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Pore-Forming and Enzymatic Activities of *Bordetella pertussis* Adenylate Cyclase Toxin Synergize in Promoting Lysis of Monocytes

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Bordetella **adenylate cyclase (AC) toxin-hemolysin (CyaA) targets myeloid phagocytes expressing the $\alpha_M\beta_2$ integrin (CD11b/CD18) and delivers into their cytosol an AC enzyme that converts ATP into cyclic AMP (cAMP). In parallel, CyaA acts as a hemolysin, forming small membrane pores. Using specific mutations, we dissected the contributions of the two activities to cytolytic potency of CyaA on J774A.1 murine monocytes. The capacity of AC to penetrate cells and deplete cytosolic ATP was essential for promoting lysis and the enzymatically inactive but fully hemolytic CyaA-AC$^-$ toxoid exhibited a 15-fold-lower cytolytic capacity on J774A.1 cells than intact CyaA. Moreover, a two- or fourfold drop of specific hemolytic activity of the CyaA-E570Q and CyaA-E581P mutants was overpowered by an intact capacity to dissipate cytosolic ATP into cAMP, allowing the less hemolytic proteins to promote lysis of J774A.1 cells as efficiently as intact CyaA. However, an increased hemolytic activity, due to lysine substitutions of glutamates 509, 516, and 581 in the pore-forming domain, conferred on AC$^-$ toxoids a correspondingly enhanced cytolytic potency. Moreover, a threefold increase in hemolytic activity could override a fourfold drop in capacity to convert cellular ATP to cAMP, conferring on the CyaA-E581K construct an overall twofold increased cytolytic potency. Hence, although appearing auxiliary in cytolytic action of the toxin on nucleated cells, the pore-forming activity can synergize with ATP-depleting activity of the cell-invasive AC enzyme and complement its action toward maximal cytotoxicity.**

The bifunctional RTX (repeat in toxin) adenylate cyclase (AC) toxin-hemolysin (CyaA, ACT, or AC-Hly) is a key virulence factor of the whooping cough agent *Bordetella pertussis* (17, 37, 56). CyaA consists of an N-terminal AC enzyme domain (first ~400 residues) and of an ~1,300-residue-long pore-forming RTX hemolysin moiety (15, 16). The latter mediates cell binding and enables the toxin to deliver its catalytic AC domain into cytosol, where the AC is activated by calmodulin and catalyzes uncontrolled conversion of cellular ATP to cyclic AMP (cAMP), a key second messenger molecule (54). Besides that, CyaA can form small cation-selective pores in target cell membranes, which accounts for its moderate hemolytic activity on erythrocytes (6, 7, 11, 19, 48, 53). The capacity of CyaA to penetrate cells, e.g., to form membrane pores and deliver the AC domain into cells, depends on covalent posttranslational fatty-acylation of proCyaA at the ε-amino groups of the internal lysine residues Lys-983 and Lys-860 by a coexpressed protein, CyaC (2–4, 23, 24, 27, 49, 52). The toxin activities further depend on binding of calcium ions to numerous sites formed in the RTX domain by the glycine- and aspartate-rich repetitions of the [X-(L/I/F)-X-G-G-X-G-(N/D)-D] nonapeptide (5, 26, 30, 39, 50).

Several reports demonstrated that CyaA is unique among the enzymatically active toxins in its capacity to cross directly the cytoplasmic membrane of cells and to reach their cytosol without the need for endocytosis. Intoxication of cells by cAMP occurs, indeed, shortly upon exposure to CyaA (14), and in vitro the CyaA can penetrate and intoxicate with detectable efficiency a variety of cell types, including mammalian erythrocytes that lack membrane endocytosis mechanisms (6, 25, 48). The most sensitive primary targets of CyaA in natural infections by *Bordetella*, however, appear to be host myeloid phagocytic cells, such as neutrophils, macrophages, or dendritic cells, which express the $\alpha_M\beta_2$ integrin (CD11b/CD18, CR3, or Mac-1) used as a receptor by CyaA (12, 21). Toxin-induced elevation of intracellular cAMP levels causes, indeed, loss of bactericidal functions of CD11b$^+$ phagocytes, such as chemotaxis, phagocytosis, or superoxide production and promotes apoptosis of macrophages (10, 13, 28, 31, 32, 36, 38, 42, 44, 47, 55, 57). The capacity to intoxicate cells by cAMP is considered to account for the cytotoxic activity of CyaA, and the contribution of the pore-forming activity to toxin potency on myeloid cells remained elusive. While the present study was approaching completion, Hewlett and coworkers (29) used our constructs that only increase cAMP or only create transmembrane pores and reported that at least two mechanisms are contributory to cytotoxicity of CyaA. In contrast to our previous report (43), however, the authors concluded that simply the production of cAMP is not enough to account for the cytotoxicity produced by AC toxin, for which the pore-forming activity also was required (29).

We used here the nonacylated proCyaA, unable to form membrane pores, and the specific CyaA mutants exhibiting a selectively reduced hemolytic activity but full capacity to elevate cAMP in cells, to corroborate the observation that enzymatic depletion of cellular ATP due to toxin action is sufficient to promote cell death and lysis. It is further shown, that pore-forming (hemolytic) activity of CyaA synergizes with the invasive AC enzyme activity in maximizing the overall cytolytic potency of the toxin.

