inhibited CXCR4-dependent macrophage chemotaxis in a dose-dependent manner. No effect was observed with CyaA-E5, while 8-CPT effectively inhibited this process. Similar effects were observed when chemotaxis was triggered by the proinflammatory chemokine MIP-1α, which binds to the CCR5 receptor (Fig. 6B).

To understand whether the inhibitory effects of CyaA on chemotaxis could be related to perturbation of MAP kinase activation by CXCR4 and CCR5, the effect of macrophage treatment with SDF-1α and MIP-1α on Erk phosphorylation was assessed. As shown in Fig. 6C, CyaA inhibited Erk activation in response to both chemokines. The inhibitory effects of the toxin were also observed with 8-CPT-cAMP. Conversely, no significant inhibition of Erk activation was observed in macrophages treated with CyaA-E5. The block in Erk activation by CyaA was partially reversed by the PKA inhibitor KT5720 (Fig. 6C). Hence, CyaA inhibits macrophage chemotaxis and perturbs chemokine recep-

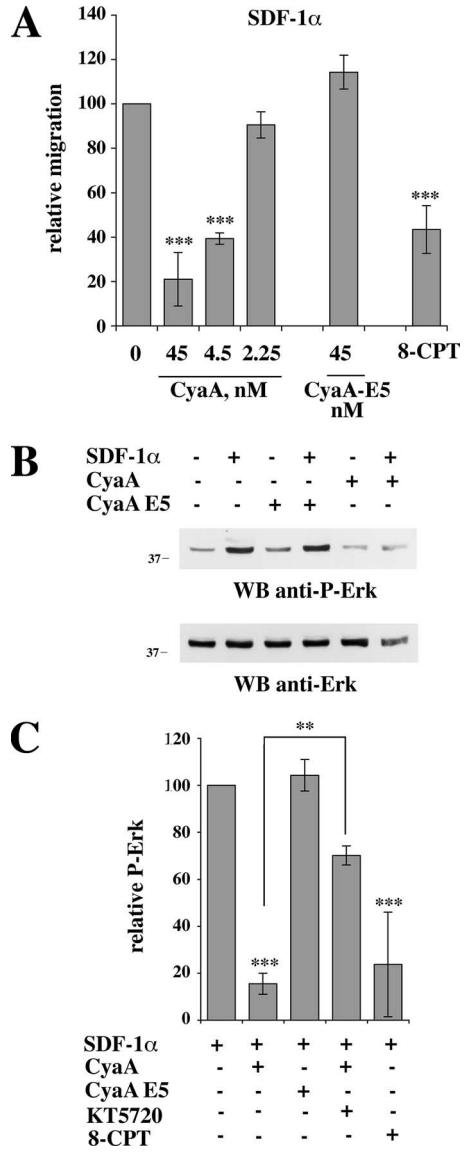

FIG. 4. CyaA inhibits SDF-1α chemotaxis and Erk phosphorylation in PBL. (A) T-cell chemotaxis induced by SDF-1α (1.2 nM) in the presence of the indicated concentrations of CyaA or CyaA-E5 or 100 μM 8-CPT-cAMP (8-CPT). The data are expressed as relative migration, and the migration index of untreated cells was defined as 100% ($n \geq 3$) The analysis was carried out using gated $CD3^+$ cells. Three asterisks, $P \leq 0.001$. (B) Immunoblot analysis, using a phosphospecific antibody, of SDF-1α-dependent Erk1/2 phosphorylation (1 min at 37°C) in postnuclear supernatants from PBL treated with 45 nM CyaA or CyaA-E5 ($n \geq 3$). Filters were stripped and reprobed with control anti-Erk antibody. The results of a representative experiment are shown. The positions of molecular mass markers are indicated on the left. (C) Quantification by laser densitometry of the relative levels of SDF-1α-dependent Erk1/2 phosphorylation (the Erk1/2 phosphorylation in control cells was defined as 100%) in postnuclear supernatants from PBL treated with 45 nM CyaA or CyaA-E5 or 100 μM 8-CPT-cAMP. Where indicated, PBL were pretreated with 56 nM KT5720 before addition of CyaA ($n \geq 3$). The error bars indicate standard deviations. Three asterisks, $P \leq 0.001$; two asterisks, $P \leq 0.01$. Neither CyaA, CyaA-E5, nor 8-CPT-cAMP affected to a significant extent the basal level of Erk1/2 phosphorylation. Furthermore, KT5720 did not affect to a significant extent either basal or SDF-1α-dependent Erk1/2 phosphorylation (data not shown).

tor signaling to MAP kinases by increasing the level of cAMP. This activity is mediated to a significant extent by PKA.

