Adenylate Cyclase Toxin Subverts Phagocyte Function by RhoA Inhibition and Unproductive Ruffling

Jana Kamanova, Olga Kofronova, Jiri Masin, Harald Genth, Jana Vojtova, Irena Lnhartova, Oldrich Benada, Ingo Just and Peter Sebo

*J Immunol* 2008; 181:5587-5597; doi: 10.4049/jimmunol.181.8.5587
http://www.jimmunol.org/content/181/8/5587

---

**References**
This article cites 55 articles, 42 of which you can access for free at:
http://www.jimmunol.org/content/181/8/5587.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Adenylate Cyclase Toxin Subverts Phagocyte Function by RhoA Inhibition and Unproductive Ruffling

Jana Kamanova,* Olga Kofronova,* Jiri Masin,* Harald Genth,† Jana Vojtova,* Irena Linhartova,* Oldrich Benada,* Ingo Just,‡ and Peter Sebo²*

Adenylate cyclase toxin (CyaA or ACT) is a key virulence factor of pathogenic Bordetella. It penetrates phagocytes expressing the αsβ2 integrin receptor (CD11b/CD18, CR3 or Mac-1), such as macrophages, neutrophils, and dendritic cells (DC). Following CD11b/CD18 binding, the toxin penetrates the cytoplasmic membrane of phagocytes and delivers into their cytosol its adenylate cyclase (AC) enzyme domain. There the AC is activated by binding of intracellular calmodulin and subverts cellular signaling by unregulated conversion of ATP to cAMP, a key second messenger molecule (see Ref. 1 for review). Rapid elevation of intracellular cAMP concentration by CyaA then yields suppression of bactericidal functions of phagocytes, such as chemotaxis, FcR-mediated phagocytosis, or superoxide production (2–5) and eventually provokes apoptosis (6). This capacity of CyaA appears to account for the key role of CyaA in virulence of Bordetella species in mammals, enabling the bacteria to escape destruction by the first-line defense of innate immune system (7, 8). Recent work further suggests that cAMP signaling of CyaA may play an even more versatile role in fooling the host defense by promoting incomplete or aberrant maturation of DCs into a so-called semimature state (9–11). This may shape the induction of adaptive immune response toward tolerance of the pathogen.

Although suppressive effects of CyaA action on bactericidal functions of myeloid phagocytes were repeatedly reported, little attention was paid to the underlying mechanisms of toxin-mediated cAMP signaling and to the corresponding downstream effectors, which remain to be identified. In general, the effects of cAMP signaling are rather complex, cell-type dependent, and quite pleiotropic, being mediated through activation of protein kinase A (PKA), the guanine exchange proteins directly activated by cAMP (Epac-1 and Epac-2) and the cAMP-activated channels, respectively. In particular, the spatio-temporal control of cAMP signaling appears to be crucial for differential regulation of cellular targets involved in various cAMP signaling cascades (see Ref. 12 for review). For example, an asymmetric distribution of active PKA is required for establishment of polarity and migration of neutrophils (13). PKA was further shown to regulate functional plasticity of DCs, via inhibition of Src-like kinases (14) and to control the synthesis and release of cytokines in macrophages (15). cAMP signaling was also demonstrated to suppress oxidative burst and FcR- and CR3-mediated phagocytosis by monocytes and macrophages (15–17). Among other cAMP effects, the actin cytoskeleton homeostasis was also found to be perturbed by elevated cAMP levels. cAMP was, indeed, shown to inhibit actin assembly on phagosomes of macrophages (18) and microfilament assembly in neutrophils (19). cAMP-induced activation of PKA caused dissolution of stress fibers and yielded a cell rounding phenotype of fibroblasts (20), or a stellate morphology of human neuroblastoma cells (21).

The key regulators of actin cytoskeletal dynamics are the Rho family GTPases. These signaling proteins act as molecular switches, being inactive when bound to GDP and becoming

Abbreviations used in this paper: DC, dendritic cell; AC, adenylate cyclase enzyme domain; PKA, protein kinase A; GEF, guanine nucleotide exchange factor; CR3, complement receptor 3; db-cAMP, 3β,6β-dibutyryladenosine 3’,5’-cyclic monophosphate; LY, lucifer yellow; BMM, bone marrow macrophage-like cells; CyaA, B. pertussis adenylate cyclase; CyaA-AC, enzymatically inactive adenylate cyclase toxoid; BOK, Rho kinase.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

*Cellular and Molecular Microbiology Division, Institute of Microbiology of the Academy of Sciences of the Czech Republic, Prague 4, Czech Republic and Institute of Toxicology, Hannover Medical School, Hannover, Germany

Received for publication January 25, 2008. Accepted for publication August 18, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the European Union 6th FP Contract LSHB-CT-2003-503582 THERAVAC (to J.V.), Deutsche Forschungs-gemeinschaft Grant SPP1130 GE1247/1-3 (to L.J. and H.G.), the Institutional Research Concept MSM0021620858 (to J.M.), and Grants I.M/06056 (to J.K.) and I.B/06161 (to L.L.) from the Ministry of Education, Youth, and Sports and concept AV0Z50200510 and the Grant No. GA310/08/0447 (to P.S.) of the National Science Foundation of the Czech Republic.

2 Address correspondence and reprint requests to Dr. Peter Sebo, Institute of Microbiology AS CR, v.v.i., Videnska 1083, Prague 4, Czech Republic. E-mail address: sebo@biomed.cas.cz

3 Abbreviations used in this paper: DC, dendritic cell; AC, adenylate cyclase enzyme domain; PKA, protein kinase A; GEF, guanine nucleotide exchange factor; CR3, complement receptor 3; db-cAMP, 3β,6β-dibutyryladenosine 3’,5’-cyclic monophosphate; LY, lucifer yellow; BMM, bone marrow macrophage-like cells; CyaA, B. pertussis adenylate cyclase; CyaA-AC, enzymatically inactive adenylate cyclase toxoid; BOK, Rho kinase.