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MATERIALS AND METHODS

Chemicals. Isobutylmethylxanthine, *N*6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (db-cAMP), and 8-bromo adenosine 3′,5′-cyclic monophos-

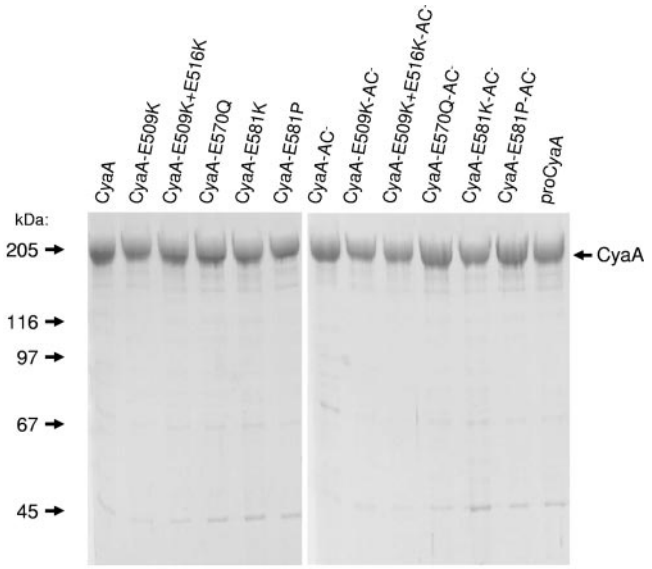

FIG. 1. SDS-PAGE analysis of the purified CyaA-derived proteins. The proteins were produced in *E. coli* XL1-Blue cells and purified from urea extracts of cell debris by a combination of ion-exchange and hydrophobic chromatography as described previously (34). Five micrograms of the purified proteins was separated by SDS-PAGE (7.5%) and visualized by Coomassie blue staining.

phate (8-bromo-cAMP) were purchased from Sigma. All other chemicals were of analytical grade.

Construction of mutant *cyaA* alleles. The site-directed substitutions were introduced into the *cyaA* gene carried on pCACT3 (8) by PCR mutagenesis as described previously (46), using suitable pairs of mutagenic primers. Prior to use in replacement of the corresponding *cyaA* gene portions on pCACT3 (46), the PCR-amplified fragments were entirely sequence verified for the absence of undesired secondary site mutations. CyaA-AC$^-$ toxoid forms of the proteins, unable to convert ATP to cAMP, were generated by placing a CysThr dipeptide between amino acid residues Asp^{188} and Ile^{189} of the ATP binding site in the catalytic domain of CyaA, as previously described (34, 45).

Expression and purification of the CyaA-derived proteins. The nonacylated proCyaA protein was expressed from pACT7 (52). The intact CyaA and its mutant derivatives were produced in the presence of the activating protein toxin acyltransferase CyaC coexpressed in *Escherichia coli* XL1-Blue (Stratagene) cells from the appropriate plasmids derived from pCACT3 (46). The proteins extracted from insoluble cell debris with 8 M urea were purified close to homogeneity by a combination of ion-exchange chromatography on DEAE-Sepharose and phenyl-Sepharose as described earlier (34). The final protein samples were eluted in 8 M urea, 50 mM Tris-HCl (pH 8.0) buffer with 0.2 mM $CaCl_2$ and stored frozen. The homogeneity of the preparations was verified by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, as documented in Fig. 1.

Hemolytic activity on sheep erythrocytes. Hemolytic activity was measured by photometric (A_{541}) determination of the amount of hemoglobin released in time from 5×10^8/ml of washed erythrocytes in the presence of 5 μg/ml of the individual toxins (6). The results represent the average of values obtained in at least three independent experiments performed in duplicate.

Cell cultures and toxin handling for activity assays. Murine monocyte/macrophage cells of the cell line J774A.1 (ATCC TIB 67) were grown in RPMI medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and appropriate antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B). Chinese hamster ovary (CHO) cells transfected for human CD11b/CD18 were obtained from D. Golenbock (Boston University School of Medicine, Boston, MA) and cultured in the presence of neomycin in F-12 medium supplemented with heat-inactivated bovine serum as described previously (33). Prior to assay, 10^5 cells per well were seeded into 96-well plates and allowed to attach for 2 h. To avoid uncontrollable chelation of calcium ions by the phosphate ions contained in RPMI medium during toxin activity assays, RPMI was replaced by 150 μl/well of Dulbecco's modified Eagle's medium (DMEM) (1.9 mM Ca^{2+}) without fetal calf serum (FCS) and the cells were allowed to rest in DMEM for 1 h at 37°C in a humidified 5% CO_2 atmosphere. Prior to addition to cells, the toxin samples were prediluted from concentrated stocks to 100 times the final indicated concentration using 8 M urea, 50 mM Tris-HCl, pH 8.0, and 0.2 mM $CaCl_2$ (UTC). Before addition to cells, the toxin solutions were first rapidly diluted 25 times in prewarmed DMEM, to reduce the urea concentration to 0.32 M, and 50-μl aliquots of the diluted toxin samples were immediately admixed with 150 μl of DMEM covering the cells. This yielded the indicated toxin concentrations and a final urea concentration of 80 mM. Appropriate blanks containing identical amounts of cells incubated with 80 mM urea in DMEM were systematically scored in all activity assays, and no effect whatsoever of the 80 mM urea concentration on cell viability and/or enzymatic activities was observed. All experiments were repeated with at least two independent toxin preparations.

