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Bordetella pertussis Adenylate Cyclase Toxin Blocks Induction of Bactericidal Nitric Oxide in Macrophages through cAMP-Dependent Activation of the SHP-1 Phosphatase

Ondrej Cerny, Jana Kamanova,1 Jiri Masin, Ilona Bibova, Karolina Skopova, and Peter Sebo

The adenylate cyclase toxin–hemolysin (CyaA) plays a key role in the virulence of Bordetella pertussis. CyaA penetrates complement receptor 3–expressing phagocytes and catalyzes uncontrolled conversion of cytosolic ATP to the key second messenger molecule cAMP. This paralyzes the capacity of neutrophils and macrophages to kill bacteria by complement-dependent oxidative burst and opsonophagocytic mechanisms. We show that cAMP signaling through the protein kinase A (PKA) pathway activates Src homology domain 2 containing protein tyrosine phosphatase (SHP) 1 and suppresses production of bactericidal NO in macrophage cells. Selective activation of PKA by the cell-permeable analog N6-benzoyladenosine-3′,5′-cyclic monophosphate interfered with LPS-induced inducible NO synthase (iNOS) expression in RAW264.7 macrophages, whereas inhibition of PKA by H-89 largely restored the production of iNOS in CyaA-treated murine macrophages. CyaA/cAMP signaling induced SHP phosphatase–dependent dephosphorylation of the c-Fos subunit of the transcription factor AP-1 and thereby inhibited TLR4-triggered induction of iNOS gene expression. Selective small interfering RNA knockdown of SHP-1, but not of the SHP-2 phosphatase, rescued production of TLR-inducible NO in toxin-treated cells. Finally, inhibition of SHP phosphatase activity by 5-methylamino-2′-O-methyladenosine-3′,5′-cyclic monophosphate (cyclic AMP) largely restored the production of iNOS in CyaA-treated murine macrophages. CyaA/cAMP signaling induced SHP phosphatase activity and may regulate numerous receptor signaling pathways in leukocytes. Hijacking of SHP-1 by CyaA action then enables B. pertussis to evade NO-mediated killing in sentinel cells of innate immunity. The Journal of Immunology, 2015, 194: 4901–4913.
transcription factors NF-κB and AP-1 in iNOS expression further appears to be modulated by the activity of the SHP-1, which was also shown to modulate the activity of the transcription factor STAT1 and of the IRF-1 (22, 23).

Production of the iNOS enzyme was previously observed to play a role in host defense against B. pertussis infection, indicating that the pathogen may be sensitive to NO-mediated killing. Indeed, naive iNOS-deficient mice were found to be more susceptible to B. pertussis respiratory challenge than were wild-type mice, and upon immunization with a whole-cell pertussis vaccine, the iNOS-deficient mice were less efficiently protected from infection than their wild-type littermates (17). It has, however, not been explored whether B. pertussis uses manipulation of iNOS expression in macrophages as part of its virulence strategy. We report that already low CyaA doses inhibit iNOS expression and NO production in mouse macrophages and that the AC enzyme activity of the CyaA toxin is essential for extended survival of B. pertussis in macrophage cells. We show that cAMP signaling leads to activation of tyrosine phosphatase SHP-1 and that this blocks TLR-induced NO production.

Materials and Methods

Abs and reagents

Escherichia coli 0111:B4 LPS, 3-isobutyl-1-methylxanthine (IBMX), and N6,N2-O dibutyryl adenosine-3′,5′-cyclic monophosphate (db-cAMP), and N6-benzoyladenosine-3′,5′-cyclic monophosphate (6-Bnz-cAMP) and 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8-ClP-cAMP) were from BIOLOG Life Science Institute. N6-β-guanosine 3′:5′-cyclic monophosphate was obtained from Sigma-Aldrich. The plasmid pSS4245 allelic exchange vector, generously provided by Dr. S. Stibitz (25), was verified by PCR sequencing of relevant portions of the gene.

Expression of introduced as well as the absence of undesired unmarked mutations in obtained clones were characterized for phenotypic change, and the presence of the pSS4245 plasmid was verified using a Safire2 microplate reader (Tecan), and data output is given in relative light units integrated over 1 h of measurement.

Determination of ROS production

ROS production was measured using a luminol-based assay as described (28). Briefly, 5 × 10⁵ RAW264.7 macrophages in HBSS supplemented with 1% glucose and 2 mM CaCl₂ were incubated for 3 min at 37°C with 150 μM luminol, before cells were transferred to wells containing the given activator (e.g., human complement–opsonized zymosan at 10 mg/ml, complement-opsonized B. pertussis at multiplicity of infection [MOI] 150), or unopsonized B. pertussis at an MOI 10:1, respectively. Luminescence was recorded using a Safire2 microplate reader (Tecan), and data output is given in relative light units integrated over 1 h of measurement.

Opsonization of bacteria with human complement

Randomized fresh human blood was purchased at the transfusion unit of the Thomayer Hospital in Prague, and human serum was obtained by centrifugation at 1200 rpm, 20 min, 17°C. Prior to use, the sera were controlled by ELISA for the absence of any detectable amounts of Abs recognizing the PT, CyaA, and FHA Ags of B. pertussis (data not shown).

For opsonization by human complement, 1 × 10⁶ heat-killed B. pertussis cells (70°C, 30 min), or 1 mg of zymosan, were incubated with 50% human serum for 30 min at 37°C under gentle shaking, and the suspensions were washed twice with serum-free HBSS.

