The Third Activity of Bordetella Adenylate Cyclase (AC) Toxin-Hemolysin

**MEMBRANE TRANSLLOCATION OF AC DOMAIN POLYPEPTIDE PROMOTES CALCIUM INFLUX INTO CD11b⁺ MONOCYTES INDEPENDENTLY OF THE CATALYTIC AND HEMOLYTIC ACTIVITIES**

Radovan Fišer†§, Jiří Mašín§, Marek Basler§, Jan Krůšek†, Veronika Špuláková†, Ivo Konopášek†§ and Peter Šebo†‡§

From the †Department of Genetics and Microbiology, Faculty of Science, Charles University, CZ-128 44, Prague 2 and the §Laboratory of Molecular Biology of Bacterial Pathogens, Institute of Microbiology, and the ‡Department of Cellular Neurophysiology, Institute of Physiology, Academy of Sciences of the Czech Republic, CZ-142 20, Prague 4, Czech Republic

The Bordetella adenylate cyclase toxin-hemolysin (CyaA) targets phagocytes expressing the α₅β₂ integrin (CD11b/CD18), permeabilizes their membranes by forming small cation-selective pores, and delivers into cells a calmodulin-activated adenylate cyclase (AC) enzyme that dissipates cytosolic ATP into cAMP. We describe here a third activity of CyaA that yields elevation of cytosolic calcium concentration ([Ca²⁺]) in target cells. The CyaA-mediated [Ca²⁺] increase in CD11b⁺ J774A.1 monocytes was inhibited by extracellular La³⁺ ions but not by nifedipine, SK&F 96365, flunarizine, 2-aminoethyl diphenylborinate, or thapsigargin, suggesting that influx of Ca²⁺ into cells was not because of receptor signaling or opening of conventional calcium channels by cAMP. Compared with intact CyaA, a CyaA-AC− toxoid unable to generate cAMP promoted a faster, albeit transient, elevation of [Ca²⁺]. This was not because of cell permeabilization by the CyaA hemolysin pores, because a mutant exhibiting a strongly enhanced pore-forming activity (CyaA-E509K/E516K), but unable to deliver the AC domain into cells, was also unable to elicit a [Ca²⁺] increase. Further mutations interfering with AC translocation into cells, such as proline substitutions of glutamate residues 509 or 570 or deletion of the AC domain as such, reduced or ablated the [Ca²⁺]-elevating capacity of CyaA. Moreover, structural alterations within the AC domain, because of insertion of various oligopeptides, differently modulated the kinetics and extent of Ca²⁺ influx elicited by the respective AC− toxins. Hence, the translocating AC polypeptide itself appears to participate in formation of a novel type of membrane path for calcium ions, contributing to action of CyaA in an unexpected manner.

Adenylate cyclase toxin-hemolysin (AC-Hly or CyaA) is a key virulence factor involved in the early stages of the respiratory tract colonization by Bordetella pertussis, a Gram-negative pathogen causing whooping cough (1). Physiologically, the most relevant cellular targets of the toxin appear to be the myeloid phagocytic cells, such as neutrophils (2). These express the α₅β₂ integrin receptor CD11b/CD18 (known also as CR3 or Mac-1) to which CyaA binds with high affinity (3). The toxin can form small, transient, and cation-selective membrane pores that permeabilize cells and account for the hemolytic activity of CyaA on erythrocytes (4–6). The major activity of the toxin on host cells, however, consists in delivery of an adenylate cyclase (AC)² enzyme domain into cells, where this is activated by binding of cytoplasmic calmodulin and catalyzes unregulated conversion of ATP to cAMP (7, 8). Dissipation of cellular ATP and signaling of cAMP then causes impairment of microbicidal functions of host phagocytes, such as inhibition of chemotaxis, oxidative burst, and phagocytosis and eventually results in cell apoptosis (7, 9–17). Recent results suggest a role for CyaA in promoting incomplete maturation of dendritic cells, possibly yielding immune tolerance of the pathogen on respiratory epithelia (18–22). Moreover, CyaA can increase cAMP levels detectably also in a variety of other cell types lacking the CD11b/CD18 receptor, including erythrocytes (4), and CyaA action can elicit release of the proinflammatory cytokine IL-6 from tracheal epithelial cells (23).