ATP level, cell viability, and lysis assays. The ATP level in J774A.1 cells was determined using the ATP bioluminescence assay kit CLS II (Roche). Cell viability was assessed spectrophotometrically as the capacity of mitochondrial dehydrogenases to reduce the WST-1 substrate (Roche) to its tetrazolium salt. Cell lysis was determined as lactate dehydrogenase (LDH) release from J774A.1 or CHO-CD11b/CD18 cells using the CytoTox 96 kit assay (Promega). The assays were performed according to manufacturer's instructions and the results represent average of values obtained in at least two independent experiments performed in triplicates ($n = 6$).

Determination of cAMP. A total of 10^5 J774A.1 cells were incubated with different concentrations of the CyaA-derived proteins for 30 min in DMEM containing 100 μM isobutylmethylxanthine. The reaction was stopped by addition of 0.2% Tween 20 in 50 mM HCl, and samples were boiled for 15 min at 100°C to denature cellular proteins (cAMP is resistant to acid and heat). The samples were neutralized by addition of 150 mM unbuffered imidazole, and the concentration of cAMP was determined by a competition immunoassay (35) performed as previously described (43). The results represent average values obtained in at least three independent experiments performed in duplicate ($n = 6$).

RESULTS

Enzymatic dissipation of ATP and not accumulation of cAMP as such accounts for cytolytic potency of CyaA on monocytic cells. We sought to analyze the respective contributions of the enzymatic and pore-forming activities to the overall cytotoxic potency of CyaA. Toward this aim, lysis of murine J774A.1 cells was assayed, since these macrophage-like monocytic cells express the $\alpha_M\beta_2$ integrin (CD11b/CD18) receptor and were previously found to be quite susceptible to the action of CyaA (22, 38).

As shown in Fig. 2A, J774A.1 cells rapidly lysed upon exposure to toxin concentrations well below 1 μg/ml and the toxin concentration causing half-maximal lysis of cells (LC_{50}) within the arbitrarily chosen interval of 3 h was 171 ± 11 ng/ml (Table 1) (43). Cell lysis was preceded by rapid accumulation of high concentrations of cAMP that were elevated already within 5 min of exposure to more than 10 ng of toxin per ml (Fig. 2B). Using 1 picoliter as the approximate volume of a single J774A.1 cell, it could be calculated from data shown in Fig. 2C that at the LC_{50} dose of CyaA (200 ng/ml) the intracellular cAMP level exceeded a 1 mM concentration within 10 min. In agreement with previous reports (1), the massive increase of cAMP level was accompanied by a concomitant decrease of intracellular concentration of ATP (Fig. 2C). In parallel, viability of J774A.1 cells, assessed as capacity of mitochondrial dehydrogenases to reduce the WST-1 substrate, was importantly compromised already within 20 min of exposure to an LC_{50} dose of CyaA (Fig. 2D).

Although there was a clear relationship between the increase in the cAMP level and depletion of the cellular ATP pool, it was important to assess the potential contribution of the pore-forming activity of CyaA to depletion of cellular ATP,