Bacterial strains

Bacterial strains were derived from B. pertussis Tohama I (B.p. cyaA-wt), obtained as strain CIP 81.32 from the Collection of Institute Pasteur, Paris, France. The B.p. ΔcyaA and B.p. cyaA-AC strains, carrying an in-frame deletion of the open reading frame on the chromosome, or secreting an enzymatically inactive CyaA-AC toxin (24), were constructed using the pSS4245 allelic exchange vector, generously provided by Dr. S. Stibitz (25). Plasmid constructs were transformed into E. coli SM10 aprI and transferred by conjugation into recipient B. pertussis cultured for 4 d under modulating conditions (Bvg−) on BGA plates (Bordet-Gengou agar, Becton Dickinson) containing 15% defibrinated sheep blood and 50 mM MgSO₄. B. pertussis clones having the plasmid construct integrated into the chromosome were selected for 5 d at 37°C on plates that contained 50 mM MgSO₄, 500 μg/ml streptomycin, 30 μg/ml ampicillin, and 40 μg/ml kanamycin and were restreaked on the same plates for an additional 5 d, before plating on BGA lacking MgSO₄ to select for allelic exchange. The obtained clones were characterized for phenotypic change, and the presence of the introduced plasmid construct as well as the absence of undesired unmarked mutations was verified by PCR sequencing of relevant portions of the cyaA gene.

Production of key virulence factors like PT, PRN, FHA, and CyaA was measured using a Safire2 microplate reader (Tecan), and data output is given in relative light units integrated over 1 h of measurement.

Cell cultures and handling

The L929 CMG cells (a kind gift of T. Bulicka, Institute of Molecular Genetics of the ASCR, v.v.i., Prague, Czech Republic) were used for M-CSF production (27). Bone marrow macrophage-like cells (bone marrow–derived macrophages [BMDMs]) were obtained from femoral and tibial bones of 6-wk-old female C57BL/6 mice by cultivating bone marrow–derived cells in DMEM containing 10% (v/v) FCS in the presence of 10% (v/v) conditioned media of L929 CMG cells and antibiotic-antimycotic solution (0.1 mg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin; Sigma-Aldrich) for 7 d at 37°C in a humidified air-CO₂ (5%) atmosphere.

RAW264.7 mouse macrophage cells (ATCC cat. no. TIB 71) were grown in RPMI 1640 medium supplemented with 10% (v/v) FCS. Prior to assays, the phosphate-buffered RPMI 1640 medium was replaced with HEPES-buffered DMEM medium with 10% (v/v) FCS, which contains 1.9 mM Ca²⁺, and the cells were allowed to rest in DMEM for 2 h before toxin addition.

Determination of ROS production

ROS production was measured using a luminol-based assay as described (28). Briefly, 5 × 10⁵ RAW264.7 macrophages in HBSS supplemented with 1% glucose and 2 mM CaCl₂ were incubated for 3 min at 37°C with 150 μM luminol, before cells were transferred to wells containing the given activator (e.g., human complement–opsonized zymosan at 10 mg/ml, complement-opsonized B. pertussis at multiplicity of infection [MOI] 150), or unopsonized B. pertussis at an MOI 10:1, respectively. Luminescence was recorded using a Safire2 microplate reader (Tecan), and data output is given in relative light units integrated over 1 h of measurement.

Determination of arginase activity

Arginase activity was measured as previously described (31). RAW264.7 cells (10⁵ per well) were seeded into 96-well plates and incubated with indicated concentrations of CyaA for 24 h and lysed in 50 μl buffer containing 50 mM Tris HCl (pH 7.5), 0.1% Triton X-100, 50 mM NaCl, 10 mM Na₂PO₄, 1 mM Na₂VO₃, 50 mM Calycin A, and the Complete Mini EDTA-free protease mixture inhibitor (Roche). After 30 min at room temperature, 10 mM MnCl₂ was added and the samples were heated for 10 min at 55°C. An equal volume of 0.5 M l-arginine was added, and the mixture was incubated for 60 min at 37°C. The reaction was stopped by the addition of 0.2% Tween 20 in 50 mM HCl. The samples were boiled for 15 min at 100°C, neutralized with 150 mM unbuffered imidazole, and cAMP concentration was determined by ELISA.

Immunodetection of proteins

RAW264.7 or BMDM cells (10⁶ per well) were cultured in 12-well plates in RPMI and replaced into DMEM medium for 2 h, and inhibitors were
added 1 h before the addition of CyaA. Cells were washed twice with ice-cold PBS and lysed with 1% Nonidet P-40 in 20 mM Tris-HCl (pH 8.0) buffer containing 100 mM NaCl, 10 mM EDTA, 10 mM Na2HPO4, 1 mM Na2VO4, 50 mM NaF, 10 mM Calcinium A, and Complete Mini protease inhibitors (Roche). SDS-PAGE–separated proteins were transferred onto nitrocellulose membranes and probed by the indicated mAbs and secondary Ab-peroxidase conjugates (1:5000; GE Healthcare); chemiluminescence was revealed using the West Femto Maximum Sensitivity Substrate (Pierce); and signals were quantified using the ImageQuant LAS 4000 imaging system (Fuji) and analyzed using AIDA software (v. 3.28; Raytest Isotopenmessgeraete).

RNA isolation and quantitative real-time PCR
RAW264.7 cells (2 × 10^6 per well) were incubated in DMEM with 100 ng/ml LPS and/or CyaA toxin at the indicated concentrations for 24 h, washed twice with ice-cold PBS, and lysed with TRIzol Reagent (Life Technologies). Total RNA was extracted using the RNasey Mini Kit (QIAGEN), including a treatment with DNase I (Ambion), and 1 μg total RNA was reverse transcribed into cDNA in a 25-μl reaction using M-MLV Reverse Transcriptase (Promega) and 0.5 μg random hexamers and oligo(dT) mixture. Quantitative PCR was performed on a Bio-Rad CFX96 instrument, using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) and gene-specific primers (Supplemental Table I). A total of 200 nM of each primer with 1× SYBR Green was used in a 20-μl qPCR reaction, with an initial step at 95˚C for 2 min, followed by 40 cycles of reaction, with an initial step at 95˚C for 2 min, followed by 40 cycles of

siRNA silencing
The siRNA transfection protocol (Dharmacon) was optimized for RAW264.7 cells, using FITC-labeled siGlo RNA interference control. A total of 2 × 10^5 cells per well were seeded into 96-well plates and grown overnight in RPMI medium without antibiotics and transfected with 50 nM siRNA using 0.05% DharmaFECT 4 reagent. Transfection was repeated 48 h later to increase silencing efficacy. After an additional 48 h, the cells were repeatedly washed with prewarmed DMEM medium and tested for NO production and/or target-protein expression. Unspecific effects were controlled in all experiments by including untreated, mock-treated (DharmaFECT 4 reagent only), and nontargeting siRNA-transfected cells, respectively.