DISCUSSION

Although *B. pertussis* infection is normally cleared by the immune system and long-lasting protective immunity against the bacterium is elicited, the temporary neutralization of the host immune defenses in the initial phases of the infection is important for *B. pertussis* to successfully colonize the airway epithelia, multiply, and spread the infection to other individuals. Among the virulence factors of *B. pertussis*, CyaA appears to be the principal factor responsible for this activity. CyaA indeed effectively prevents bacterial killing by inhibiting recruitment of polymorphonuclear cells to the site of infection and by suppressing phagocytosis, the oxidative burst, and production of proinflammatory cytokines (29, 44). Furthermore, CyaA delays the development of a systemic immune response in the host by driving differentiation of dendritic cells to a semimature state which indirectly inhibits T-cell activation by promoting the development of regulatory T cells (2). The data presented in this report demonstrate that CyaA has a direct inhibitory effect on the process of T-lymphocyte activation and proliferation. This finding can explain, at least in part, the hyporesponsiveness of T cells purified from the lungs of mice in the acute phase of infection with *B. pertussis* (32). Furthermore, we show that CyaA effectively blocks T-cell chemotaxis. The pleiotropic activities of CyaA on T cells may contribute to the suppression of the development of pathogen-specific adaptive responses at multiple steps of the process, from activation of naive T cells to the recruitment and effector function of antigen-specific effector T cells in the infected tissue, thereby further delaying clearance of the infection. Hence, using a virulence factor which acts on multiple cell targets in the immune system, *B. pertussis* effectively paralyzes both the innate and adaptive immune defenses of the host.

A major consideration is whether the toxin concentrations used in this and other studies can be considered physiological. To our knowledge, the range of CyaA concentrations that can be found in vivo during *B. pertussis* infection is not known. However, the affinity of CyaA for its receptor, CD11b/CD18, has been measured on macrophages and neutrophils, as well as on CHO cells transfected with CD11b/CD18-encoding constructs. In these cells the equilibrium dissociation constant (K_D) of the CD11b/CD18 binding was found to range from 5 to 20 nM CyaA (14, 19). Hence, if the interaction of CyaA with the CD11b/CD18 integrin has physiological significance, a possibility that is widely accepted, one can deduce that such concentrations of toxin should be found in vivo. Our data show that CyaA has profound physiological effects on T cells at even lower concentrations (0.5 to 10 nM), notwithstanding the fact that these cells do not express the CD11b/CD18 receptor. This suggests that during infection T cells are likely to be exposed to biologically functional concentrations of CyaA. Although entry of CyaA into T cells was not directly addressed in this study, CyaA is likely to enter these cells through a receptor-independent mechanism involving membrane insertion, as documented for other cell types (29, 38). The high sensitivity of T cells to CyaA in the absence of the known CyaA receptor is, however, intriguing. Unless these cells express an alternative,