The bi-functional toxin molecule consists of an N-terminal ~400-residue-long AC enzyme domain that is linked to a characteristic RTX hemolysin moiety (Hly) of ~1300 residues (24). The Hly itself consists of several functional domains (25). It contains a hydrophobic channel-forming domain, including residues 500–800 (6), and an acylation subdomain of Hly

---

2 The abbreviations used are: AC, adenylate cyclase; 2-APB, 2-aminoethyl diphenylborinate; 2DG, 2-deoxy-o-glucose; AC-domain, N-terminal enzymatic adenylate cyclase domain; AM, acetoxymethyl ester; CyaA, adenylate cyclase toxin; CyaC, acyltransferase; CyaA-AC−, enzymatically inactive adenylate cyclase toxin; [Ca²⁺], free cytosolic calcium concentration; Br₂cAMP, dibutylryl cyclic AMP; FCCP, carbonyl cyanide p-(trifluoromethyl)oxyphenylhydrazone; HBSS, Hank's balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; PMCA, plasma membrane Ca²⁺ -ATPase; SERCA, sarcoplasmic reticulum Ca²⁺ -ATPase; SKG6 96365, 1-(1-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole hydrochloride; DMEM, Dulbecco’s modified Eagle’s medium; OVA, ovalbumin.

---

*This work was supported by Grant 146/2005/3-B IO/PrF from Charles University (to R.F.), Grant 1M0506 from the Ministry of Education, Youth, and Sports (to J.M. and M.B.), European Union 6th FP Contract LSBB-CT-2003-503582 THERAVAC (to P.S.), and Grant IAAS020406 (to J.K.) and the Institutional Research Concept 50200510 from the Academy of Sciences of the Czech Republic. 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.

†‡§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

‡ To whom correspondence should be addressed. Tel.: 420-241-062-762; Fax: 420-241-062-152; E-mail: sebo@biomed.cas.cz.
located between residues 800 and 1000 carries the post-translational fatty acyl modifications of CyaA, which are essential for toxin activity (26, 27). Finally, a typical calcium-binding RTX domain occupies the C-terminal half of Hly between residues 1008 and 1706, where glycine- and aspartate-rich nonapeptide repeats are located that form the numerous (~40) calcium-binding sites of CyaA (28, 29). Both toxin activities, penetration across target cell membranes, and formation of cation-selective pores strictly depend on covalent post-translational palmitoylation of the ε-amino groups of internal lysine residues 860 and 983 (30) and on binding of calcium ions into sites within the glycine- and aspartate-rich repeats (29).

A unique feature of CyaA is its capacity to translocate the enzymic AC domain directly across the cytoplasmic membrane of target cells, without the need for receptor-mediated endocytosis (31, 32). Translocation across, but not insertion of, CyaA into the cytoplasmic membrane of cells as such appears to be driven by negative (inside) membrane potential (33). The path and mechanism of direct passage of the ~40-kDa AC domain across the lipid bilayer of target membranes remain, however, poorly understood. The AC domain does not appear to enter cells through the CyaA hemolysin pore, which exhibits a diameter of only 0.6–0.8 nm (6). Formation of pores and translocation of the AC domain indeed appear to be two parallel and independent activities of the membrane-inserted forms of CyaA and can be dissociated and manipulated independently by mutations, fatty-acylation status, and assay conditions, such as temperature and calcium loading (26, 34–37).

Recent work suggests that the pore forming activity contributes to cytotoxic action of CyaA by synergizing with enzymic conversion of ATP to cAMP in promoting cell death (38, 39). Aside from perturbing ion homeostasis and promoting colloid-osmotic lysis of permeabilized cells, the cytotoxic contribution of the pore forming activity of CyaA might also be expected to consist of elevating the cytoplasmic levels of calcium ions ([Ca\(^{2+}\)]), as has been observed with many other bacterial pore-forming toxins (40). Indeed, modulation of [Ca\(^{2+}\)], belongs to the most prominent mechanisms of cellular signaling, and [Ca\(^{2+}\)], regulates many cellular pathways, including those leading to cell death through apoptosis or necrosis (41–44). Therefore, [Ca\(^{2+}\)], is tightly controlled and maintained at a low level (~100 nm), as prolonged elevation of [Ca\(^{2+}\)], is lethal to most cell types. Toxin action of CyaA leading to an increase of cellular cAMP level was indeed shown to promote massive calcium influx and increase of [Ca\(^{2+}\)], in cardiac myocytes and βTC3 cells through opening of cAMP-regulated L-type calcium channels (33, 45). However, it has not been examined whether CyaA can elevate [Ca\(^{2+}\)], also in its natural targets that lack the L-type Ca\(^{2+}\) channels, such as the CD11b-expressing myeloid phagocytes.