*Corresponding author.

www.jimmunol.org
activated upon a GDP/GTP exchange mediated by guanine nucleotide exchange factors (GEFs). RhoA, Rac1, and Cdc42 are the most extensively characterized members of this protein family, where RhoA regulates formation of contractile actin/myosin stress fibers in fibroblasts and actin cables in macrophages, Rac is involved in formation of lamellipodia and membrane ruffles, and Cdc42 governs formation of filopodia and microspikes in both fibroblasts, as well as macrophages (22). The Rho GTPases exert crucial mechanistic functions in innate and adaptive immunity and have a pivotal role in the biology of infection (see Ref. 23 for review). For example, FcR-mediated phagocytosis is initiated by engagement of the Ig receptor FcR and depends on the function of Cdc42 and Rac. In turn, the other phagocytic mechanism mediated by complement receptor 3 (CR3) engagement depends on RhoA (24). Not surprisingly then, targeting of Rho GTPases represents an important strategy used by bacterial pathogens in subversion of host cell functions (23).

In this study, we show for the first time that CAMP signaling effects of low doses of CyaA toxin cause rapid and transient inactivation of RhoA, massive actin cytoskeleton rearrangements, nonproductive membrane ruffling, inhibition of macroinocytic uptake and most importantly, an instantaneous and near-complete inhibition of CR3-mediated phagocytosis in macrophages.

Materials and Methods

Reagents

In brief, 3-isobutyl-1-methylxanthin, N6,2’-O-dibutyryladenosine 3’,5’-cyclic monophosphate (db-cAMP), PMA, Wortmannin, and Cs-deficient serum were obtained from Sigma-Aldrich and Y-27632 was obtained from Calbiochem. The Abs used in this study were anti-Rac1 (610650, BD Transduction Laboratories), anti-Rac2 (07-604, Upstate), anti-RhoG (sc-1007, Santa Cruz Biotechnology), anti-RhoA (2117, Cell Signaling Technology), anti-NTAL (NAP-07, gift of P. Angelisova, Institute of Molecular Genetics, Prague, Czech Republic), anti-cofilin (3318, Cell Signaling Technology), anti-phospho-cofilin (ser3, 3531, Cell Signaling Technology), and anti-shear RBC IgM Abs (CL9000-M, Cedarlane Laboratories).

Cell cultures, growth conditions, and handling of cells

J774A.1 mouse macrophages (ATCC TIB 67), RAW 264.7 mouse macrophages (ATCC TIB 71) and bone marrow macrophage-like cells were used. The J774A.1 and RAW 264.7 cells were maintained in RPMI 1640 medium containing 10% FCS (FCS, Life Technologies) and antibiotic antimycotic solution (0.1 mg/ml streptomycin, 1000 U/ml penicillin and 0.25 mg/ml amphotericin, Sigma-Aldrich). Bone marrow macrophage-like cells were obtained from femoral and tibial bones of 6- to 8-wk-old female C57BL/6 mice by cultivating bone marrow–derived cells in RPMI 1640 medium supplemented with 10% FCS, 20 mg/ml GM-CSF and antibiotic antimycotic solution for 7 days, as previously described by (25). At day 7, the nonadherent cells were removed, whereas adherent cells were physically scrapped and washed before use. The expression of surface Ags (% positive of adherent cell population) was 47% for F4/80 (FITC-anti-mouse-F4/80 Ab, clone BM8, BioLegend), 20% for Gr-1 (FITC-anti-mouse-Gr-1 Ab, clone RB6-8C5, eBioscience), 92% for CD11c (APC-anti-mouse-CD11c Ab, clone N418, eBioscience), and 99% for CD11b (PE-anti-mouse-CD11b Ab, clone M1/70, BD Pharmingen) as determined using a FACS LSR II instrument (BD Biosciences) and gating for live cells. During all experiments the RPMI 1640 medium used for cultivating of cells was replaced by DMEM (1.9 mM Ca2+ and 2 mM Mg2+) in liquid cultures in the presence of the activating protein CyaA by using the Escherichia coli strain XL-1 Blue (Stratagene) transformed with the appropriate constructs derived from pT7CACT1 (27), and purified as described (28). In the final step, the proteins were eluted with 8 M urea, 2 mM EDTA, 50 mM Tris-HCl (pH 8.0), and stored at −20°C. The endotoxin content in the samples was determined by the LAL assay (QCL-1000; Cambrex), according to the manufacturer’s instructions and was below 50 EU/mg of purified protein. The CyaA proteins were precluded to 100 or 1000 times the final indicated concentration using 50 mM Tris-HCl (pH 8.0), 8 M urea, and 0.2 mM CaCl2 buffer. Before addition of CyaA to cells, it was diluted in DMEM without FCS and mixed with cell suspension to reach its final indicated toxin concentration. This resulted in a final concentration of 80 mM or less, which had no effect on cell physiology or morphology whatsoever, as verified by appropriate controls with cells incubated at identical urea concentrations.

Determination of intracellular cAMP, phagocytic, and macroinocytic activities

For determination of intracellular cAMP, J774A.1 cells (2 × 106 per well) were incubated with 100 or 10 ng/ml CyaA in DMEM without FCS for indicated time intervals before the reaction was stopped by addition of 0.2% Tween 20 in 50 mM HCl and the cAMP concentration was determined by immunoassay as described elsewhere (29).

FcR-mediated phagocytosis was assessed using parformaldehyde-inactivated, FITC-labeled E. coli BioParticles (E-2861, Molecular Probes) opsonized with E. coli-specific rabbit polyclonal IgG (E-2870, Molecular Probes) according to the manufacturer’s instructions. Opsonized IgG-DNP-E. coli were added at a ratio of ~10 particles per macrophage cell. Upon incubation in the dark at 37°C for 30 min, the unengaged particles were washed-out with PBS and fluorescence of extracellularly attached IgG-FITC-E. coli particles was quenched by treatment with trypan blue (300 μg/ml, Molecular Probes) for 1 min. Macrophage cells were rinsed in PBS and the fluorescence of phagocytosed E. coli was determined upon J774A.1 cell lysis in 50 mM Tris-HCl (pH 9.0), 150 mM NaCl, 5 mM EDTA and 0.5% Nonidet P-40 using a microparticle reader (483/525 nm, Safire2, Schoeller Instruments). Data were corrected for the fluorescence of unquenched, extracellular bacteria, determined as fluorescence of cells incubated with IgG-FITC-E. coli at 4°C.