In vitro killing assay
B. pertussis suspensions were grown in liquid Stainer–Scholte medium (33) to OD600 = 1. DAE-NONOate or hydrogen peroxide was added, and after 2 h of incubation at 37˚C, the serial dilutions were plated on BGA for determination of viable CFUs after 5 d of growth.

RAW264.7 cell infection by B. pertussis
Cell infection assays were performed as described by Lamberti et al. (15). Briefly, RAW264.7 cells (10^5 per well) were seeded overnight into 24-well plates in RPMI medium with 10% heat-inactivated FCS, but without antibiotics. Prior to infection, RPMI 1640 medium was replaced by DMEM (containing 1.9 mM Ca^2+ and 10% [v/v] HIFCS), and exponentially growing B. pertussis cells expressing GFP from the pBBRcyaGFP plasmid were added at MOI 10:1. The bacteria were gently spun onto RAW264.7 cells expressing GFP from the pBBRcyaGFP plasmid were added at MOI 10:1. The bacteria were gently spun onto

Fluorescence microscopy of cell-associated bacteria
After 1 h of incubation with B. pertussis/pBBRcyaGFP bacteria, the RAW264.7 cells were washed three times with prewarmed DMEM medium and fixed with 4% paraformaldehyde in PBS for 20 min. Non-phagocytosed bacteria attached to the surface of macrophage cells were decorated with rabbit polyclonal antiserum against B. pertussis (a kind gift of Dr. B. Vecerek) and stained with goat anti-rabbit IgG conjugated with Cy5. F-actin was stained with tetramethylrhodamine isothiocyanate–conjugated phalloidin (0.5 μg/ml; Sigma-Aldrich) and DNA with DAPI (10 ng/ml; Sigma-Aldrich) in PBS supplemented with 3% BSA (v/v) for 30 min. Samples were mounted on glass coverslips in Mowiol, and images were captured using the CellIR Imaging Station based on the Olympus IX81 fluorescence microscope using a 100×/1.35 oil objective. Images were captured in 0.1-μm Z-stack layers across the complete cell, and the three-dimensional image was reconstructed using three-dimensional deconvolution. Internalized bacteria, emitting only green fluorescence (GFP*), and cell surface–associated yellow bacteria (emitting both red [Cy5] and green [GFP] fluorescence), were counted for at least 50 cells per sample and experiment.

SHP-1 activity assay
A total of 5 × 10^5 RAW264.7 cells were incubated in DMEM with 100 ng/ml LPS and/or 10 ng/ml CyaA and washed twice with ice-cold PBS. Cells were lysed and SHP-1 was immunoprecipitated from postnuclear fractions using the Pierce Co-Immunoprecipitation (Co-IP) Kit. The columns with bound SHP-1 were washed six times with reaction buffer (25 mM imidazole pH 7.2, 45 mM NaCl, 1 mM EDTA in phosphate-free water). Then 250 μM tyrosine phosphopeptide (RLLIEDAEpYAARG; Upstate Biotechnologies) was loaded on the columns. After 30 min at 37˚C, the free phosphopeptide was determined in column eluates using Malachite Green Phosphate Assay (ScienCell). SHP-1 was next eluted from the columns and detected by immunoblotting. Specific SHP-1 activity was calculated as the amount of free phosphate normalized to the total eluted SHP-1 protein amount.

NF-κB translocation assay
Translocation of NF-κB into the nucleus was analyzed by confocal microscopy. A total of 10^5 RAW264.7 cells were seeded onto glass coverslips 1 d before the experiment and after incubation with or without LPS (100 ng/ml) and CyaA (10 ng/ml) for 3 h; the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Upon blocking with PBS-5% BSA (v/v), NF-κB staining was performed at 4˚C overnight in PBS-2% BSA (v/v) buffer with anti-p65 Ab (dilution 1/100) and revealed using goat anti-rabbit secondary Ab conjugated with FITC (Sigma-Aldrich). Glass coverslips were mounted in Mowiol, and images were captured using Leica confocal microscope TCS SP2 (Wetzlar).

NF-κB reporter assay
RAW264.7 cells were transfected using the FuGENE HD transfection reagent according to the manufacturer’s instructions. Briefly, cells (10^5 per well) were seeded into 96-well plates in RPMI medium without antibiotics. On the next day, a mixture of pNF-κB-d2EGFP with pEGFP/mCherry-N1 and FuGENE HD in the ratio 2:8 was added to the cell culture. After 18 h, cells were washed with preheated DMEM medium and incubated in the presence or absence of CyaA (10 ng/ml) and LPS (100 ng/ml). After 6 h, the ratio of mCherry and GFP expression was analyzed using flow cytometry (LSR II; BD).

Ethics statement
All animal experiments were approved by the Animal Welfare Committee of the Institute of Microbiology of the Academy of Sciences of the Czech Republic. Animals were handled according to the Guidelines for the Care and Use of Laboratory Animals, the Act of the Czech National Assembly, Collection of Laws no. 149/2004, inclusive of the amendments, on the Protection of Animals against Cruelty, and Public Notice of the Ministry of Agriculture of the Czech Republic, Collection of Laws no. 207/2004, on care and use of experimental animals.

Statistical analysis
The significance of differences in values was assessed by the Student t test.