Here we report that CyaA causes elevation of [Ca\(^{2+}\)], in CD11b\(^+\) J774A.1 monocytes by a novel mechanism that is independent of its enzymatic or pore forming activities and that requires structural integrity and membrane translocation of the AC domain polypeptide as such.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—2-APB, 2-deoxy-D-glucose (2DG), Bt\(_2\)cAMP, EGTA, FCCP, flunarisin, IBMX, LaCl\(_3\), nifedipine, SK&F 96365, calmodulin, cytochalasin D, and lipopolysaccharide from *Escherichia coli* serotype 0111:B4 were purchased from Sigma. Fura-2/AM was purchased from Molecular Probes and thapsigargin from ICN. All other chemicals were of analytical grade. Purified monoclonal anti-CD11b antibody (M1/70, rat IgG2b, κ) was purchased from Pharmingen.

**Mutagenesis of cyaA**—The site-directed substitutions were introduced into the *cyaA* gene by PCR mutagenesis as described previously (36). CyaA-AC\(^-\) forms of the proteins, unable to convert ATP to CAMP, were generated by placing a Cys-Thr dipeptide between amino acid residues Asp\(^{188}\) and Ile\(^{189}\) of the ATP-binding site in the catalytic domain of CyaA, as described previously (46). The mutated or truncated CyaA constructs CyaA-247LQ (ACT247), CyaA-K58Q, CyaA-108OVA-AC\(^-\), CyaA-336OVA-AC\(^-\), CyaA-ΔAC, and AC (CyaAΔC1306) were described earlier (25, 46–48).

**Production and Purification of the CyaA-derived Proteins**—The wild type CyaA and its mutant derivatives were produced in the presence or absence of the activating protein CyaC, using the E. coli strain XL1-Blue (Stratagene) transformed with the appropriate plasmid construct, derived from pCACT3 (49). Bacteria were grown at 37 °C in LB medium supplemented with ampicillin (150 μg/ml). Recombinant CyaA synthesis was induced in exponential 500-ml cultures by 1 mM isopropyl β-D-thiogalactopyranoside for 3 h. Cells were disrupted by ultrasound; the insoluble cell debris was extracted with 8 M urea in 50 mM Tris-HCl (pH 8.0) and 0.2 mM CaCl\(_2\), and the proteins were further purified by ion-exchange chromatography on DEAE-Sepharose followed by hydrophobic chromatography on phenyl-Sepharose, as described previously (50, 51). The integrity of all proteins was systematically controlled by SDS-PAGE (supplemental Fig. 1).

CyaA and derived proteins were added to J774A.1 cells by direct dilution from the concentrated stock solutions containing 8 M urea. Unless indicated otherwise, the final working concentration of the toxin was 3 μg/ml, and the final concentration of urea was 80 mM in all samples. Appropriate controls showed that at this concentration the urea had no effect on cell physiology whatsoever. The lipopolysaccharide content of CyaA preparations was between 10 and 15 EU/μg CyaA, as determined by the chromogenic *Limulus* amebocyte lystate assay (BioWhittaker).

**Assay of Hemolytic Activity on Sheep Erythrocytes**—Hemolytic activity of the CyaA and mutant toxin variants was measured by photometric (A\(_{541}\)) determination of the amount of hemoglobin released upon prolonged incubation (5 h) of erythrocytes (5 × 10\(^6\)/ml) with 5 μg/ml toxin (4).

**Assay of Adenylate Cyclase Activity**—Adenylate cyclase activities were measured as described previously (52). One unit of AC activity corresponds to 1 pmol of cAMP formed per min in the presence of 1 μM calmodulin at 30 °C, pH 8.0.