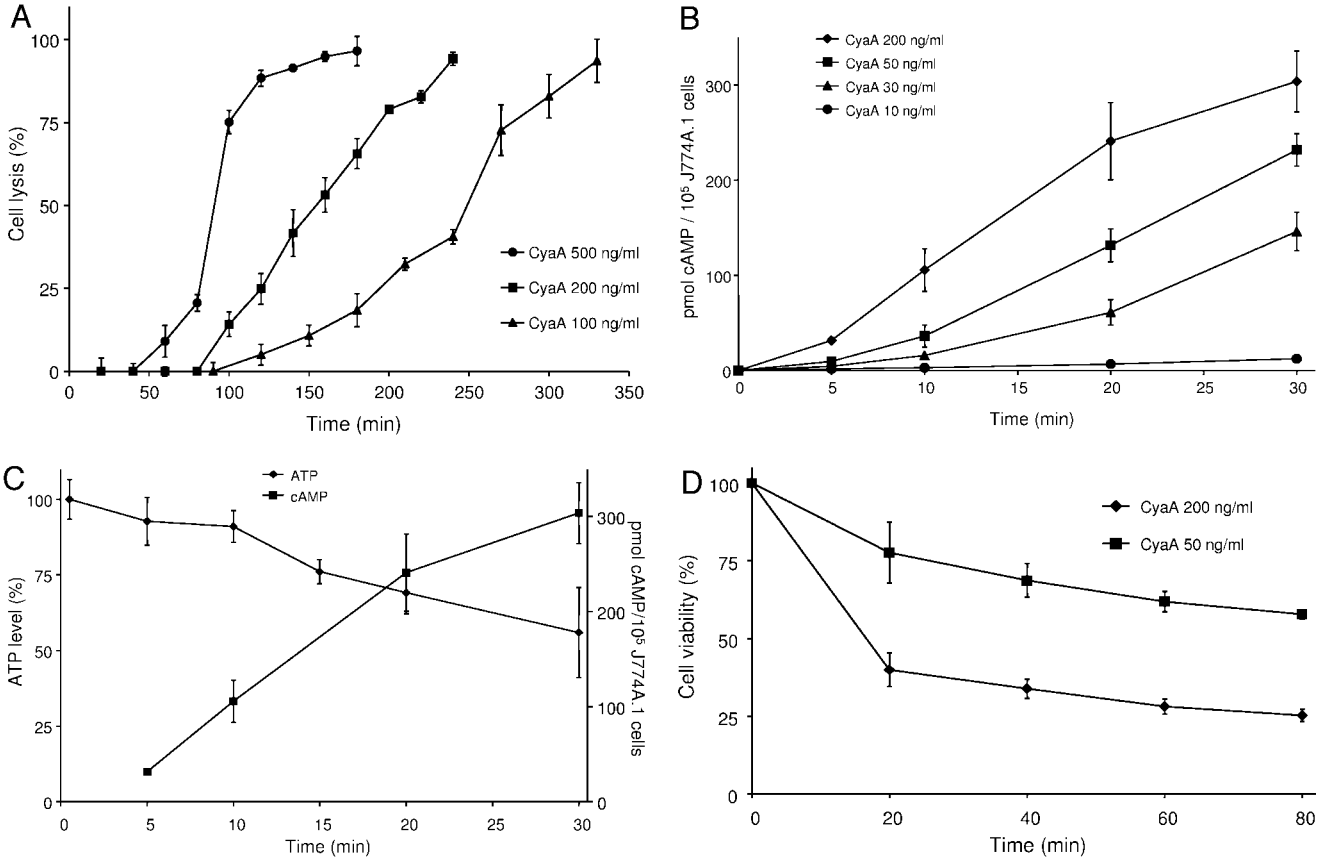

FIG. 2. J774A.1 murine monocytes are highly susceptible to CyaA toxin activity. (A) J774A.1 cells rapidly lyse upon exposure to low concentrations of CyaA. A total of 10^5 J774A.1 cells were incubated with CyaA at 37°C in DMEM, and the extent of cell lysis was determined at the indicated time points as the amount of LDH released into culture media, using a Cytotox 96 assay kit (Promega). (B) Low CyaA concentrations cause massive elevation of cAMP level in cells. A total of 10^5 J774A.1 cells were incubated at 37°C with indicated concentrations of toxin in DMEM. The reaction was stopped after 5, 10, 20, and 30 min by addition of 0.2% Tween 20 in 50 mM HCl; the samples were boiled for 15 min at 100°C, neutralized by addition of 150 mM unbuffered imidazole; and the cAMP concentration was determined by immunoassay (35). (C) cAMP accumulation is accompanied by depletion of cellular ATP. A total of 10^5 J774A.1 cells were incubated with 200 ng/ml of CyaA in DMEM, and the ATP level was monitored over time in cell aliquots using the ATP Bioluminescence Assay kit CLS II (Roche). (D) CyaA activity causes loss of cell viability. A total of 10^5 J774A.1 cells were incubated with CyaA, and cell viability was assessed spectrophotometrically at the indicated time points as the amount of the WST-1 substrate (Roche) reduced to its tetrazolium salt by mitochondrial dehydrogenases. The given results are representative of at least two independent determinations performed in triplicate.

since permeabilization of cell membrane by CyaA could be expected to activate ATP-consuming membrane transporters involved in maintenance of ion homeostasis. Therefore, the level of cellular ATP was followed over the time of cell exposure to an enzymatically inactive CyaA-AC$^-$ construct unable to convert ATP to cAMP (41). This toxoid was previously shown to possess a full capacity to bind CD11b/CD18, to be fully hemolytic, and to deliver the modified AC domain into cell cytosol, respectively (12, 21, 45, 51). As, however, demonstrated in Fig. 3A, no measurable decrease of ATP level was observed in cells exposed to 200 ng/ml of CyaA-AC$^-$ over time, while the intact toxin caused a reduction of cellular ATP to ~25% of the initial level within 60 min. Hence, at an LC_{50} concentration of the toxin, the contribution of pore-forming activity of CyaA to depletion of cellular ATP pool was negligible.

It was further important to ascertain whether intracellular accumulation of cAMP, a key signaling molecule, was itself contributing to the rapid killing and lysis of J774A.1 cells independently of ATP depletion. This was unlikely, since highly supraphysiological cAMP concentrations were reached in 30 min also in cells exposed to ≤50 ng/ml of CyaA (cf. Fig. 2B), while no J774A.1 lysis was observed at these toxin concentrations within 3 h, as shown in Fig. 3B. In line with that, no lysis was observed upon exposure of cells to high concentrations of the membrane-permeable cAMP analogues (10 mM db-cAMP or 3 mM 8-Br-cAMP; not shown). Neither did the presence of the cAMP analogues enhance the residual (~10%) cytolytic capacity of the CyaA-AC$^-$ toxoid (Fig. 3B). It can, hence, be concluded that neither mere accumulation of cAMP nor signaling of the CD11b/CD18 receptor upon engagement by CyaA or permeabilization by the pore-forming activity was accounting for rapid lysis of cells at CyaA concentrations exceeding 100 ng/ml. In contrast, the capacity of the invasive AC enzyme to dissipate cellular ATP into cAMP appeared as central to the cytolytic action of CyaA.

TABLE 1. Toxin activities of CyaA constructs[a]

Toxin	Results for J774A.1 cells				Results for erythrocytes (hemolytic activity [% of WT] in AC^+/AC^-)[d]
	cAMP intoxication ($C_{10\_pmol\_cAMP}$ [ng/ml])[b]		Cell lysis (CL_{50} [ng/ml])[c]		
	AC^+[e]	AC^-[f]	AC^+[e]	AC^-[f]	
CyaA	15 ± 2	ND	171 ± 11	2,812 ± 457	100 ± 8
CyaA-E509K	24 ± 7	ND	112 ± 7	536 ± 47	220 ± 14
CyaA-E509K+E516K	956 ± 117	ND	367 ± 91	485 ± 96	500 ± 28
CyaA-E581K	61 ± 7	ND	97 ± 6	643 ± 56	320 ± 23
CyaA-E570Q	12 ± 2	ND	169 ± 12	>5,000	49 ± 11
CyaA-E581P	20 ± 4	ND	273 ± 47	>5,000	25 ± 11
proCyaA[g]	493 ± 107	Not determined	6,273 ± 766	Not determined	<1

[a] Site-directed substitutions were introduced into the *cyaA* gene carried on pCACT3 (8) using PCR mutagenesis as previously described (46). Intact CyaA and its mutant derivatives were produced in *E. coli* XL1-Blue (Stratagene) in the presence of the activating toxin acyltransferase, CyaC, coexpressed from the appropriate plasmid constructs derived from pCACT3, as previously described (46). All activity determinations were repeated with at least two independent toxin preparations. The results represent average of values obtained in at least three independent experiments performed in duplicate ± standard deviation. ND, not detectable.