CR3-mediated phagocytosis was assessed as internalization of C3b-opsinized sheep RBCs by J774A.1 cells as described (24). In brief, 5 × 105 of RBCs/ml were incubated for 30 min at 37°C in the presence of anti-sheep RBCs IgM Abs (1/250). RBCs were washed and IgM-coated RBCs were opsonized by incubation in 10% (v/v) C5-deficient human serum for 30 min at 37°C. Under these conditions, C3b is rapidly fixed to IgM-coated RBCs and is completely converted into C3bi (30). Opsonization with C3bi was checked by flow cytometry following incubation with goat anti-C3 (Sigma-Aldrich) and staining by FITC-conjugated anti-goat IgG (H+L) (Jackson ImmunoResearch), followed by cell lysis with 2,7-diaminofluorene. J774A.1 cells were induced for efficient CR3-mediated phagocytosis by incubation in 20% FCS at 37°C (as previously described (31)). C3b-RBCs were added to cells at a ratio of ~25 RBCs per J774A.1 cell and incubated for 30 min at 37°C to allow phagocytosis. Unbound RBCs were washed away with ice-cold PBS, whereas adherent but nonphagocytosed RBCs were hypotonically lysed by a 30 s incubation pulse with distilled H2O, followed by wash in PBS and incubation in trypan blue, repeated hypotonic lysis, and removal of noninternalized RBCs. The quantity of phagocytosed C3b-RBCs was next determined by measuring the amount of fluoresce blue formed from 2,7-diaminofluorene (Sigma-Aldrich) by the pseudoperoxidase activity of hemoglobin released from RBCs upon lysis of J774A.1 cells (32). Absorbance was read at 610 nm using a microparticle reader (Safire2, Schoeller Instruments) and corrected against a blank of lysed J774A.1 cells incubated with 2,7-diaminofluorene alone.

To determine the level of fluid-phase uptake (macronucleocytosis), J774A.1 cells were incubated with either lucifer yellow (LY, 500 μg/ml, Molecular Probes) or FITC-dextran (1 mg/ml, Mw 4 KDa, Sigma-Aldrich) for 30 min at 37°C. LY or FITC-dextran uptake was stopped by three washes with ice-cold PBS and fluorescence of cell surface-bound LY or FITC-dextran was quenched in trypan blue as above. Cells were then rinsed in PBS and lysed as above. The fluorescence of internalized LY or FITC-dextran was then read in a microplate reader (405/580nm for LY or 483/525nm for FITC-dextran, Safire2, Schoeller Instruments) and data were corrected for the fluorescence of unquenched, extracellular LY, or FITC-dextran (fluorescence of cells incubated with LY or FITC-dextran at 4°C).

Transfection experiments

Transfections of pEGFP-C1 constructs bearing cDNA of Rac1, Cdc42, RhoA and their mutants Rac1-T17N, Cdc42-T17N and RhoA-G14V, or mock pEGFP-C1 construct, were performed at 50% confluence of RAW 264.7 macrophages using the FuGENE-6 transfection reagent (Roche) according to the manufacturer’s instructions. Imaging of actin cytoskeleton

Downloaded from http://www.jimmunol.org/ by guest on October 6, 2017
and determination of cell surface level of CD11b/CD18 were performed 12 h after transfection. Expression of CD11b/CD18 on transfected cells was analyzed by flow cytometry after incubation with PE-conjugated anti-CD11b Ab (clone M1/70, BD Pharmingen) using a FACS LSR II instrument (BD Biosciences) and gating for live GFP-positive cells. Level of CD11b/CD18 was deduced from the mean fluorescence intensity and expressed as the percentage of the CD11b level of control (nontransfected) cells.

Microscopy of J774A.1, RAW 264.7, and bone marrow macrophage-like cells

For scanning electron microscopy, the cells were fixed with 3% glutaraldehyde and washed with a cacodylate buffer. The samples were dehydrated in an alcohol series followed by absolute acetone, and dried in a critical-point device (Balzers 010). Finally, the samples were sputter-coated with gold in a Polaron sputter-coater (Series 11HD) and examined in an Aquasem scanning electron microscope (Tescan) at 15 kV.

For fluorescence microscopy of the actin cytoskeleton, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS (20 min, room temperature). After three washes with PBS (3 × 5 min), the cells were permeabilized with 0.1% Triton X-100 in PBS (5 min), and washed with PBS (3 × 5 min). F-actin was stained with TRITC-conjugated phalloidin (0.5 μg/ml, Sigma-Aldrich) in PBS supplemented with 2% BSA for 30 min. Images were taken using an Olympus BX60 fluorescence microscope.

Quantification of ruffling

Quantification of ruffling was performed according to Cox et al. (33). Ruffling was defined by the presence of F-actin-rich submembranous folds using fluorescence microscopy. The extent of ruffling of each cell was scored using a scale of 0–2, where 0 indicates that no ruffles were present, 1 indicates that ruffling was confined to one area of the cell only (<25% of the cell’s circumference), and 2 indicates that two or more discrete areas of the cell contained ruffles. The ruffling index was recorded as the sum of the ruffling scores of 100 cells.