**Determination of cAMP**—To determine the intracellular levels of cAMP in cells exposed to toxin, the J774A.1 cells (10\(^5\) per well) were incubated with different concentrations of the
CyaA-derived protein for 30 min in DMEM containing 100 µM 3-isobutyl-1-methylxanthine (IBMX) as inhibitor of phosphodiesterase activity. The reaction was stopped by addition of 100 mM HCl solution containing 0.2% Tween 20, and the samples were boiled for 15 min at 100 °C to denature cellular proteins (cAMP is heat- and acid-resistant). The samples were neutralized by addition of 150 mM unbuffered imidazole, and cAMP concentration was determined by an antibody competition immunoassay as described elsewhere (53).

Cellular ATP Level and Cell Lysis—ATP level in J774A.1 cells (10⁵ per well) was determined using the ATP bioluminescence assay kit CLS II (Roche Applied Science). Cell lysis was determined as lactate dehydrogenase release from J774A.1 cells (10⁵ per well) using the CytoTox 96 kit assay (Promega). Assays were performed according to the manufacturer’s instructions, and the results represent the average of values obtained in at least three independent experiments performed in triplicate.

Cell Culture and Handling—J774A.1 murine macrophages (ATCC, number TIB-67) were cultured at 37 °C in a humidified air/CO₂ (19:1) atmosphere in RPMI medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 UI/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml). For fluorescence measurements, cells were mechanically harvested, seeded on glass coverslips in 6-well plates, and grown to 30% confluence. In some experiments, J774A.1 cells were pretreated at room temperature for 20–40 min with the calcium channel blockers 2-APB (100 µM), LaCl₃ (100 µM), nifedipine (10 µM), flunarizine (30 µM), thapsigargin (1 µM), or SK&F 96365 (10 µM), respectively.

Fluorescence Measurement of Cytosolic Ca²⁺—Cells grown on glass coverslips were washed in modified HBSS (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 3 mM MgCl₂, 10 mM Hepes-Na, 50 mM glucose (pH 7.4)). After washing, cells were loaded with 3 µM Fura-2 acetoxyethyl ester (Fura-2/AM) for 30 min at 25 °C in the dark, rinsed, and allowed to rest in HBSS for 30 min prior to fluorescence measurements of [Ca²⁺], at 25 °C. Ratimetric rapid screening and multiply repeated measurements were performed using FluoroMax-3 spectrofluorometer equipped with DataMax software (Jobin Yvon Horriba, France). The observed area of coverslip mounted in the 1-cm cuvette was about 10 mm², corresponding to approximately 10⁶ cells. Fluorescence intensity of Fura-2 (excitation wavelengths 340 and 380 nm, emission wavelength 510 nm) was recorded every 15 s, and integration time for each wavelength was 3 s. The measured fluorescence intensity was not corrected for background intensity (<10%).

Intracellular calcium fluctuations were further verified and corroborated by following calcium entry into individual cells using an Olympus microscope IX 50-based microspectrofluorometer (VisiRon Systems, Puchheim, Germany), equipped with a cooled digital CCD camera (MicroMAX RTE/CCD-512EFT, Princeton Instruments, Monmouth Junction, NJ). Fluorescence of Fura-2 was excited at wavelengths of 340 and 380 nm switched by a Polychrome II (Till Photonics, Planegg, Germany) illumination device. Emitted light at λ > 420 nm (filter U-MWU) was recorded every 10 s, and integration time for each wavelength was 300 ms. MetaFluor 2.76 software (Universal Imaging Corp., West Chester, PA) was used to control synchronization of excitation and data acquisition and for the visualization of the relative calcium concentration based on the ratiometric measurement. The curves in the shown graphs correspond to average kinetics of a random sample of at least 30 individual cells, and the curves are representative of typical average kinetics from more than three independent measurements. The background fluorescence of the coverslip area without cells (about 50% of total area) was monitored and subtracted for each measurement. In vivo calibrations for determination of the calcium concentration were performed as described elsewhere (54), using 10⁻⁵ M ionomycin and a Kᵣ = 224 nm value for Fura-2. The minimal cytotoxic concentration of Ca²⁺ was determined by measurements in calcium-free HBSS, pH 7.4, containing 4 mM EGTA instead of 2 mM CaCl₂. The maximum of [Ca²⁺] was determined in HBSS supplemented with 10 mM CaCl₂.