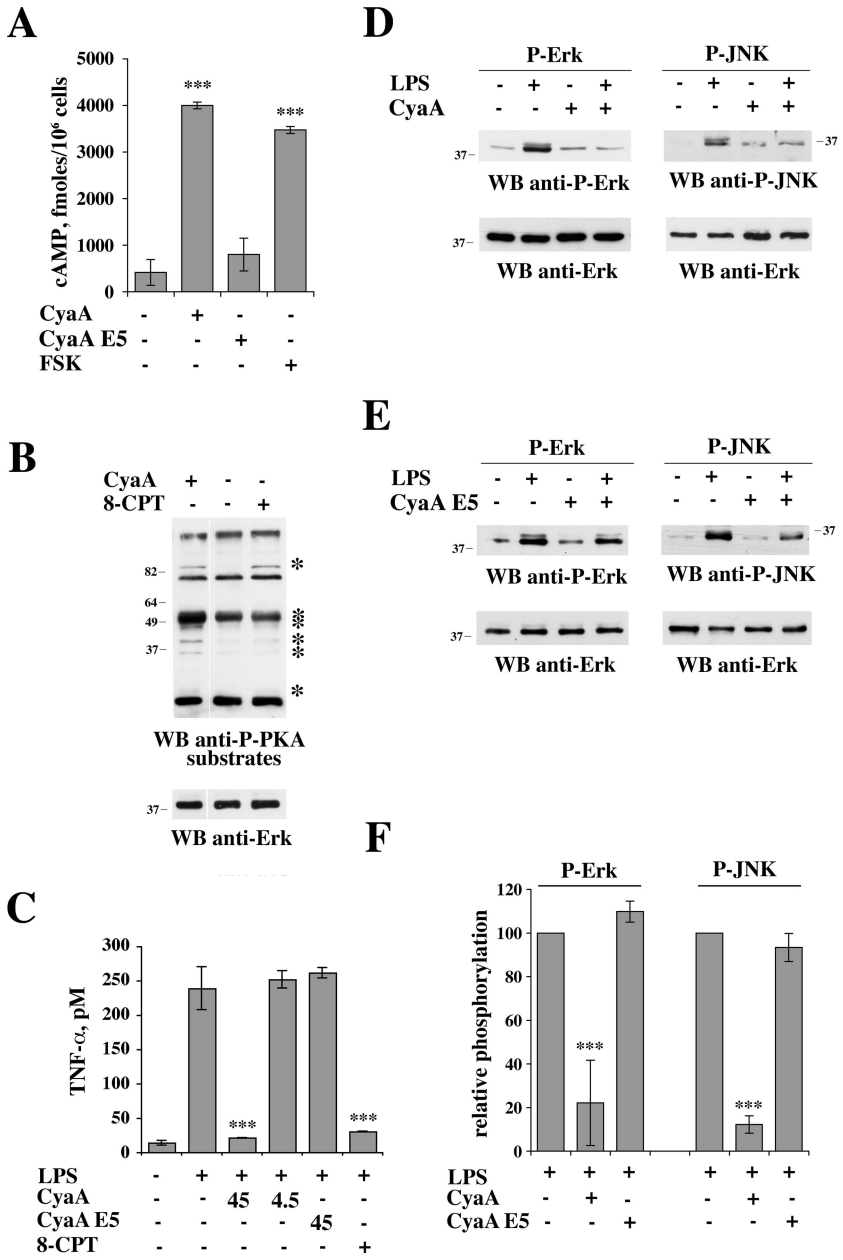

FIG. 5. Inhibition of LPS-dependent macrophage activation and phosphorylation of MAP kinases by CyaA. (A) Quantification of cAMP in lysates of macrophages treated with 45 nM CyaA or CyaA-E5 for 2 h or with 20 μM forskolin (FSK) for 30 min. The results for duplicate samples from a representative experiment are shown ($n \geq 3$). (B) Immunoblot analysis of the phosphorylation state of PKA substrates in postnuclear supernatants of macrophages treated with 45 nM CyaA for 2 h or with 100 μM 8-CPT-cAMP for 30 min. Filters were stripped and reprobed with control anti-Erk antibody. The asterisks indicate the proteins whose phosphorylation by PKA is either induced or enhanced by CyaA or 8-CPT-cAMP. The results of a representative experiment are shown ($n = 3$). The positions of molecular mass markers are indicated on the left. (C) Quantification of TNF-α in the supernatants of duplicate samples of macrophages from two independent donors activated for 30 h with 10 μg ml^{-1} LPS in the presence or absence of 4.5 or 45 nM CyaA or CyaA-E5 or 100 μM 8-CPT-cAMP (8-CPT). (D and E) Immunoblot analysis of postnuclear supernatants from macrophages activated for 10 min with 10 μg ml^{-1} LPS in the presence of 45 nM CyaA (D) or CyaA-E5 (E). Immunoblotting was performed using an antibody against the phosphorylated, active form of Erk (left blot) or JNK (right blot). Filters were stripped and reprobed with an anti-Erk antibody. The results of representative experiments are shown. (F) Quantification by laser densitometry of the relative levels of LPS-dependent Erk1/2 and JNK phosphorylation (the Erk1/2 or JNK phosphorylation in control cells was defined as 100%) in postnuclear supernatants from macrophages treated with 45 nM CyaA or CyaA-E5 ($n \geq 3$). The error bars indicate standard deviations. Three asterisks, $P \leq 0.001$. Neither CyaA nor CyaA-E5 affected to any significant extent the basal level of Erk1/2 or JNK phosphorylation (data not shown).

as-yet-unidentified receptor, one could hypothesize that they might be more sensitive to small amounts of CyaA than neutrophils or macrophages due to their small volume.