Results
B. pertussis is sensitive to NO-mediated killing and blocks inOS expression and NO production in macrophages through CyaA-catalyzed formation of cAMP
CyaA toxin production was previously found to be required for persistence of unopsonized B. pertussis in macrophages (14). It remained, however, unclear whether the pore-forming hemolytic activity of CyaA contributed to survival of B. pertussis in macrophages, or whether this depended entirely on the capacity of the cell-invasive AC enzyme of CyaA to elevate cAMP concentrations.
in cells. Therefore, survival in murine RAW264.7 macrophage cells of a hemolytic *B. pertussis* strain producing a catalytically inactive CyaA-AC<sup>−</sup> toxoid unable to convert cellular ATP to cAMP (*B. pertussis* cyaA<sup>−</sup> AC<sup>−</sup>) was compared at MOI 10:1 with persistence of the parental *B. pertussis* Tohama I strain (*B. pertussis* cyaA<sup>+</sup>-wt) producing intact CyaA. As a negative control, a mutant not producing CyaA owing to the deletion of the entire cyaA open reading frame (*B. pertussis* ΔcyA) was used. As shown in Fig. 1, association of cyaA-AC<sup>−</sup> or of ΔcyA bacteria with the RAW264.7 macrophages after 1 h of coincubation was not impaired by the inability to produce enzymatically active CyaA toxin. When compared with the cyaA-wt strain, internalization of bacteria producing the AC<sup>−</sup> toxoid (cyA-AC<sup>−</sup>), or not producing CyaA at all (ΔcyA), was even enhanced (∼120%). Although the differences were not statistically significant, this observation would go well with the previously shown capacity of CyaA to provoke cAMP-triggered unproductive actin cytoskeleton rearrangements and macrophage ruffling (11). In contrast to enhanced initial uptake, the survival of the cyaA-AC<sup>−</sup> and ΔcyA bacteria within murine macrophages was significantly impaired in 24 h, as shown in Fig. 1B. After 48 h of incubation, about two orders of magnitude lower viable counts of the mutants were recovered from RAW264.7 cells. Hence, the enzymatic AC activity of CyaA was crucial for extended survival of unopsonized *B. pertussis* inside murine macrophages.

CyaA-catalyzed elevation of cAMP was previously shown to efficiently block oxidative burst in neutrophils (8, 10). However, as shown in Fig. 2A, despite responding by production of ROS to stimulation by complement-opsonized zymosan, the RAW264.7 cells produced little or no ROS upon incubation with heat-killed *B. pertussis* cells, irrespective of whether these were opsonized by complement or not. It hence appeared unlikely that the unopsonized bacteria, eliciting even lower ROS production than opsonized bacteria, were killed in RAW264.7 cells by oxidative burst. Macrophage cells are, however, known to produce substantial amounts of bactericidal NO (34). Moreover, *B. pertussis* was shown to persist much better inside activated macrophages from iNOS-deficient mice (17). Therefore, we tested whether suppression of bactericidal NO production by CyaA contributed to survival of unopsonized *B. pertussis* inside murine macrophages.

As shown in Fig. 2B, indeed, *B. pertussis* is rather susceptible to NO-mediated killing in vitro, because exposure to 400 μM DEA-NONOate as a donor of NO radicals (35, 36) significantly decreased viability of the bacteria, albeit less than upon oxidative killing by 100 μM H<sub>2</sub>O<sub>2</sub> (37). As further shown in Fig. 2C, significantly decreased NO production was observed in RAW264.7 macrophages infected with bacteria producing intact CyaA (*B. pertussis* cyaA-wt), compared with RAW264.7 cells coincubated with heat-inactivated *B. pertussis*, or with macrophages infected with *B. pertussis* cyaA-AC<sup>−</sup> or *B. pertussis* ΔcyA bacteria, respectively. This suggested that signaling of CyaA-produced cAMP interfered with TLR-triggered induction of NO production. As indeed shown in Fig. 2D, a detectable increase of intracellular cAMP concentration in RAW264.7 cells was observed already in 5 min of exposure to CyaA concentrations as low as 10 ng/ml that correspond to toxin amounts detected in nasopharyngeal fluids from *B. pertussis*-infected humans and primates (38). The CyaA-catalyzed cAMP production in cells then peaked within 1 h, and intracellular cAMP concentrations remained elevated for 24 h, irrespective of whether the cells were activated by the addition of *E. coli* LPS (100 ng/ml) or not. Hence, signaling of LPS used for induction of NO production did not interfere with cAMP elevation in macrophage cells. The peak amounts of cAMP produced upon exposure to 10 ng/ml of CyaA were fully comparable to cAMP levels resulting from infection of macrophages by wild-type *B. pertussis* for 24 h at the MOI 10:1, as used in the intracellular survival assay (cf. Fig. 1). It should, however, be noted that some increase of cAMP levels was observed also upon prolonged macrophage infection by the AC enzyme-deficient cyaA-AC<sup>−</sup> mutant (Fig. 2D). This was most likely due to the action of the pertussis toxin (PT) produced...
FIGURE 2. *B. pertussis* is sensitive to oxidative killing. (A) RAW264.7 macrophages were activated with complement-opsonized zymosan, with complement-opsonized *B. p.* _cyaA-wt_, or with unopsonized *B. p.* _cyaA-wt_ bacteria. Production of ROS was measured over a period of 1 h. Means ±SD from three independent experiments performed in triplicates (n = 9) are given. **p < 0.001 versus negative control. (B) Exponentially growing cultures of *B. pertussis* (OD600 = 1) were divided into aliquots to which corresponding volumes of solvent control (ethanol), or of 400 μM DAE-NONOate solution, or of 100 μM H2O2 were added for 2 h. Following plating on BGA, the numbers of viable CFUs were counted upon 5 d of growth. The values are means ±SD from at least five independent experiments performed in duplicates (n = 10). **p < 0.001 versus control. (C) RAW264.7 macrophages were incubated with heat-inactivated (HI) *B. p.* _cyaA-wt_, or live *B. p.* _cyaA-wt_, *B. p.* _cyaA-AC2_, or *B. p.* _ΔcyaA_ cells for 24 h before NO production was measured in culture supernatants. Values represent the means ±SD from three experiments performed in triplicates (n = 9). *p < 0.001 versus HI control. (D) RAW264.7 macrophages were treated with 10 ng/ml CyaA, in the presence or absence of 100 ng/ml of LPS for the indicated times, or were infected for 24 h with the indicated *B. pertussis* strains, as described in Fig. 1. Concentration of cAMP was measured in cell lysates by ELISA. One representative result out of three independent experiments performed in triplicates is shown. (E and F) RAW264.7 macrophages or BMDM, respectively, were treated in the presence or absence of 100 ng/ml of LPS from *E. coli* with the CyaA variants (CyaA concentrations are given in ng/ml) or with 1 mM db-cAMP for 24 h, 10 μM IBMX was added 1 h prior to db-cAMP addition to cells, and the produced NO was measured by the Griess reagent or by the DAF-FM probe, as indicated in Materials and Methods. Values represent means ±SD from three independent experiments performed in triplicates (n = 9). **p < 0.001 versus LPS-treated control. (G) RAW264.7 cells or BMDM were isolated from three animals, and one blot representative out of three experiments is shown (n = 3). Mean ±SD iNOS amounts were determined by densitometric analysis. *p < 0.01, **p < 0.001 versus LPS-treated control. (H) (Figure legend continues)
by the mutant strain, as PT can deregulate the activity of the cellular adenyl cyclase enzymes through inhibitory ADP-ribosylation of the Gαi subunits of the trimeric G proteins (39). The PT-triggered cAMP elevation occurs, however, several hours after PT penetration into cells and requires external stimuli activating the endogenous AC enzyme, while yielding substantially lower levels of cAMP than what CyaA does produce within minutes of contact with phagocytes (40). Hence, likely because of the delay in the onset of PT-mediated cAMP elevation, which takes 8–12 h to manifest (40), the PT-mediated elevation of cAMP did not prevent the rapid killing of the cyaA-AC− bacteria inside RAW264.7 cells (cf. Fig. 1).