RESULTS

CyaA Promotes Elevation of Cytosolic Calcium Level in J774A.1 Monocytes by a Mechanism That Does Not Involve cAMP Signaling—Elevation of cellular cAMP levels upon exposure to CyaA was shown previously to cause massive influx of calcium ions into excitatory types of cells possessing the cAMP-regulated L-type Ca²⁺ channels (33, 45). These channels, however, appear to be absent in natural targets of the toxin, such as the CD11b-expressing myeloid phagocytes, and the impact of CyaA action on [Ca²⁺], in myeloid cells has not been addressed previously. Because CyaA possesses the capacity to form small cation-selective membrane pores that account for hemolytic activity of CyaA and thus might potentially permeabilize cells for entry of Ca²⁺, we first examined whether exposure to CyaA causes alterations of [Ca²⁺] in CD11b⁺ J774A.1 monocyctic cells.

As documented in Fig. 1 by a typical result of a ratiometric measurement employing Fura-2/AM as a Ca²⁺-sensitive probe
CyaA-dependent Entry of \( \text{Ca}^{2+} \) into J774A.1 Monocytes

![Image](230x26 to 257x38)

**FIGURE 2.** Elevation of intracellular cAMP level does not account for \( \text{Ca}^{2+} \) increase. After loading of J774A.1 cells with Fura-2/AM, the indicated proteins (3 \( \mu \text{g/ml} \)) and the membrane-permeable cAMP analog, 1 \( \text{mM} \) (Bt2cAMP), were added, and calcium entry was followed and evaluated as described in Fig. 1. All incubations were performed in the presence of 100 \( \mu \text{M} \) phosphodiesterase inhibitor IBMX.

accumulating in cell cytosol, exposure of cells to CyaA resulted in \( \text{Ca}^{2+} \) increase, with the kinetics and final \( \text{Ca}^{2+} \) level being a function of toxin concentration. At 3 \( \mu \text{g/ml} \) of CyaA, the \( \text{Ca}^{2+} \) level increased shortly upon exposure of J774A.1 cells to toxin, and it continued to rise for about 8–12 min, until a maximal \( \text{Ca}^{2+} \) level was reached. Based on calibration experiments employing the calcium ionophore ionomycin (supplemental Fig. 2), it could be estimated that exposure of cells to 3 \( \mu \text{g/ml} \) CyaA resulted in a \( \text{Ca}^{2+} \) increase from a basal level of \( \sim 80 \text{ nM} \) to a persistent final level of about 2 \( \mu \text{M} \). As further shown in Fig. 1, no elevation of \( \text{Ca}^{2+} \) was observed upon exposure of cells to 3 \( \mu \text{g/ml} \) of the comparably pure but nonacylated pro-CyaA, which binds CD11/CD18 in a nonproductive manner and exhibits only a residual biological activity (53), and neither did an appropriate lipopolysaccharide control (45 EU/ml) that caused no elevation of \( \text{Ca}^{2+} \) (supplemental Fig. 3). Therefore, it can be concluded that the \( \text{Ca}^{2+} \) increase was specifically due to action of the toxin itself and was not provoked by any potential traces of contaminating bacterial components that might still have been present in the purified toxin preparation. Moreover, although the presence of a control isotype antibody had no effect on \( \text{Ca}^{2+} \) increase (supplemental Fig. 3), the capacity of the toxin to elevate \( \text{Ca}^{2+} \), in J774A.1 cells was completely abrogated in the presence of an excess of the CD11b-specific antibody M1/70 that competes with CyaA for binding to the CD11b/CD18 receptor (Fig. 1). This showed that elevation of \( \text{Ca}^{2+} \) by CyaA required binding of the toxin to its integrin receptor CD11b/CD18.

However, as further documented in Fig. 2, the capacity of CyaA to cause a \( \text{Ca}^{2+} \) increase in J774A.1 cells was not primarily because of its capacity to elevate cellular cAMP concentration. Exposure of the CD11b\(^+\) monocytes to the cell-permeable cAMP analog Bt\(_{2}\)cAMP (1 \( \mu \text{M} \)) had no effect on \( \text{Ca}^{2+} \), and more importantly, the enzymatically inactive CyaA-AC\(^-\) construct, unable to catalyze any cAMP synthesis because of disruption of the ATP-binding site, was still able to promote the \( \text{Ca}^{2+} \) increase in cells (Fig. 2).