The capacity of CyaA to inhibit central processes in different cellular components of the mammalian immune system stems from its capacity to catalyze the production of supraphysiological amounts of cAMP, a key second messenger whose levels are kept, in physiological conditions, under tight spatiotempo-

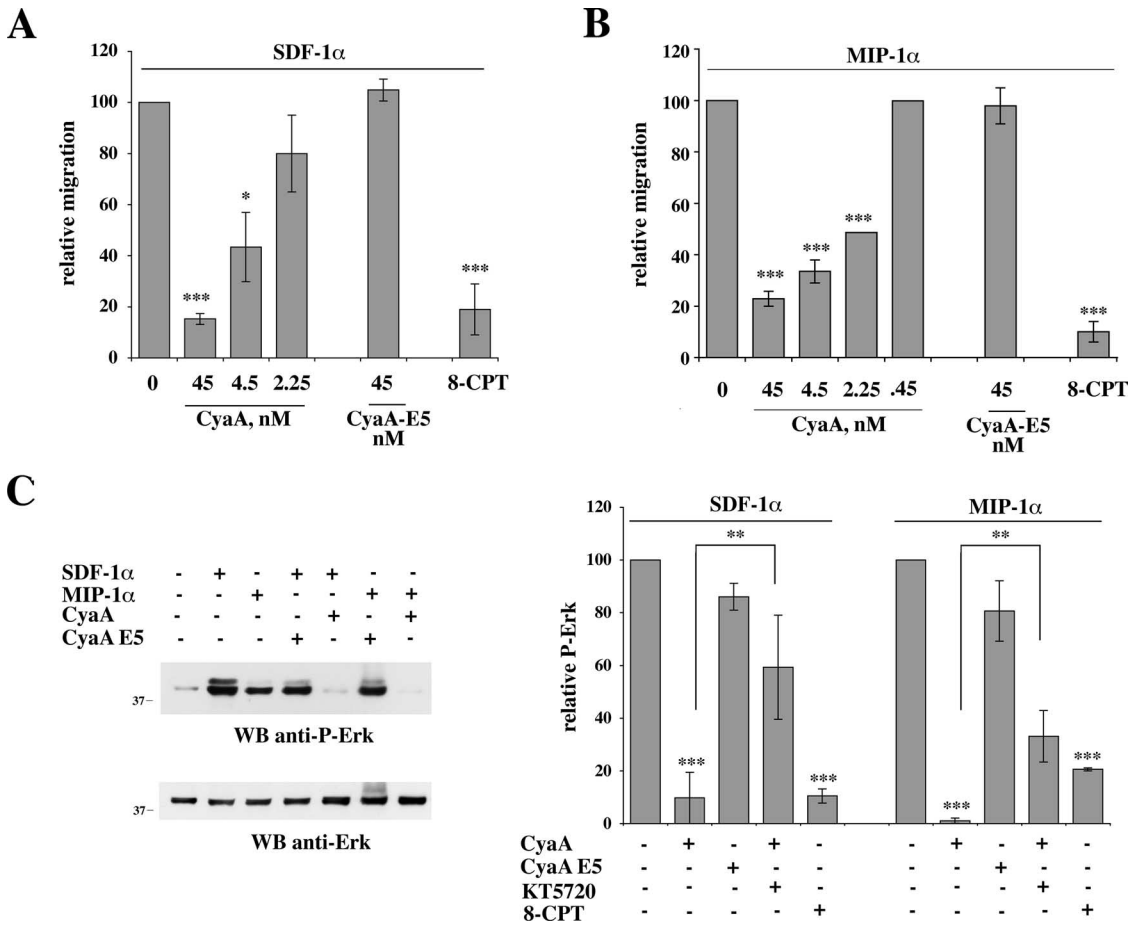

FIG. 6. CyaA inhibits SDF-1α- and MIP-1α-dependent chemotaxis and Erk phosphorylation in macrophages. (A) Macrophage chemotaxis induced by SDF-1α (1.2 nM) after treatment with the indicated concentrations of CyaA or CyaA-E5 or with 100 μM 8-CPT-cAMP (8-CPT) ($n \geq 3$). The data are expressed as relative migration, and the migration index of untreated cells was defined as 100%. (B) Macrophage chemotaxis induced by MIP-1α (3.2 nM) after treatment with the indicated concentrations CyaA or CyaA-E5 or with 100 μM 8-CPT-cAMP ($n \geq 3$). (C) (Left panel) Immunoblot analysis, using a phosphospecific antibody, of SDF-1α-dependent or MIP-1α-dependent Erk1/2 phosphorylation (1 min at 37°C) in postnuclear supernatants from macrophages treated with 45 nM CyaA or CyaA-E5. Filters were stripped and reprobed with control anti-Erk antibody. The results of representative experiments are shown. The positions of molecular mass markers are indicated on the left. (Right panel) Quantification by laser densitometry of the relative levels of SDF-1α-dependent or MIP-1α-dependent Erk1/2 phosphorylation (the Erk1/2 phosphorylation in control cells was defined as 100%) in postnuclear supernatants from macrophages treated with 45 nM CyaA or CyaA-E5 ($n > 3$) or 100 μM 8-CPT-cAMP ($n = 2$). Where indicated, macrophages were pretreated with 56 nM KT5720 before addition of CyaA ($n = 2$). The error bars indicate standard deviations. Three asterisks, $P \leq 0.001$; two asterisks, $P \leq 0.01$; one asterisk, $P \leq 0.05$. Neither 8-CPT-cAMP nor KT5720 affected to any significant extent the basal levels of Erk1/2 phosphorylation. Furthermore, KT5720 did not affect to a significant extent either SDF-1α-dependent or MIP-1α-dependent Erk1/2 phosphorylation (data not shown).