As next shown in Fig. 2E, exposure to as little as 1 ng/ml of CyaA did significantly decrease LPS-induced NO production in RAW264.7 macrophages, as detected by the nitrite-determining Griess reaction and corroborated by the use of the fluorescence probe DAF-FM, which would also detect the nitrite eventually converted to nitrate. The inhibition of NO production was clearly specific and specifically due to the elevation of cAMP concentration by the AC enzyme activity of the CyaA toxin, as the LPS-inducible NO production was also inhibited upon RAW264.7 cell exposure to the membrane-permeable cAMP analog db-cAMP (1 mM). In contrast, cell exposure to the nonenzymatic CyaA-AC− toxoid, unable to raise cAMP levels even at concentrations as high as 100 ng/ml, had no effect on induction of NO production by LPS. As shown in Fig. 2F, CyaA also inhibited NO production in BMDM cells, confirming that primary murine macrophages were highly susceptible to CyaA-provoked inhibition of bactericidal NO induction.

As further revealed by immunodetection with specific Abs in Fig. 2G, exposure to 1 ng/ml of CyaA already resulted in a significant decrease of iNOS production in LPS-stimulated RAW264.7 cells, or primary BMDMs. Moreover, no iNOS protein was detected in cells exposed to higher toxin concentrations (10 or 100 ng/ml) or to 1 mM db-cAMP. Indeed, a strong decrease of iNOS mRNA level was detected by quantitative PCR in so treated RAW264.7 cells, as shown in Fig. 2H. Hence, treatment with the cell-permeable cAMP analog fully reproduced the impact of CyaA toxin action, whereas cell exposure to the CyaA-AC− toxoid had no effect.

Previously, Cheung and coworkers (41) suggested that CyaA might interfere with bactericidal NO production through induction of arginase expression. A cAMP-dependent increase of arginase activity was, indeed, observed in CyaA-treated RAW264.7 cells, as shown in Fig. 3A. However, as shown in Fig. 3B, the production of NO was not restored in CyaA-treated macrophages either upon selective inhibition of arginases by 100 μM nor-NOHA or at saturating concentrations of the arginase substrate L-arginine (12.8 mM), as shown in Fig. 3C. It can thus be concluded that rather than by enhancement of arginase activity and degradation of the L-arginine substrate, the CyaA-produced cAMP signaling suppressed NO production in LPS-stimulated RAW264.7 cells through inhibition of iNOS gene expression.

Intriguingly, induction of arginase would be indicative of macrophage polarization toward the M2 phenotype; however, induction of the M1 phenotype–associated cyclooxygenase-2 enzyme activity in murine macrophages by CyaA was previously reported (42) and we did not observe any alteration in the protein levels of the Mox macrophage markers NRF2 and HO-1 (Supplemental Fig. 1) (43). It appears, therefore, that the subversive cAMP-dependent signaling elicited by CyaA does not yield any clear macrophage polarization.

**CyaA blocks iNOS expression by cAMP-dependent activation of protein kinase A**

cAMP activates signaling of protein kinase A (PKA) and of the exchange protein directly activated by cAMP (Epac) (44). To determine whether PKA or Epac activities were involved in suppression of LPS-induced iNOS expression by cAMP signaling, RAW264.7 cells were preincubated with 10 μM of the phosphodiesterase inhibitor IBMX and stimulated by LPS in the presence of 1 mM cell-permeable cAMP analogs that either activate both PKA and Epac, such as db-cAMP, or activate selectively only the PKA (6-Bnz-cAMP) or only Epac (8-CPT-cAMP), respectively. As shown in Fig. 4A, signaling of 1 mM db-cAMP or of the PKA-specific activator 6-Bnz-cAMP provoked as significant an inhibition of LPS-triggered NO production as the exposure of cells to CyaA toxin (10 ng/ml). In contrast, treatment with the Epac-selective activator 8-CPT-cAMP had little effect on LPS-triggered NO production. The PKA-activating 6-Bnz-cAMP and db-cAMP treatments, but not signaling of 8-CPT-cAMP (Epac activation), also blocked iNOS protein production in RAW264.7 macrophages, as shown in Fig. 4B. An insignificant effect of the Epac activator was, however, also observed. It remains to be clarified if it was due to a low level of nonspecific activation of PKA by 8-CPT-cAMP or whether Epac signaling also contributed to some extent to suppression of iNOS gene expression. Nevertheless, as shown in Fig. 4C, activation of PKA by toxin-produced cAMP appeared to be the dominant pathway through which the CyaA-produced cAMP suppressed iNOS-mediated NO production in macrophages. The inhibition could be reversed, albeit not fully, by preincubation of cells with the PKA inhibitor H-89 (10 μM). Besides possibly incomplete inhibition of PKA by H-89 at high cAMP levels produced by CyaA, the incomplete restoration of iNOS expression in H-89–treated cells was likely due to some nonspecific off-target effects of H-89. As also shown in Fig. 4C, the H-89 inhibitor (10 μM) itself caused some observable reduction of LPS-stimulated iNOS expression. H-89 is, indeed, known to be not entirely specific for PKA and to influence activities of other AGC kinases at the concentrations used in this study (45). Moreover, the more specific inhibitors of PKA, such as mPKI or cAMPS, were less potent in reversal of the overwhelming cAMP-mediated impact of CyaA action on iNOS expression (data not shown). It remains, therefore, possible that H-89 may also interfere with induction of iNOS expression at some step downstream of PKA signaling. Collectively, these results show that the CyaA toxin inhibits iNOS expression predominantly through cAMP-mediated hijacking of the PKA signaling pathway.