It should be noted, however, that the time course of the \( \text{Ca}^{2+} \) increase promoted by CyaA-AC\(^-\) differed markedly from that induced by the enzymatically active CyaA. The latter caused a progressive \( \text{Ca}^{2+} \) increase that continued over 10 min, and the CyaA-AC\(^-\) toxoid (3 \( \mu \text{g/ml} \)) promoted a strikingly faster \( \text{Ca}^{2+} \) increase with an earlier onset and a maximum of \( \text{Ca}^{2+} \), reached within 3 min followed by a progressive decrease of \( \text{Ca}^{2+} \), to an intermediate level in about 5 min from toxoid addition. Moreover, the time course of \( \text{Ca}^{2+} \) increase produced by CyaA-AC\(^-\) was not affected in the presence of 1 \( \mu \text{M} \) Bt\(_{2}\)cAMP (Fig. 2), suggesting that it was the capacity of CyaA to dissipate ATP into cAMP, rather than the cAMP itself, which accounted for the observed difference in time courses of the \( \text{Ca}^{2+} \), increase induced by CyaA and CyaA-AC\(^-\) proteins, respectively.

**Depletion of Cellular ATP Enables CyaA to Cause Permanent Elevation of Cytosolic Calcium Levels in J774A.1 Monocytes**—To analyze in more detail the mechanistic basis of the difference in \( \text{Ca}^{2+} \) increase, we examined the respective effects of their action on intracellular ATP levels. CyaA was expected to cause ATP deprivation of cells, and as documented in Fig. 3A and in agreement with previous work (38, 39), at a concentration of 3 \( \mu \text{g/ml} \) the enzymatically active CyaA caused massive dissipation of cellular ATP into cAMP. This yielded a reduction of the cellular ATP level by more than 80% already within the first 3 min of toxin action. In turn, exposure to the same amount of CyaA-AC\(^-\) had no effect on cellular ATP level. This indicated that the wild type CyaA could provoke permanent elevation of \( \text{Ca}^{2+} \) because of causing ATP depletion, as compared with CyaA-AC\(^-\) that caused only transient elevation of \( \text{Ca}^{2+} \). To test this hypothesis, ATP depletion was induced in J774A.1 cells prior to exposure to CyaA-AC\(^-\) by treating cells with the combination of an uncoupler of oxidative phosphorylation, FCCP (inhibiting ATP synthesis in mitochondria), and by replacing glucose in the incubation media with 2-DG (a nonmetabolizable sugar analog inhibiting ATP generation through the glycolysis pathway). As indeed shown in Fig. 3B, pretreatment of cells for 20 min with 10 \( \mu \text{M} \) 2DG, followed by addition of 2.5 \( \mu \text{M} \) FCCP and incubation for further 5 min prior to exposing the cells to CyaA-AC\(^-\), provoked a drastic drop of cellular ATP levels to about 5% of that found in untreated cells. As further shown in Fig. 3C, when such ATP-depleted cells were exposed to CyaA-AC\(^-\), the \( \text{Ca}^{2+} \) increase induced by the enzymatically inactive toxoid exhibited a slower onset and resulted in permanent elevation of \( \text{Ca}^{2+} \), and hence was not followed by the usual decrease of calcium concentration in the 3rd minute following addition of CyaA-AC\(^-\). The same pretreatment of cells with 2DG and FCCP, however, did not provoke any \( \text{Ca}^{2+} \) increase in cells on its own, nor did it affect the time course of calcium influx induced by wild type CyaA toxin, thus showing that ATP depletion as such was not sufficient to promote calcium entry into the cytosol of J774A.1 cells (supplemental Fig. 4). These results suggest that the stable elevation of \( \text{Ca}^{2+} \), in cells exposed to the relatively high concentrations of intact CyaA (3 \( \mu \text{g/ml} \)) was because of a combination of the capabilities of CyaA to mediate entry of calcium ions into the cytosol of cells and to cause depletion of cytosolic ATP in parallel. In contrast, upon exposure to the same amount of the CyaA-AC\(^-\) toxoid, which was only able to mediate \( \text{Ca}^{2+} \) entry into cytosol, the J774A.1 cells were still
CyaA-dependent Entry of Ca\(^{2+}\) into J774A.1 Monocytes