Affinity precipitation of cellular GTP-Rac1/Rac2, GTP-RhoG, and GTP-RhoA

GTP-bound active forms of Rac1/2, RhoG, and RhoA were detected using a pull-down assay using Cdc42/Rac interactive-binding region of PAK (GST-CRIB), RhoG docking protein (GST-ELMO1), and RhoA interactive-binding region of rhoetkin (GST-RBD), respectively. In total, 2 × 10⁶ J774A.1 cells were treated with the indicated agent (CyaA, CyaA-AC*, db-cAMP) at 37°C for the indicated time, washed with ice-cold PBS and lysed by three cycles of freezing and thawing followed by three passages through a 25-gauge needle. After pelleting of unlysed cells (10 min, 10 000 × g, 4°C), the membranes were collected by ultra-centrifugation (30 min, 100 000 × g). The membrane pellet was resuspended in 1% Nonidet P-40, the membrane lysates were treated with 100 μM GTPγS as a nonhydrolysable GTP analog, as follows. Lysates were treated with 100 μM GTPγS in the presence of 10 mM EDTA for 15 min at 30°C. The reaction was terminated and GTPγS was locked into the proteins by addition of 60 mM MgCl₂ on ice.

Preparations of cell membranes for determination of RhoA content

For preparation of the fraction enriched in plasma membrane, 2 × 10⁶ J774A.1 cells were washed with PBS and incubated in ice-cold hypotonic buffer containing 20 mM HEPES (pH 7.5), 2 mM EDTA, and Complete Mini protease inhibitors. After 30 min of incubation on ice the cells were lysed by three cycles of freezing and thawing followed by three passages through a 25-gauge needle. After pelleting of unlysed cells (10 min, 800 × g, 4°C), the membranes were collected by ultra-centrifugation (30 min, 100 000 × g). Membrane pellet was resuspended in 1% Nonidet P-40, the membrane lysates were treated with 100 μM GTPγS as a nonhydrolysable GTP analog, as follows. Lysates were treated with 100 μM GTPγS in the presence of 10 mM EDTA for 15 min at 30°C. The reaction was terminated and GTPγS was locked into the proteins by addition of 60 mM MgCl₂ on ice.

Preparation of cell lysates for determination of phospho-cofilin

Preparations of cell lysates for determination of phospho-cofilin

Two × 10⁶ J774A.1 cells were washed with PBS and lysed in 150 μl of ice-cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 10 mM Na₃PO₄, 1 mM NaN₃, 50 mM NaF, 10 nM CalyculinA, and Complete Mini protease inhibitors per well of a 6-well culture dish. After scraping, lysates were incubated on ice for 15 min and briefly vortexed. Samples were clarified by centrifugation (3 min, 10 000 × g) and analyzed by Western blot.

Western blot analysis

The proteins were separated by 15% SDS-PAGE, transferred onto nitrocellulose membrane, and probed overnight with primary Abs, as indicated in the figure legends and revealed by HRP-conjugated secondary Ab (dilution 1:4,000, GE Healthcare) using the West Femto Maximum Sensitivity Substrate (Pierce). Western blot signals were visualized using the LAS-1000 (Luminiscence Analyzing System, Fujifilm) and AIDA 1000/1D Image Analyzer software, version 3.28 (Raytest Isotopeanalysegeraete GmbH).

Statistical analysis

Significance of differences in values was assessed by Student’s t test.

Results

cAMP signaling of CyaA toxin rapidly halts complement-mediated phagocytosis

We first analyzed the effects of toxin action on phagocytosis in model mouse J774A.1 monocyte/macrophage-like cells (29). As samples were mixed with Laemmli buffer, heated for 5 min at 95°C and analyzed by Western blots.

Preparation of cell lysates for determination of phospho-cofilin

Two × 10⁶ J774A.1 cells were washed with PBS and lysed in 150 μl of ice-cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 10 mM Na₃PO₄, 1 mM NaN₃, 50 mM NaF, 10 nM CalyculinA, and Complete Mini protease inhibitors per well of a 6-well culture dish. After scraping, lysates were incubated on ice for 15 min and briefly vortexed. Samples were clarified by centrifugation (3 min, 10 000 × g) and analyzed by Western blot.
previously reported, already at as low toxin concentrations as 10 ng/ml, a rapid accumulation of supraphysiological concentrations of cAMP in J774A.1 cells was observed, as shown in Fig. 1A. At this toxin concentration, a moderate but statistically significant impairment of FcR-mediated phagocytosis of IgG-opsonised *E. coli* bioparticles by J774A.1 cells was seen and the extent of phagocytosis inhibition further increased at higher toxin doses (Fig. 1B).

Importantly, as further shown in Fig. 1B, the impact of CyaA action on CR3-mediated phagocytic capacity of macrophages was much more pronounced. This pathway depends on opsonization of pathogens by the C3bi component of complement and is mediated by the CR3 (known also as αβ_2 integrin, CD11b/CD18, or Mac-1). Intriguingly, CD11b/CD18 serves also as the cellular receptor of the CyaA toxin itself. However, the observed inhibitory effect of CyaA on CR3-mediated phagocytosis at the used toxin dose was clearly not due to competition of CyaA for the receptor with the C3bi-opsonized particles. No effect on CR3-mediated phagocytosis was, indeed, seen at 100 ng/ml of the enzymatically inactive CyaA-AC toxoid (Fig. 1B), that lacks the capacity to convert ATP into cAMP, while being fully competent in receptor binding (34). In turn, a statistically significant inhibition of CR3-mediated phagocytosis was observed already at 1 ng/ml CyaA and the effect of CyaA could be mimicked by exposing J774A.1 cells to 1 mM db-cAMP, a membrane-permeable cAMP analog. Collectively, hence, these results unambiguously show that it was the capacity of CyaA to elevate cAMP levels in J774A.1 cells that accounted for the inhibition of CR3-mediated phagocytosis by the toxin.