**cAMP signaling through PKA causes dephosphorylation of AP-1**

iNOS expression depends to large extent on the activity of the transcription factor AP-1 (20, 21). Therefore, we examined whether cAMP signaling of CyaA affected the regulatory phosphorylation of the c-Fos subunit of AP-1. As shown in Fig. 5, no activating phosphorylation of c-Fos was detected in untreated cells, whereas activation of RAW264.7 macrophages by LPS resulted in hyperphosphorylation of c-Fos, detected as two bands in P–c-Fos immunoblots. Exposure of LPS-activated RAW264.7 cells to 10 ng/ml of CyaA then provoked a near-complete disap-

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RAW264.7 cells were treated as in (E). After 24 h, iNOS mRNA was quantified by quantitative RT-PCR. Relative iNOS mRNA levels normalized to iNOS mRNA extracted from LPS-treated control cells are expressed as fold change. Means ± SD of three independent experiments performed in duplicates (n = 6) are given. **p < 0.001 versus LPS-treated control. N.C., negative control.
The appearance of the upper hyperphosphorylated c-Fos isoform, whereas the hypophosphorylated lower band of P–c-Fos was detected at comparable levels as in LPS-activated and mock-treated cells. In contrast, specific activation of Epac with 1 mM 8-CPT-cAMP did not affect TLR-(LPS)–triggered phosphorylation of c-Fos, whereas the action of CyaA was almost fully reproduced by the PKA-activating analogs db-cAMP (1 mM) and 6-Bnz-cAMP (1 mM), respectively. Compared with CyaA action, the activation of PKA by the cAMP analogs yielded a less comprehensive effect.

**FIGURE 3.** CyaA induces increase in arginase activity. (A) RAW264.7 cells were incubated with CyaA (ng/ml) and 100 ng/ml LPS for 24 h before total arginase activity was measured in cell lysates. The values are means ± SD from three independent experiments performed in triplicates (n = 9). (B) RAW264.7 cells were preincubated for 1 h in the presence or absence of 100 μM nor-NOHA prior to activation with 100 ng/ml of LPS and/or addition of 10 ng/ml CyaA for 24 h. Values represent means ± SD from three independent experiments performed in triplicates (n = 9). (C) RAW264.7 cells were incubated with 100 ng/ml of LPS and 10 ng/ml of CyaA in the presence of the indicated concentration of L-arginine for 24 h. Values represent the means ± SD from three independent experiments performed in triplicates (n = 9). **p < 0.001 versus LPS-treated control.

**FIGURE 4.** PKA activation by CyaA inhibits NO production. (A) RAW264.7 cells were preincubated for 1 h in the presence or absence of 10 μM IBMX before LPS (100 ng/ml) and CyaA (10 ng/ml), 8-CPT-cAMP, 6-Bnz-cAMP, or db-cAMP (1 mM) were added, respectively. NO production was detected after 24 h. Values represent the means ± SD from three independent experiments performed in triplicates (n = 9). (B) RAW264.7 cells were treated as in (A). CyaA was added to the cells concomitantly with LPS, and iNOS was detected by immunoblotting. One representative blot out of three independent experiments is shown. (C) RAW264.7 cells were preincubated for 1 h, with or without the PKA inhibitor H-89 (10 μM), prior to stimulation with 100 ng/ml LPS and 10 ng/ml of CyaA. After 24 h, the levels of iNOS protein were detected by immunoblotting. One representative blot out of three independent experiments is shown. The means ± SD of densitometric analysis from three experiments are shown (n = 3). **p < 0.001 versus LPS-treated control, †p < 0.01 versus each other.
active hypophosphorylated form (lower band) were detected by immunoblotting. After 24 h, the active hyperphosphorylated P–c-Fos form (slower migrating upper band) and the inactive hypophosphorylated form (lower band) were detected by immunoblotting and are indicated by arrows, respectively. **p < 0.01, † † † p < 0.001 versus LPS treated control.

CyaA/cAMP blocks iNOS expression through activation of the SHP-1 phosphatase

No protein phosphatases directly activated by cAMP signaling have as yet been identified, but the tyrosine phosphatase SHP-1 is known to be involved in the regulation of numerous receptor signaling pathways in leukocytes and it was previously implicated also in the regulation of iNOS gene expression in LPS-activated macrophages. CyaA/cAMP provokes dephosphorylation of the hyperphosphorylated P–c-Fos subunit of the AP-1 transcription factor that plays a crucial role in iNOS gene expression in LPS-activated macrophages.

**FIGURE 5.** CyaA provokes dephosphorylation of the P–c-Fos subunit of AP-1 via PKA-mediated signaling. RAW264.7 macrophages were preincubated with or without 10 μM IBMX for 1 h before addition of LPS (100 ng/ml) and/or CyaA (10 ng/ml), or db-cAMP, 6-Bnz-cAMP, or 8-CPT-cAMP (1 mM), respectively. After 24 h, the active hyperphosphorylated P–c-Fos form (slower migrating upper band) and the inactive hypophosphorylated form (lower band) were detected by immunoblotting and are indicated by arrows, respectively. **p < 0.01, † † † p < 0.001 versus LPS treated control.