ral control by compartmentalization and modulation of cellular adenylate cyclases and phosphodiesterases (3). As an agonist of PKA, which is activated by many surface receptors and phosphorylates a multitude of substrates, cAMP controls intracellular pathways governing both rapid and reversible responses, such as transient changes in metabolism and remodeling of the actin cytoskeleton, and gene expression, which may result in irreversible changes in the cellular phenotype (15). The production of supraphysiological levels of cAMP by CyaA can therefore be hypothesized to underlie the perturbation of signaling by receptors as diverse as antigen receptors, chemokine receptors, and TLRs, all of which are modulated by cAMP, and the resulting suppression of cell activation and chemotaxis in both T cells and macrophages. In agreement with this hypothesis, these biological effects could be mimicked by the nonhydrolyzable cAMP analogue 8-CPT-cAMP. This possibility is further supported by the failure of the adenylate cyclase-defective CyaA-E5 mutant to reproduce the effects of CyaA. It should be emphasized that this mutant retains both the pore-forming and Ca^{2+}-elevating activities of CyaA, which are independent of the adenylate cyclase activity of the toxin but require binding to CD11b/CD18 (16). The finding that this mutant does not affect to a significant extent any of the biological characteristics assessed indicates that the principal factor responsible for the effects of CyaA on macrophages is the enzymatic activity of the toxin. Consistent with a major role for the cAMP-elevating activity of CyaA in the immunosuppressive activities of the toxin, similar effects were shown to be elicited by edema factor, the Ca^{2+}/calmodulin-dependent adenylate cyclase toxin of *Bacillus anthracis* (35, 41).