Phagocytosis inhibition is accompanied by actin cytoskeleton rearrangements and membrane ruffling

Because phagocytic functions depend on the coordinated rearrangements of actin cytoskeleton, we examined the morphological consequences of toxin treatment using two different murine macrophage cell lines (J774A.1 and RAW 264.7) and primary bone marrow-derived macrophage-like cells (BMM), respectively. As revealed by scanning electron micrographs (upper panels) and TRITC-phalloidin staining of F-actin (lower panels) shown in Fig. 2, exposure of all three macrophage types to CyaA resulted in
massive formation of sheet-like cell membrane extensions commonly referred as lamellipodia, or membrane ruffles. Moreover, in primary BMM also disappearance of actin cables and podosomes was observed to result from CyaA action. In turn, no ruffling was observed with cells exposed to the enzymatically inactive CyaA-AC\textsuperscript{−} toxoid purified in the same way as CyaA, thus excluding the possibility that cell ruffling might have been promoted by traces of some contaminating bacterial components present in the CyaA and CyaA-AC\textsuperscript{−} preparations. Moreover, comparable membrane ruffling was also observed upon exposure of macrophage cells to 2 mM db-cAMP, demonstrating that elevation of cAMP in cells due to CyaA action was itself accounting for the massive ruffling of murine macrophages.

As further shown in Fig. 2 and determined by calculating the ruffling index using the counting of actin-rich submembrane folds according to Cox et al. (33), all three examined macrophage types responded by similar morphological changes to CyaA or db-cAMP exposure, albeit at differing toxin doses and at a differing percentage of responding cells. The J774A.1 cells and BMM were comparably sensitive to CyaA action and ruffled vigorously already at 10 ng/ml CyaA, with however only about half of the cell population responding by ruffling to CyaA or db-cAMP treatment. Moreover, this proportion of ruffling cells was not enhanced even at a 10-fold higher (100 ng/ml) concentration of CyaA (see Fig. 3 for J774A.1 cells). In contrast, while the RAW 264.7 cells were less sensitive to CyaA action and up to 100 ng/ml of the toxin had to be used to observe ruffling in 10 min of exposure to toxin, the ruffling response of RAW 264.7 cells was quite homogeneous with essentially all cells in the population responding to CyaA or db-cAMP treatment by ruffling. A likely explanation of these differences in homogeneity of ruffling responses would be that it depends on the proportion of cells that was differentiated into...
macrophages. While the RAW 264.7 cell population is quite homogeneous, consisting essentially of macrophage-like cells, the J774A.1 cell population consists of a mixture of monocyte- and macrophage-like cells, similarly to the primary BMM culture, the latter comprising also bone marrow DCs and granulocytes (see Materials and Methods for phenotype characterization of BMM culture). Alternatively, also the cell-cycle dependence of the ruffling response of the RAW 264.7 and J774A.1 cell and BMM population may differ.

CyaA-induced actin rearrangements and cell ruffling are transient and wane faster at higher toxin concentration

To characterize the CyaA-induced macrophage ruffling phenotype in more detail, kinetics of morphological and actin cytoskeleton alterations of J774A.1 cells were examined in time at a high (100 ng/ml) and a low (10 ng/ml) toxin concentration, respectively. As determined by quantification of F-actin accumulation in cell periphery and confirmed by scanning electron micrographs (Fig. 3), membrane ruffling of J774A.1 cells was transient and could be observed already in 5 min of exposure to CyaA at both 10 ng/ml and 100 ng/ml concentrations of CyaA. However, while cells treated with 10 ng/ml CyaA still formed membrane ruffles at 30 min and started to lose them only after 60 min of exposure to toxin, cells exposed to 100 ng/ml CyaA had already retracted the ruffles in 30 min of toxin treatment, showing that ruffling waned faster at higher CyaA concentration. The hypothesis would be that at higher toxin concentrations an inhibitory signaling threshold, such as activation of cAMP-dependent protein phosphatases interfering with cAMP signal transmission, and/or exhaustion of actin rearrangement machinery is reached earlier than at lower toxin concentrations.

CyaA-induced membrane ruffling subverts macropinocytic fluid phase uptake

Membrane ruffling is a typical feature of myeloid phagocytes activated by TLR signaling of pathogen components, which serves to

---

**FIGURE 5.** CyaA action does not yield detectable activation of Rac1, Rac2, or RhoG. Active cellular Rac1, Rac2, and RhoG were affinity precipitated from lysates of toxin-treated J774A.1 cells using GST-CRIB or GST-ELMO1-coated agarose beads. Rac1-GTP, total Rac1, Rac2-GTP, total Rac2, RhoG-GTP, and total RhoG were detected by Western blot using anti-Rac1, anti-Rac2 or anti-RhoG Abs. Relative activities of Rac1, Rac2, and RhoG were determined as the amounts of CRIB or ELMO1-bound Rac1, Rac2, or RhoG by normalization to total detected amounts of Rac1, Rac2, or RhoG, respectively. Results represent the means ± SD for at least five independent experiments (n = 5). No statistically significant differences (p < 0.05) of amounts of active Rac1, Rac2, or RhoG were observed between toxin-treated and untreated cells.

**FIGURE 6.** Rac1 activity is required for induction of actin cytoskeleton rearrangements by CyaA. RAW 264.7 cells were transfected to express wild-type or dominant negative (d.n.) variants of Rac1 or Cdc42 fused to GFP, or GFP alone (mock treatment). Production of the proteins was allowed to proceed for 12 h before the cells were exposed to 100 ng/ml CyaA for 10 min. Cell morphology was assessed by F-actin staining with TRITC-phalloidin and transfected cells were visualized by GFP expression. The morphology of transfected cells in the shown micrographs is representative of images from three independent experiments for which the mean ± SD ruffling index was calculated (n = 3). Cell surface level of CD11b/CD18 on GFP-positive Rac1-T17N-transfected cells was determined by flow cytometry and expressed as percentage of CD11b level of control (nontransfected) cells. No statistically significant difference (p < 0.05) of cell surface level of CD11b/CD18 was observed between control and Rac1-T17N-transfected cells.
enhance environment sampling through macropinocytic fluid phase uptake and allows enhanced Ag presentation and induction of adaptive immune response against infection (35). Moreover, active enhancement of membrane ruffling and macropinocytosis is exploited by numerous bacterial pathogens for cell entry. Therefore, we examined whether CyaA-induced ruffling of J774A.1 cells was also accompanied by enhancement of macropinocytic activity.