Inhibition of SHP phosphatase activity abrogates B. pertussis survival in macrophages

It was next important to examine whether SHP-1 activation by CyaA accounted for the extended survival of unopsonized B. pertussis inside mouse macrophages. As shown in Fig. 7A, treatment with the SHP-1/2 inhibitor NSC87877 (500 nM) enhanced by about 2- to 3-fold the total number of B. pertussis cells associated with and internalized by the RAW264.7 macrophages. This finding matches with an earlier observation that inhibition of the SHP-1 phosphatase enhances the phagocytic activity of macrophages (47, 48). In sharp contrast, the presence of the SHP inhibitor completely abrogated the capacity of B. pertussis to survive within macrophages, as documented in Fig. 7B. Essentially no viable bacteria could be recovered from macrophages incubated with NSC87877 already at 24 h post infection, whereas in the absence of the SHP inhibitor, the bacterial viability inside cells was decreasing gradually over 72 h. Collectively, these data show that activation of the SHP-1 phosphatase by CyaA-elicited signaling of cAMP enables unopsonized B. pertussis bacteria to extend their survival inside macrophage cells (Fig. 8).

Discussion

We show that cAMP-activated signaling through PKA yields activation of the tyrosine phosphatase SHP-1. Hijacking of this novel cAMP-regulated signaling pathway by the adenylate cyclase toxin then enables B. pertussis to evade rapid NO-mediated killing inside macrophage cells. As summarized in the model shown in Fig. 8, the CyaA-cAMP–triggered activation of SHP-1 leads to dephosphorylation of the P–c-Fos subunit of the transcription factor AP-1 and results in loss of TLR-induced expression of the iNOS enzyme and loss of production of bactericidal levels of NO. This novel mechanism adds to the broad spectrum of immuno-
subversive outcomes of CyaA-catalyzed synthesis of cAMP in host phagocytes. Somewhat controversial results were previously reported on the role of cAMP in the regulation of iNOS expression in different cell types and tissues. Although cAMP was found to be a strong inducer of iNOS gene expression in rat vascular smooth muscle and mesangial cells, elevation of cAMP in other rodent cells yielded reduction of iNOS expression (49–51). It has, indeed, been previously observed that modulation of cAMP levels in RAW264.7 macrophages may impact on LPS-induced expression of iNOS and

![Figure 6](image-url)

**FIGURE 6.** SHP-1 mediates CyaA-provoked inhibition of NO production. (A) RAW264.7 macrophages were pretreated, with or without the 500 nM NSC87877 inhibitor of SHP-1/2 phosphatases, for 1 h before 100 ng/ml LPS and 10 ng/ml of CyaA were added for 24 h and the phosphorylation status of P–c-Fos was analyzed by immunoblotting of cell lysates. One representative blot out of three independent experiments is shown. The mean values ± SD from densitometric analysis are shown (n = 3). (B) RAW264.7 macrophages were treated as above, and after 24 h the production of NO was detected. Values represent the means ± SD from three independent experiments performed in triplicates (n = 9). (C) RAW264.7 cells were treated as in (A), and after 24 h, the levels of iNOS protein were detected by immunoblotting. One representative blot out of three independent experiments is shown. The means ± SD from densitometric analysis of three experiments is shown (n = 3). (D) RAW264.7 cells were transfected with nontargeting, SHP-1–, or SHP-2–specific siRNA, respectively. LPS (100 ng/ml) or CyaA (10 ng/ml) or both were added, and incubation was continued for 24 h. The production of SHP proteins was analyzed by immunoblotting. Values represent the means ± SD from three independent experiments performed in triplicates (n = 9). (E) RAW264.7 or BMDM cells were treated with 100 ng/ml LPS and CyaA (10 ng/ml) or both were added, and phosphorylation status of SHP-1 was analyzed by immunoblotting. One representative blot out of three independent experiments is shown. Mean values ± SD from densitometric analysis of three experiments is given (n = 3). **p < 0.001 versus LPS-treated control, †p < 0.01 versus each other, ‡p < 0.001 versus NSC87877 control.
We show that as little as 1 ng/ml does produce sufficient amounts of cAMP to cause a strong reduction of iNOS expression in LPS-activated macrophage cells (cf. Fig. 2G). In agreement with a previous report (54) and in contrast to CyaA, the PT produced by the mutant cyaA-AC<sup>2</sup>bacteria was unable to increase cAMP concentrations through deregulation of the endogenous AC to a level that would provoke a block of induction of bactericidal NO production in LPS-activated macrophage cells (cf. Fig. 2B). Indeed, PT action takes hours to translate into cAMP increase in cells, and it depends on additional receptor signaling–mediated activation of the endogenous AC enzyme. It is, therefore, plausible to propose that the steep increase of cAMP concentration, accomplished early upon bacterial contact with phagocytes by the CyaA toxin, was required for prevention of TLR-activated iNOS expression. This activity is likely made possible by two unique features of CyaA. First, CyaA exhibits an extraordinarily rapid mechanism of target cell penetration. Translocation of the AC domain into cells occurs by an endocytosis-independent mechanism directly across the cytoplasmic membrane of cells, and it proceeds with a half-time of only ∼30 s (55–58). Second, the calmodulin-activated catalytic domain of CyaA possesses an extremely high specific AC enzyme activity, with a turnover number of ∼2000 s<sup>−1</sup> (58). As a result, CyaA concentrations as low as those used in this study (10 ng/ml), and detected in mucosal fluids of infected infants and experimentally challenged baboons (38), are not only sufficient for ablation of superoxide production and inhibition of neutrophil extracellular trap release by host neutrophils (8–10) but also provoke inhibition of bactericidal NO production in macrophages. This finding explains why CyaA expression is crucial for the extended survival of unopsonized <i>B. pertussis</i> bacteria in macrophages. These results further highlight the unique role played by CyaA in subversion of innate immunity mechanisms, explaining the importance of the role played by CyaA in the early phases of bacterial colonization of host airways (2–4, 59).