[b] $C_{10\_pmol\_cAMP}$ corresponds to the concentration of added toxin that caused accumulation of 10 pmol of cAMP in 10^5 J774A.1 cells incubated with toxin for 30 min at 37°C in DMEM. Intracellular cAMP concentrations were determined by enzyme-linked immunosorbent assay as described in the legend to Fig. 2.

[c] CL_{50} was determined as the concentration of toxin (ng/ml) causing half-maximal (50%) release of lactate dehydrogenase from 10^5 J774A.1 cells upon 3 h of incubation at 37°C in DMEM.

[d] Lysis of sheep erythrocytes (RBC) was determined as the amount of hemoglobin (6) released in time upon incubation of 5×10^8 RBC at 37°C in the presence of 2 mM Ca^{2+} with 5 μg/ml of the given toxin. The hemolytic activity of intact CyaA was taken as 100% activity. The enzymatically active (AC^+) and inactive (AC^-) proteins exhibited the same specific hemolytic activities. Therefore, results obtained with both protein forms were pooled, and the average of values from at least three separate experiments performed in duplicate are given ± standard deviation.

[e] AC^+, enzymatically active CyaA-derived proteins capable to catalyze conversion of cellular ATP to cAMP.

[f] AC^-, toxoid CyaA variants unable to convert ATP to cAMP due to ablation of enzymatic activity by a CysThr dipeptide insertion between residues Asp^{188} and Ile^{189} of the ATP binding site within the AC domain (34, 45).

[g] Toxin activity values for nonacylated proCyaA were taken from Masin et al. (43).

Enhanced pore-forming activity of CyaA can on its own promote lysis of CD11b/CD18-expressing cells. The AC^- toxoid exhibited only about 10% of the specific cytolytic potency of intact CyaA (Fig. 3B), and this was most likely due to the conserved pore-forming (hemolytic) activity of CyaA-AC^-. Cell lysis occurring at the high concentrations of CyaA-AC^- appeared, indeed, to proceed by a different mechanism from lysis of cells induced at the much lower concentrations of intact CyaA, as witnessed by the quite different appearance of cells treated by LC_{50} doses of CyaA or of its AC^- toxoid. As shown in Fig. 4A, the ATP-depleting CyaA reproducibly induced massive vacuolization of J774A.1 cells in 90 min, while no vacuolization of cells was observed upon treatment with LC_{50} amounts of CyaA-AC^- that caused only mild decrease of cellular ATP over time (Fig. 4B).

To address in more detail the role of the pore-forming activity in the overall capacity of CyaA to kill and lyse J774A.1 cells, we characterized the cytolytic activities of a subset of CyaA constructs exhibiting reduced or enhanced specific pore-forming activities (46). These CyaA variants were recently generated in frame of a screening for mutations affecting the hemolytic activity of CyaA (M. Basler et al., in preparation), and their cell-invasive AC and cytolytic activities are summarized in Table 1. Interestingly, the CyaA-E509K, CyaA-E581K, and CyaA-E509K+E516K proteins, bearing positively charged lysine residues in place of the negatively charged glutamates at positions 509, 516, or 581 of the pore-forming domain, respectively, exhibited about two, three, and five times higher specific hemolytic activities on sheep erythrocytes than intact CyaA. In contrast, as further shown in Table 1, the CyaA-E570Q and CyaA-E581P constructs carrying a neutral glutamine, or an α-helix-breaking proline residue in place of the glutamates 570 and 581, respectively, displayed about two- and four-times-reduced specific hemolytic activity due to forming pores of decreased size (M. Basler et al., in preparation).

To assess the proper contribution of the enhanced or reduced pore-forming capacity to the cytolytic potency of the mutant constructs, their biasing capacity to dissipate cellular ATP into cAMP was ablated and the cytolytic activity of thus obtained AC^- toxoids was determined. As also documented in Table 1, indeed, a clear relationship between the relative hemolytic activity of a given toxoid and its half-lytic concentration on J774A.1 cells was observed. The less hemolytic CyaA-E570Q-AC^- and CyaA-E581P-AC^- constructs failed to produce any J774A.1 lysis even at the highest concentration tested (5,000 ng/ml). In turn, the AC^- toxoids having the pore-forming (hemolytic) activity increased by E509K, E509K+E516K, or E581K substitutions, exhibited a correspondingly enhanced specific cytolytic activity on J774A.1 cells.