Unexpectedly, however, as shown in Fig. 4A and measured as uptake of LY and of FITC-labeled dextran, the basal, as well as enhanced fluid phase uptake induced by addition of bacterial lysate to J774A.1 cells, or the membrane-associated RhoA, were detected by Western blotting and normalized to total RhoA detected in whole cell lysates, or to amounts of the integral membrane protein marker NTAL, respectively. The results represent the means ± SD from at least five independent experiments (n = 5). *p < 0.05 vs untreated cells. B, Expression of constitutively active RhoA prevents CyaA-induced membrane ruffling. RAW 264.7 cells were transfected to express wild-type or the constitutively active (c.a.) variant of RhoA fused to GFP. Production of RhoA-GFP was allowed to proceed for 12 h before the cells were exposed to 100 ng/ml CyaA for 10 min. Cell morphology was assessed by F-actin staining with TRITC-phalloidin and transfected cells were visualized by GFP expression. The morphology of transfected cells in the shown micrographs is representative of images from three independent experiments for which the mean ± SD ruffling index was calculated (n = 3). Cell surface level of CD11b/CD18 on GFP-positive RhoA and RhoA-G14V-transfected cells was determined by flow cytometry and expressed as percentage of CD11b level of control (nontransfected) cells. No statistically significant difference (p < 0.05) of cell surface level of CD11b/CD18 was observed between control, RhoA, and RhoA-G14V-transfected cells.

**FIGURE 7.** CyaA-induced membrane ruffling is due to transient inactivation of RhoA. A, CyaA transiently inactivates RhoA and provokes its relocalization from cell membranes. Isolation of cell membranes and affinity-precipitation of active cellular RhoA-GTP on GST-RBD-coated agarose beads were performed on J774A.1 cells treated with db-cAMP (2 mM, 10 min) or 10 or 100 ng/ml CyaA (for indicated times). RhoA-GTP pulled-down from whole cell lysates, or the membrane-associated RhoA, were detected by Western blotting and normalized to total RhoA detected in whole cell lysates, or to amounts of the integral membrane protein marker NTAL, respectively. The results represent the means ± SD from at least five independent experiments (n = 5). *p < 0.05 vs untreated cells. B, Expression of constitutively active RhoA prevents CyaA-induced membrane ruffling. RAW 264.7 cells were transfected to express wild-type or the constitutively active (c.a.) variant of RhoA fused to GFP. Production of RhoA-GFP was allowed to proceed for 12 h before the cells were exposed to 100 ng/ml CyaA for 10 min. Cell morphology was assessed by F-actin staining with TRITC-phalloidin and transfected cells were visualized by GFP expression. The morphology of transfected cells in the shown micrographs is representative of images from three independent experiments for which the mean ± SD ruffling index was calculated (n = 3). Cell surface level of CD11b/CD18 on GFP-positive RhoA and RhoA-G14V-transfected cells was determined by flow cytometry and expressed as percentage of CD11b level of control (nontransfected) cells. No statistically significant difference (p < 0.05) of cell surface level of CD11b/CD18 was observed between control, RhoA, and RhoA-G14V-transfected cells.

**Steady-state Rac1 activity is required for CyaA/cAMP-induced actin rearrangements**

The CyaA-induced actin rearrangements suggested that cAMP-signaling modulated the activity of Rho family GTPases controlling the homeostasis of actin cytoskeleton. As a first choice, we examined the activation of Rac subfamily proteins in cells treated with CyaA, as activation of Rac-like GTPases is known to be involved in formation of membrane ruffles in macrophages (22). However, as determined by pull-down assays for active (GTP-loaded) Rac1, Rac2, and RhoG with the Cdc42/Rac interactive-binding region of PAK (GST-CRIB) and the RhoG effector protein as did treatment with 10 ng/ml CyaA, showing that it was the elevation of intracellular cAMP concentration by toxin action that accounted for the inhibition of macropinocytosis. As further confirmed by fluorescence microscopy, action of CyaA at 100 ng/ml caused a comparable block of FITC-dextran uptake as did 100 nM wortmannin, a potent inhibitor of macropinosome formation (Fig. 4B).

**Steady-state Rac1 activity is required for CyaA/cAMP-induced actin rearrangements**

The CyaA-induced actin rearrangements suggested that cAMP-signaling modulated the activity of Rho family GTPases controlling the homeostasis of actin cytoskeleton. As a first choice, we examined the activation of Rac subfamily proteins in cells treated with CyaA, as activation of Rac-like GTPases is known to be involved in formation of membrane ruffles in macrophages (22). However, as determined by pull-down assays for active (GTP-loaded) Rac1, Rac2, and RhoG with the Cdc42/Rac interactive-binding region of PAK (GST-CRIB) and the RhoG effector protein...
ELMO1 (GST-ELMO1), respectively, no detectable activation of Rac1, Rac2, or RhoG was observed in CyaA-exposed cells at the time points at which pronounced cell ruffling occurred in response to CyaA treatment, as shown in Fig. 5.

To further corroborate these observations, the capacity of CyaA to induce ruffling was examined on macrophages transiently transfected with constructs for expression of the wild-type and dominant negative variants of Rac1 fused to GFP. Because J774A.1 cells are particularly resilient to transfection, this set of experiments was performed using RAW 264.7 murine macrophages that responded by identical morphological alterations to toxin treatment as did J774A.1 cells, although at increased CyaA concentrations (100 ng/ml). As documented in Fig. 6, the mock-transfected cells, or cells transfected with wild type Rac1-GFP, both responded to CyaA treatment by typical ruffling. In contrast, expression of the dominant negative variant of Rac1-GFP (Rac1-T17N) efficiently protected cells from undergoing CyaA-induced ruffling. This requirement for Rac1 function for CyaA-induced ruffling to occur appeared to be specific, as expression of the dominant negative variant of Cdc42-GFP (Cdc42-T17N) used as control had a modest effect on toxin induced ruffling, as also shown in Fig. 6. Moreover, the loss of ruffling response to CyaA action upon Rac1-T17N expression was not a secondary effect of a decrease in toxin receptor expression in transfected cells, as flow cytometric analysis confirmed that the amounts of surface-exposed CyaA receptor, CD11b/CD18, on Rac1-T17N-transfected and nontransfected cells were identical (Fig. 6). Hence, while no activation of Rac1 was detected in CyaA-exposed cells, a steady state level of Rac1 activity still appeared to be specifically required for CyaA-induced cell ruffling to occur.