Although major differences exist in iNOS expression regulation in humans and mice, the expression of bactericidal iNOS in human phagocytes and the role of NO in innate immunity in humans are now well established (60). iNOS expression in human macrophages, however, requires, besides activation by LPS, a simultaneous in-
volvement of several cytokine signals (e.g., IFN-γ, TNF-α, and IL-1β). This requirement makes the deciphering of signaling pathways leading to iNOS expression in human phagocytes difficult. In contrast, LPS alone is sufficient for strong iNOS expression in murine macrophages (21). Therefore, mouse cells like the RAW264.7 macrophages are preferentially used for analysis of signaling that leads to bactericidal iNOS expression in phagocytes (52, 53, 61). Using these cells, we reveal in this study the prominent role played by cAMP signaling in the regulation of activity of the transcription factors involved in iNOS expression in phagocytes. This remains scarcely documented in the literature. Activation of the NF-kB transcription factor in human keratinocytes by db-cAMP or forskolin has previously been reported (62), which accords with the observed triggering of NF-kB translocation into cell nuclei by the cAMP-elevating activity of CyaA (cf. Supplemental Fig. 2). However, NF-kB activation alone does not lead to iNOS expression, and Hickey and coworkers (63) observed previously that a CyaA production by CyaA caused a decrease of levels of both IRF1 and IRF8 transcription factors. Similarly, CAMP elevation by cholera toxin was found to cause inhibition of IRF1–IRF8 interaction (64). Furthermore, IRF1 and STAT1 activities were also found to be inhibited upon VPAC1-mediated PKA activation by a mechanism independent of suppressor of cytokine signaling 1/3 (65). Hence, the previous observations that IRF1 and STAT1 activities are regulated by cAMP agree with our results showing that CyaA activity concomitantly decreased STAT1 phosphorylation and IRF1 levels (cf. Supplemental Fig. 2D). Although STAT1 activation was previously found to be inhibited via cAMP/PKA signaling–triggered activation of SHP-2 (66), we observed in this study a highly SHP-1–specific effect of cAMP increase on LPS-triggered iNOS expression in murine macrophage cells. Moreover, inhibition of STAT1 by CyaA occurred later than inhibition of iNOS expression (cf. Supplemental Fig. 2E), showing that STAT1 inhibition did not account for the inhibition of iNOS expression. In contrast, the effects of CyaA-provoked cAMP signaling on phosphorylation of the c-Fos subunit of AP-1 correlated in time with the observed inhibition of iNOS expression. The c-Fos subunit of the transcription factor AP-1 was, indeed, shown to undergo hyperphosphorylation at multiple sites upon macrophage activation by LPS. This increases its activity (67) and accounts for the appearance of several P–c-Fos bands, or a smear of hyperphosphorylated P–c-Fos, in immunoblots with P–c-Fos–specific Abs. The analyzed CyaA-produced cAMP signaling then caused dephosphorylation of the hyperphosphorylated P–c-Fos into the hypophosphorylated c-Fos. This finding suggests that P–c-Fos was, indeed, the primary target of PKA activation–dependent signaling in the course of CyaA action and that dephosphorylation of P–c-Fos accounted for inhibition of iNOS expression. Intriguingly, manipulation of iNOS expression through a very different mechanism of protease-dependent activation of SHP-1 and a subsequent modulation of c-Fos activity has previously been observed in mouse macrophages infected with Leishmania donovani (46, 68, 69). Moreover, LPS–triggered iNOS expression in murine macrophages could previously be blocked by activation of SHP-1 (70). To the best of our knowledge, however, we describe in this article for the first time the activation of SHP-1 by a mechanism dependent on cAMP elevation and PKA activity. Intriguingly, although the cAMP levels increased with time of toxin action (cf. Fig. 2D), and presumably the PKA activity remained high over the duration of cell exposure to CyaA, the activation of SHP-1 by toxin-produced cAMP signaling was only transient. The mechanism underlying downregulation of SHP-1 activity after prolonged cell exposure to CyaA (60 min; cf. Fig. 6E) remains unknown but correlates in time with the cAMP-provoked transient inhibition of the small GTPase RhoA activity in CyaA-treated murine macrophages, which was maximal at 30 min from toxin addition (11). It will therefore be of interest to determine if the same regulatory circuit is involved in modulation of both RhoA and SHP-1 activities by toxin-produced cAMP signaling. PKA, indeed, likely exerts its effect on SHP-1 activity by an indirect mechanism, as no phosphorylation consensus sequence for PKA (−RRXS/T− where X stays for any amino acid) is found in the SHP-1 sequence, in contrast to SHP-2. We then conclude that it is selectively the cAMP-activated PKA signaling that leads to activation of SHP-1. This is based on the observation that the Epac-specific cell-permeable activator 8-CPT-cAMP exerted little, if any, effect on c-Fos phosphorylation, iNOS expression, and NO production. In contrast, the PKA-specific activator 6-Bnz-cAMP mimicked to a large extent the impact of CyaA action on cells. Hence, cAMP-dependent activation of PKA clearly played a dominant role in CyaA-provoked suppression of iNOS expression in macrophages. However, it cannot be definitively concluded at present that only PKA signaling was involved and Epac did not play any role in the control of TLR-induced iNOS expression. PKA inhibition with H-89 did not fully reverse the impact of CyaA action on iNOS expression. Moreover, H-89 is known to exert some off-target effects (45), and H-89 on its own was found to partially impair iNOS expression (cf. Fig. 4C). It is thus possible that owing to high levels of cAMP produced in cells by CyaA, the H-89 inhibitor at the usual concentration of 10 μM was just unable to completely inhibit the fully activated PKA signaling.

In summary, modulation of iNOS expression by a cAMP/PKA-regulated SHP-1 activity, as reported in this article, indicates that SHP-1 is playing a rather central role in the control of iNOS expression and bacterial survival in phagocytes. Along this line, selective silencing of SHP-1 expression led to restoration of NO production in CyaA toxin–treated RAW264.7 cells, and inhibition of SHP-1/2 phosphatases by NSC87877 provoked a steep decrease of B. pertussis survival inside phagocytes. These observations reveal an as yet undescribed cAMP/PKA-regulated signaling mechanism manipulated by the CyaA toxin that the whooping cough agent B. pertussis uses for hijacking of the tyrosine phosphatase SHP-1. These findings open the way for development and testing of novel SHP-1–specific inhibitors as potential drugs for treating pertussis in the early phases of B. pertussis infections.

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