Since the cytolytic potency of the superhemolytic toxoids on J774A.1 cells was only about two to three times lower than that of the intact CyaA, it was important to assess whether also these toxoids provoked extensive depletion of cellular ATP. As, however, shown in Fig. 4B, compared to the rapid depletion of ATP caused by an LC_{50} amount of intact CyaA, the LC_{50} doses of less hemolytic (CyaA-E570Q-AC^-), intact (CyaA-AC^-), or superhemolytic (CyaA-E509K+E516K-AC^-, CyaA-E581K-AC^-) toxoids all produced a comparably mild decrease of cellular ATP level. This suggests that it was the permeabilization of cell membrane by AC^- toxoids that provoked lysis of the J774A.1 monocytes. In line with that, at higher protein concentrations (5,000 ng/ml), a similar pattern of specific cytolytic activity of the mutant AC^- toxoids was observed also when nonmyeloid CHO cells sensitized by expression of the toxin receptor CD11b/CD18 were used as targets, as documented in Fig. 5.

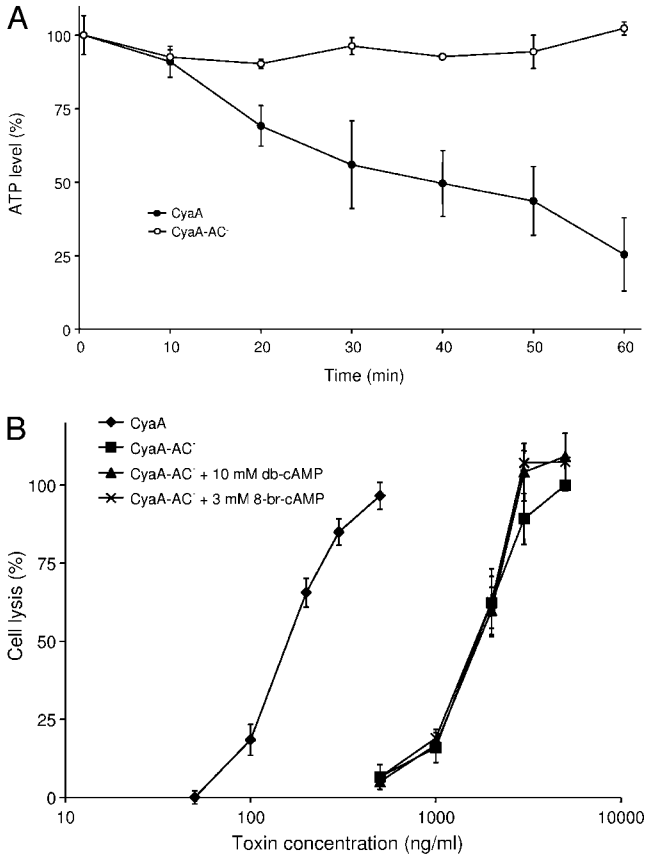

FIG. 3. Enzymatic depletion of ATP and not accumulation of cAMP as such accounts for rapid lysis of J774A.1 cells by CyaA. (A) Depletion of ATP in J774A.1 monocytes is due to enzymatic activity of CyaA. A total of 10^5 J774A.1 cells were incubated with 200 ng/ml of CyaA or CyaA-AC$^-$ in DMEM, and the ATP concentration over the time of incubation was monitored in cell aliquots using the ATP Bioluminescence Assay kit CLS II (Roche). (B) Accumulation of cAMP as such does not promote cell lysis. A total of 10^5 J774A.1 cells were incubated for 3 h in DMEM and with different concentrations of intact CyaA, its enzymatically inactive CyaA-AC$^-$ toxoid alone, or with CyaA-AC$^-$ plus 10 mM db-cAMP or 3 mM 8-bromo-cAMP, respectively. The extent of cell lysis was determined by LDH release assay as above. The results are representative of two independent determinations performed in triplicate.