**cAMP signaling of CyaA causes transient inactivation of RhoA in macrophages**

We reasoned that in the absence of Rac activation, the observed ruffling might have been due to inhibition of the signaling pathway of the Rac antagonist, RhoA. Indeed, formation of membrane protrusions in human THP1 monocytes was previously seen upon inhibition of the RhoA effector kinase ROK (36). Therefore, RhoA activity was first examined in whole cell lysates of CyaA-treated cells. As documented in Fig. 7A by results of pull-down assays for active RhoA using RhoA interactive-binding region of rhotekin (GST-RBD), exposure of J774A.1 cells to 10 ng/ml CyaA yielded a transient inactivation of RhoA. At this low toxin concentration a significant decrease in the pulled-down amounts of active RhoA was observed at 5 and 30 min of cell exposure to CyaA, with a recovery of active RhoA levels in the cells by 60 min of exposure to toxin. At the higher CyaA concentration (100 ng/ml), the decrease in active RhoA-GTP level was observed only at 5 min after toxin addition and restoration of active RhoA levels occurred already in 30 min. This observation was further corroborated by analyzing the content of RhoA in membrane fraction, as RhoA in its active GTP-bound conformation is associated with cellular membranes, while inactive GDP-bound RhoA can be sequestered in the cytosol by Rho-GDI proteins. As further shown in Fig. 7A, the exposure of J774A.1 cells to CyaA resulted in a transient decrease of RhoA amounts associated with the membrane fraction of cells, supporting the conclusion that activity of RhoA was transiently decreased upon cell exposure to CyaA. Hence, a similarly transient and toxin concentration-dependent effect of CyaA action on RhoA-GTP levels was observed as when cellular morphology and ruffling in response to CyaA action was examined (cf. Fig. 3).

To test the hypothesis that inactivation of RhoA accounted for actin rearrangements and cell ruffling in response to elevation of cAMP in cells by CyaA, we examined the ruffling of toxin-treated RAW 264.7 macrophages that were transfected with wild-type or constitutively active RhoA variants fused to GFP. Although again the levels of CD11b/CD18 receptor on cells were not altered upon transfection with the used RhoA constructs and the expression of wild-type RhoA-GFP in transfected cells had a modest impact on toxin-induced cell ruffling, the expression of the constitutively active RhoA-GFP variant (RhoA-G14V) effectively protected cells from undergoing CyaA-induced ruffling (Fig. 7B). Altogether, these results show that inactivation of RhoA by a cAMP-dependent signaling mechanism was both a consequence of the toxin action, as well as a prerequisite for the ruffling response to occur.

To further corroborate this result, activity of cofilin, an effector downstream of RhoA was assessed in CyaA-treated cells. Cofilin has been identified as an important actin-filament severing and...
depolymerizing protein that is regulated by inhibitory phosphorylation at its Ser 3 residue and is involved in initiation of actin-driven membrane protrusions in regions of rapid actin assembly (see Ref. 37 for review). As shown in Fig. 8A, when lysates from cells exposed to CyaA or to db-cAMP were probed with Abs selectively recognizing only the inactive phosphocofilin, or detecting both phosphocofilin and active cofilin, a significant cofilin activation (dephosphorylation) was observed in lysates of CyaA or db-cAMP treated cells. Moreover, as shown in Fig. 8B, cells treated with the inhibitor of the RhoA effector ROK kinase, Y-27632, exhibited a clear albeit somewhat delayed enhancement of membrane protrusive activity and cofilin dephosphorylation, thus mimicking the outcome of CyaA action. These results go well with the conclusion that cAMP signaling provokes phagocyte ruffling by a mechanism that involves RhoA inactivation and activation of cofilin.

Discussion

The present study unravels the capacity of CyaA to suppress the bactericidal activities of macrophages through unproductive membrane ruffling and shows for the first time that cAMP signaling of low, likely physiologically relevant, concentrations of the CyaA toxin causes a rapid and complete inhibition of CR3-mediated phagocytosis. This may be of crucial importance in the early stages of bacterial colonization of naive (unvaccinated) infants that lack specific Abs to B. pertussis, because this bacterium resists lysis by complement itself (38) and would benefit from evading destruction ensuing CR3-mediated phagocytosis, to which it is in turn highly sensitive (39, 40).

We report that the molecular mechanism of the repeatedly documented capacity of CyaA to undermine bactericidal activities of αMβ2 integrin-expressing myeloid phagocytic cells may well rely primarily on RhoA inactivation as a result of cAMP signaling, as summarized in the model proposed in Fig. 9. An essential role of RhoA GTPase in innate immunity functions of macrophages has, indeed, been previously established through the work of several groups. An active RhoA signaling pathway was found to be essential for CR3-mediated phagocytosis (24), where the RhoA-dependent activation of ROK allows recruitment of myosin-IIA and initial assembly of actin and Arp2/3 complex in the phagocytic cup (31). Furthermore, inactivation of RhoA signaling was found to affect chemotactic properties of primary monocytes, probably by deregulation of actin cytoskeleton coordination (36). Moreover, RhoA was also found to be involved in signaling leading to superoxide formation through CR3- and Fcy-receptor stimulation (41). Hence, the repeatedly observed interference of CyaA with all three of the above mentioned activities would go well with a mechanism of toxin action relying on inactivation of RhoA through CyaA/cAMP signaling observed in this study.

RhoA is, indeed, a frequent target of virulence factors of several pathogenic bacteria. For example, covalent modification of the RhoA switch I domain by large clostridial toxins (C. difficile A and B, C. sordellii hemorrhagic toxins and C. novyi α toxins) results in inhibition of RhoA effector binding, whereas spatial regulation of RhoA is targeted by the C3 transferase of C. botulinum, or YopT of Yersinia (see Ref. 42 for review). In this study, we show that CyaA of Bordetella inactivates RhoA by yet another, cAMP-signaling dependent mechanism. This sets a new paradigm, because the same mechanism may be used also by other toxins potentially targeting macrophages and manipulating the intracellular concentration of cAMP, such as the edema factor of Bacillus anthracis, ExoY of Pseudomonas aeruginosa, or the adenylyl cyclase of Yersinia pestis.