Although cAMP can act as an agonist of pathways regulated by proteins other than PKA, such as cyclic nucleotide-gated ion channels and the Rap-specific guanine nucleotide exchangers EPACs, PKA is its principal ubiquitous and multifunctional target (15). The effects of increases in the cAMP levels in lymphocytes and macrophages appear to be largely dependent on the capacity of the toxin to activate PKA. Indeed, PKA has been reported to inhibit cytokine and chemokine production by macrophages and dendritic cells (1). Furthermore, similar to CyaA, PKA blocks monocyte differentiation of dendritic cells at a semimature state which promotes IL-10 production, thereby favoring regulatory T-cell development (40). The role of PKA in the inhibitory activity of CyaA on Erk activation in T cells and macrophages is strongly supported by the significant recovery of MAP kinase activation by the PKA inhibitor KT5720, even at the very low concentrations used in our experiments, which allow selective inhibition of PKA (26). The results do not, however, rule out the possibility that cAMP effectors other than PKA contribute to the immunosuppressive effects of the toxin. A potential candidate is the Rap1 activator EPAC1, which has recently been reported to inhibit FcR-dependent phagocytosis in macrophages (8) and to mediate the inhibitory effects of anthrax edema toxin on endothelial cell chemotaxis (21).

The data presented in this report show that CyaA suppresses signal transduction by surface receptors with a completely different organization. The TCR is indeed a tyrosine kinase-coupled receptor (24), CXCR4 and CCR5 are coupled to adenylate cyclase through heterotrimeric Gi proteins (47), and TLR4 is coupled to the serine/threonine kinase IRAK through the adaptor MyD88 (20). The specific mechanisms underlying the inhibitory activity of PKA on these receptors are only beginning to be unraveled. Initiation of TCR signaling is crucially dependent on phosphorylation of the CD3 ζ chain by Lck (24). By potentiating the activity of the inhibitory kinase Csk, PKA contributes to maintaining Lck in an inactive state, thereby preventing firing of the TCR signaling cascade (43). PKA also interferes with heterotrimeric G protein-coupled receptors, such as chemokine receptors, at a very early step in the pathway. This is strikingly exemplified by the autoregulatory loop of the β2 adrenergic receptor. This receptor, normally coupled to a Gs protein, activates adenylate cyclase following ligand binding, thereby promoting PKA activation. PKA in turn phosphorylates specific serine residues in the receptor, which results in a switch of receptor coupling from Gs to Gi, leading to attenuation of PKA activity and sustained activation of a tyrosine phosphorylation-dependent pathway regulated by Src (10). On the other hand, there is at present no evidence indicating that PKA might interfere with the early steps of TLR signaling, suggesting that inhibition of TLR4 by CyaA might occur at a downstream step. Whatever the mechanism of surface receptor coupling to the intracellular signaling machinery, MAP kinase cascades are also direct targets of PKA, as exemplified by the PKA-dependent phosphorylation of a negative regulatory serine residue of Raf, the most upstream component in the MAP kinase cascade leading to Erk activation (15), indicating that these signaling modules can be inhibited through both receptor-proximal and receptor-distal pathways regulated by PKA. The recent demonstration that PKA activity is compartmentalized within the cell by specific adaptors, known as AKAPs (3), suggests that distinct pools of active PKA may act independently on the multiple effector pathways which control immune cell activation and chemotaxis. Bacterial adenylate cyclases which enter the cell through different routes, thereby generating local cAMP gradients, may be instrumental in tracking the role of compartmentalized pools of active PKA in signaling by different receptors.

Mammals have developed an extremely effective and versatile immune system, and a key to successful infection by pathogens is the neutralization or at least evasion of these immune defenses for the time required to colonize the host, multiply, and spread to other individuals. In this respect, it is noteworthy that virulence factors of completely unrelated bacteria, such as *B. anthracis* (35, 41), *Helicobacter pylori* (5), and *B. pertussis*, have been independently shaped by evolution to target highly conserved signaling modules, the MAP kinase cascades, which represent central nodes in the orchestration of immune responses. In the immune system MAP kinase cascades are implicated in signaling by antigen receptors, cytokine receptors, chemokine receptors, and TLRs and, as such, represent an attractive target for neutralizing both innate and adaptive immune responses. As mentioned above, cAMP/PKA acts as a potent and ubiquitous suppressor of MAP kinases by affecting multiple steps of the signal transduction cascades triggered by a wide array of surface receptors. The widespread use of cAMP-elevating toxins by many different bacteria (4) may indicate that there is an evolutionary advantage in the inactivation of MAP kinases, which results in effective and systemic paralysis of the host immune defenses.

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