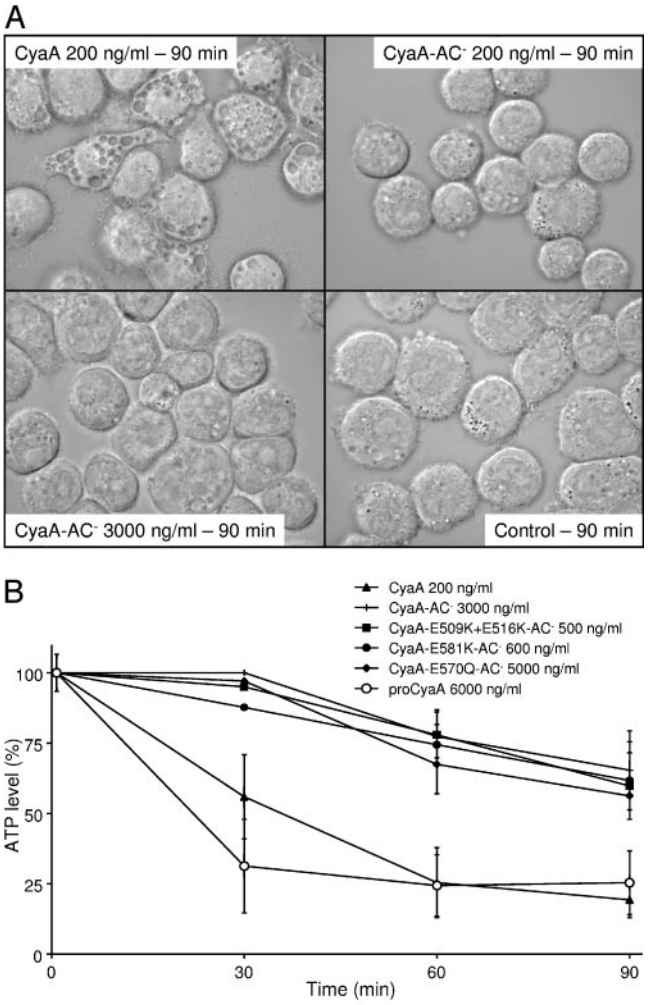

FIG. 4. Enzymatic activity and not the pore-forming capacity of CyaA induces depletion of ATP and vacuolization of J774A.1 cells. (A) Monocytes were grown overnight on glass coverslips in RPMI medium; the medium was changed for DMEM 2 h prior to addition of the toxins at indicated concentrations. After 90 min of exposure to toxin in DMEM at 37°C, the J774A.1 cells were viewed at a magnification of ×100 using Nomarski differential interference contrast optics with an Olympus BX60 microscope. The experiment was repeated twice, and representative images from a series of micrographs are shown. (B) Pore-forming activity of CyaA causes only moderate ATP depletion even at LC_{50} doses of AC$^-$ toxoids. A total of 10^5 J774A.1 cells were incubated with mutant CyaA-AC$^-$ toxoids, intact acylated CyaA, or the nonacylated proCyaA at protein concentrations representing the respective LC_{50} (see Table 1) in DMEM. The ATP level in toxin-treated cells over time was determined in cell aliquots using the ATP Bioluminescence Assay kit CLS II (Roche). The results are representative of at least three independent determinations performed in triplicate.

Pore-forming activity of CyaA synergizes with the cell-invasive AC enzyme action that by itself is capable to promote cell lysis. Despite a difference of almost 2 orders of magnitude in the respective capacity to raise cellular cAMP levels, however, the intact CyaA and the superhemolytic CyaA-E509K+E516K construct were comparably potent in causing lysis of J774A.1 cells (Table 1). Thus, the increased pore-forming capacity of CyaA-E509K+E516K could rather efficiently compensate for the essentially nil capacity of the mutant to deliver the AC domain into cells and convert cellular ATP to cAMP. Moreover, the other superhemolytic proteins, CyaA-E509K and CyaA-E581K, which exhibited only a moderately decreased capacity to deliver the AC enzyme into cells and dissipate cellular ATP into cAMP, both scored reproducibly as almost two times more potent at causing J774A.1 cell lysis than intact CyaA was (Table 1). These results, hence, clearly show that a severalfold-enhanced specific pore-forming activity of the CyaA-E509K or CyaA-E581K mutants could synergize with even a reduced capacity to convert cellular ATP to cAMP, overriding the latter defect and bringing about an overall increase of the specific cytolytic potency of the toxin.

On the other hand, it needs to be stressed that the contribution of pore-forming activity to the overall cytolytic potency of CyaA was only seen with mutants exhibiting either a reduced capacity to elevate cAMP in cells or an enhanced spe-

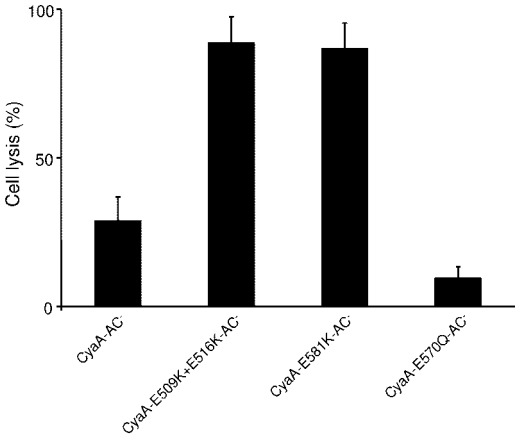

FIG. 5. CyaA promotes lysis of nonmyeloid CHO cells expressing CD11b/CD18. CHO transfectants expressing the CD11b/CD18 receptor (10^5 cells) were incubated with 5,000 ng/ml of CyaA variants at 37°C in DMEM for 3 h, and the extent of cell lysis was determined as the amount of LDH released into culture media using the Cytotox 96 assay kit (Promega). The results are representative of at least three independent determinations performed in triplicate.

cific hemolytic activity. When the capacity of CyaA mutants to dissipate cellular ATP into cAMP was intact, as observed with the CyaA-E570Q and CyaA-E581P proteins, it overpowered the substantial loss of specific pore-forming (hemolytic) activity and the less hemolytic proteins exhibited the same cytolytic potency on J774A.1 cells as intact CyaA (Table 1). Furthermore, despite not exhibiting any hemolytic activity and possessing only ~0.1% of the specific pore-forming capacity of CyaA (7, 43), the nonacylated proCyaA was still capable to cause rapid lysis of J774A.1 cells at concentrations exceeding 5 μg/ml (Table 1). At this high protein concentration (LC_{50}, ~6,000 ng/ml), the about 30-times-less-potent proCyaA caused, indeed, massive depletion of ATP in J774A.1 cells, as did LC_{50} amounts (200 ng/ml) of acylated CyaA (cf. Fig. 4B). Thus, the pore-forming (hemolytic) activity was dispensable for cell killing and played an auxiliary, synergic role in the cytolytic action of CyaA.

DISCUSSION

We report here dissection of the complex cytolytic action of CyaA into the individual contributions of its cell-invasive AC enzyme and pore-forming activities and show that CyaA produces rapid cell death primarily through enzymatic dissipation of ATP into cAMP, while permeabilization by the pore-forming activity of CyaA can also contribute to cell death.