Membrane ruffling in macrophages is known to be mediated by activation of Rac-like protein subfamily signaling (22), while activation of RhoA and of its downstream effector, ROK may suppress this membrane protrusive activity of Rac-like GTPases (43). Indeed, Rac and RhoA appear to exhibit an antagonistic relationship, with the two proteins counterbalancing each other’s activity. For example, in migrating leukocytes a low RhoA activity was detected at the protruding, leading edge of the cell, while high levels of RhoA activity were detected by RhoA biosensors at the rear and at the sides of the cell, where formation of protrusions is suppressed (44). Furthermore, inhibition of the RhoA/ROK signaling pathway enhances the membrane protrusive activity of monocytes and results in competing membrane lamellipodia (36). Thus, the observed requirement for a functional steady-state Rac1 signaling (Fig. 6) in the presence of cAMP signaling-mediated inactivation of RhoA (Fig. 7A) is well compatible with the observed induction of membrane ruffling in macrophages in response to toxin action of CyaA toxin (Fig. 2).

RhoA was, indeed, reported to be phosphorylated by cAMP-activated PKA at the Ser188 residue near its C terminus (45) and this appears to inhibit RhoA function, possibly by impairing RhoA interaction with RhoGDI (Rho guanine nucleotide dissociation inhibitor) (45, 46). Effects of cAMP elevation on stress fiber dissolution and cell morphology could, indeed, be prevented by overexpression of both, a phosphorylation-resistant S188A RhoA mutant (46) and of ROKα (21). In our hands, however, expression of the S188A RhoA mutant did not protect cells from undergoing CyaA-induced membrane ruffling (data not shown). This was only prevented by expression of the constitutively active G14V RhoA mutant (Fig. 7B). It remains, hence, to be clarified whether expression of S188A RhoA in the RAW 264.7 cells did not exert a sufficiently dominant phenotype on the background of the native RhoA produced in the cells, or whether other mechanisms than direct RhoA phosphorylation at Ser 188 are involved in RhoA pathway inactivation and induction of membrane ruffling in macrophages by CyaA/cAMP signaling. Recent studies indicate that PKA can also regulate the activity of upstream activators of RhoA, such as Gα13 (47) and/or promote also the inhibition of RhoA-GEF activity of the AKAP-Lbc signaling complex (48). Besides that, also the cAMP/Epac1/Rap1 pathway may potentially contribute to cAMP signaling-mediated inhibition of RhoA. It has, indeed, been demonstrated that
the RhoA-GAP activity of ARAP3 is activated upon binding of Rap (49). It should be also noted that the ROK inhibitor alone induced ruffles that were more localized and brush-like, while CyaA induced sheet-like ruffles covering the complete cell surface (Fig. 2 vs Fig. SB), suggesting that CyaA/CAMP signaling-induced ruffling of macrophage cells may involve mechanisms additional to RhoA-mediated decrease of ROK activity. 

As a part of their colonization strategies, various pathogenic bacteria (e.g., Salmonella typhimurium and Shigella spp.) inject effectors into cells to trigger membrane ruffling and exploit enhanced formation of macropinosomes for cell invasion. Intriguingly, the primacy of the extracellular pathogen Bordetella pertussis appears to down-modulate its own invasion into the tracheal epithelial cells by the colonization by CyaA. The results reported in this study indicate that this may be due to the capacity of CyaA to promote subversive membrane ruffling and to inhibit at the same time the macropinocytic activity of cells. It will, hence, be of interest to elucidate the details of the mechanism by which CyaA/CAMP signaling leads to inhibition of macropinocytosis.

Membrane ruffling is intimately linked to the stimulation of macropinocytosis via formation of macropinosomes, which originate primarily as actin-rich ruffles that close to form intracellular vesicles. Although ruffling is a prerequisite for macropinosome formation, indeed, additional activities may be required to transform a ruffle into a closed intracellular vesicle. For example, Araki et al. showed that PI3K activity is necessary for the completion of macropinocytosis but not for the initial ruffling phase in macrophages (51). Further, Rah, a small GTPase of the Rab family, was shown to be required for efficient macropinosome formation from membrane ruffles (52). In the light of the results reported in this study, it is plausible to hypothesize that CAMP/CyaA signaling may target also the signaling pathway(s) accounting for closure of macropinosomes and phagosomes and their transformation into intracellular vesicles. This would go well with the here observed persistence of inhibition of CR3-mediated phagocytosis also upon restoration of the initial RhoA levels at 30 min of macrophage exposure to the higher CyaA dose of 100 ng/ml (Fig. 7 and data not shown). Alternatively, the latter phagocytic impotence of CyaA-treated cells may, however, be also caused by depleted ATP levels due to CyaA action (29), as well as due to mislocalization of components needed to be recruited for productive assembly of phagocytic machinery by the preceding massive ruffling.

Macropinocytosis was also shown to account for an important part of Ag internalization and presentation on MHC molecules by professional APCs, such as DCs and macrophages (53–55). Moreover, a burst of cell macropinocytic activity upon encounter of TLR ligands was shown to direct an enhanced Ag presentation by DCs (35). It is, hence, plausible to propose that inhibiting macropinocytosis through CyaA action might contribute to Bordetella survival on tracheal epithelia also by hampering induction of adaptive immune response. Experiments are underway to test the hypothesis that CyaA activity inhibits Ag uptake and presentation by DCs.

Acknowledgments
We thank Johannes Huelsenbeck and Hana Kabinova for assistance. The gift of bone marrow cells and analysis of their phenotype by Irena Adkins is gratefully acknowledged.

Disclosures
The authors have no financial conflict of interest.

References