Using specific toxin mutants with two activities manipulated one at a time, only partial synergy of the enzymatic and pore-forming activities in promoting rapid lysis of the CD11b-expressing monocytes was, however, observed. A full capacity to dissipate cellular ATP into cAMP appeared, indeed, to be sufficient for conferring on less hemolytic CyaA mutants full potency to promote rapid lysis of J774A.1 cell lysis. Moreover, lysis of monocytes in vitro could not be induced by high concentrations of cell-permeable cAMP analogues alone, nor could cAMP enhance lysis produced by cell-permeabilizing CyaA-AC^- toxoid. This strongly suggests that it was the overwhelming depletion of cellular ATP resulting from action of the cell-invasive AC enzyme activity and not the signaling of accumulated cAMP or activation of the apoptotic program that accounted for rapid death and lysis of monocytes. This interpretation differs somewhat from conclusions reached in previous studies and derived from results obtained under somewhat different experimental conditions (9, 29, 36).

In this respect, it is noteworthy that precautions applied in this study during toxin purification and especially in toxin handling while setting up the cytotoxicity assays allowed us to observe high specific toxin activities, in terms of capacity of CyaA to elevate cAMP and to promote cell lysis at low toxin concentration (~200 ng/ml). It remains, nevertheless, an open question whether toxin levels similar to the LC_{50}s of CyaA determined here may be encountered under physiological conditions by phagocytes entering the *Bordetella*-colonized tracheal epithelia. Local in vivo concentrations of active toxin surrounding a phagocyte in contact with adhering *Bordetella* bacteria are difficult to estimate. When *B. pertussis* is grown in vitro, the amounts of CyaA in exponential cultures typically do not exceed 100 to 200 ng of total CyaA protein per ml, or 10^9 bacteria (P. Sebo, unpublished results). Moreover, most of the accumulated CyaA appears to be tightly associated with bacterial surface and unable to penetrate and intoxicate target cells. Only toxin molecules newly secreted by the adhering bacteria and representing a fraction of total accumulated CyaA at a given moment were found to be effective in intoxication of phagocytic cells (18). We attempted here to mimic the process of folding and formation of active toxin in the vicinity of target cell membrane, which the unfolded toxin precursor has to undergo in vivo after being excreted by the bacteria through the "channel-tunnel" type I secretory apparatus (40). Toward this aim, rapid dilution of urea-unfolded toxin directly into cell suspensions was performed, in order to allow renaturation of CyaA to proceed in the presence of target cells. Despite that, the observed LC_{50}s of CyaA (~200 ng/ml) still appear rather high. Thus, rather than direct cell killing by toxin-induced ATP depletion, the requirement for CyaA in *B. pertussis* virulence would reflect the capacity of CyaA to induce phagocyte impotence by signaling of formed cAMP and its synergy with the effects of other bacterial components, such as lipopolysaccharide and other toxins, in inducing phagocyte apoptosis (20, 36).

The other important finding of the present study is that the second activity of CyaA, the pore-forming (hemolytic) activity, may be contributing to the overall cytotoxic action of the toxin more importantly than anticipated earlier. The results obtained with CyaA mutants exhibiting an enhanced specific hemolytic activity clearly show that at fairly low protein concentrations (≤600 ng/ml), the capacity of the superhemolytic CyaA mutants to permeabilize cells by forming membrane channels was by itself sufficient to contribute a cytolytic activity toward myeloid cells expressing CD11b/CD18. Moreover, in the case of the CyaA-E581K protein, a clear synergy between the enhanced pore-forming and the reduced cell-invasive AC enzyme activities was observed, yielding an overall two-times-superior cytolytic potency of the mutant toxin. This synergy of the two activities may, indeed, be quite relevant in vivo for the cytotoxic activity of CyaA naturally made by *Bordetella.* It should be noted that in this study we have used recombinant CyaA produced in *E. coli* (r-$CyaA_{Ec}$) because of the technical

difficulty of producing sufficient amounts of purified CyaA and of its mutant derivatives in *B. pertussis* strains. The r-CyaA$_{Ec}$ was, however, previously shown to be aberrantly acylated by unsaturated palmitoleil (*cis* Δ9 $C_{16:1}$) fatty-acyl groups and to exhibit an about four times lower specific hemolytic activity than the native CyaA produced by *B. pertussis* (CyaA$_{Bp}$).The latter appears to be acylated exclusively by saturated palmitoyl (C16:0) residues, while both r-CyaA$_{Ec}$ and CyaA$_{Bp}$ were found to exhibit the same capacity to deliver the AC domain into target cells (3, 4, 7, 8, 24, 27, 52). In this light, the E581K substitution brought back the hemolytic potency of the r-CyaA$_{Ec}$ close to that of the native CyaA$_{Bp}$ toxin. As such enhancement of the hemolytic activity resulted in doubling of the overall cytolytic potency of the r-CyaA$_{Ec}$ toxin, despite reduction of the cell-invasive AC activity of the construct, the higher pore-forming (hemolytic) activity of CyaA$_{Bp}$ may, indeed, be making an important contribution to the capacity of the native toxin to neutralize host phagocytes in vivo.

The pore-forming (hemolytic) capacity of CyaA is generally considered to be relatively weak, compared to that of true pore-forming toxins, and its contribution to toxin activity and *Bordetella* virulence remained, indeed, largely disregarded. The physiological role of the pore-forming activity of CyaA could not be conclusively examined so far, since the CyaA-E570Q construct described here appears to be the first toxin variant exhibiting a selectively reduced specific hemolytic activity, while retaining a full capacity to penetrate cells and elevate cellular cAMP levels. Its achievement opens the way to assessment of the contribution of the pore-forming activity of CyaA to the key role played by this toxin in the virulence of *Bordetella pertussis.*

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