FIGURE 3. Depletion of cellular ATP by high amounts of toxin allows for permanent elevation of cytosolic calcium level in J774A.1 monocytes by CyaA. A, CyaA causes depletion of intracellular ATP level in J774A.1 cells. 10\(^7\) J774A.1 cells per well were incubated with 3 \(\mu\)g/ml of CyaA or CyaA-AC\(^-\) in HBSS at room temperature for the indicated time, and intracellular ATP content was determined using the ATP bioluminescence assay kit CLS II (Roche Applied Science). B, 2DG and FCCP treatment leads to depletion of cellular ATP. 10\(^7\) J774A.1 cells per well were incubated for 20 min in HBSS or in HBSS containing instead of glucose the glycolysis inhibitor 2DG (10 mM), before 2.5 \(\mu\)M FCCP (inhibitor of mitochondrial ATP synthesis) was added where applicable, and incubation was continued for additional 5 min. Cellular ATP level was determined as above. C, Ca\(^{2+}\) influx under the conditions of ATP depletion. J774A.1 cells loaded with Fura-2/AM were preincubated for 20 min in HBSS containing 10 mM 2DG instead of glucose before 2.5 \(\mu\)M FCCP was added for 5 min prior to addition of CyaA or CyaA-AC\(^-\) (arrow). The shown curves are representative of at least three independent measurements of [Ca\(^{2+}\)].

CyaA-mediated Increase of [Ca\(^{2+}\)]\(_i\) in Monocytes Results from Influx of Extracellular Calcium Ions—It was important to define the origin of Ca\(^{2+}\) ions entering the cytoplasm of toxin-treated cells, because elevation of [Ca\(^{2+}\)]\(_i\), can occur both by direct influx of calcium ions into cell cytosol from external medium, via calcium channels in plasma membrane, and it can result from Ca\(^{2+}\) release from intracellular stores through calcium channels in the membrane of the endoplasmic or sarcoplasmic reticulum (55). Therefore, prior to exposure to CyaA, the cells were pretreated for the indicated times with a comprehensive set of specific inhibitors of the various types of known cellular calcium channels at their respective individual effective concentrations (Fig. 4). By comparison with the no inhibitor control, pretreatment of cells with any of the used inhibitors had any discernible effect on CyaA-promoted elevation of [Ca\(^{2+}\)]\(_i\) in J774A.1 monocytes, as shown in Fig. 4. Toxin-induced [Ca\(^{2+}\)]\(_i\) increase could not be blocked with 1 \(\mu\)M thapsigargin, an inhibitor of ATPase pumps (SERCA) of the endoplasmic reticulum (Fig. 4A), nor was the elevation of [Ca\(^{2+}\)]\(_i\) blocked by the selective calcium antagonist and L-type channel inhibitor 10 \(\mu\)M nifedipine (Fig. 4B) nor was the T-type channel inhibitor 30 \(\mu\)M flunarizine (Fig. 4C), both compounds being known to block voltage-gated channels present in certain types of nonexcitable cells (56–58). Similarly, no effect on [Ca\(^{2+}\)]\(_i\) increase was observed with the antagonist of receptor-operated calcium channels 10 \(\mu\)M SK&F 96365 (Fig. 4D) nor with 100 \(\mu\)M 2-APB, an inhibitor of store-operated channels (Fig. 4E). In parallel, the same type of analysis was also performed on cells exposed to the enzymatically inactive CyaA-AC\(^-\) protein, and again no effect whatsoever of the specific calcium channel inhibitors on toxoid-induced calcium entry into cells was observed (supplemental Fig. 5). These results suggested that entry of Ca\(^{2+}\) into the cytosol of CyaA-treated J774A.1 monocytes was not mediated by any conventional cellular calcium channels that might be expected to be present in these cells and that the [Ca\(^{2+}\)]\(_i\) increase was because of the influx of extracellular Ca\(^{2+}\) into cells through a path formed by the CyaA molecule itself.

To test this hypothesis, we examined the capacity of CyaA to promote [Ca\(^{2+}\)]\(_i\) increase in the presence of extracellular La\(^{3+}\). This ion acts as a general calcium channel blocker, obtruding the channel, while being unable to cross the plasma membrane of cells. It was important to ascertain that the presence of 100 \(\mu\)M La\(^{3+}\) during exposure of cells to CyaA did not interfere with ability to counteract the toxin-mediated [Ca\(^{2+}\)]\(_i\) burst and could reduce the [Ca\(^{2+}\)]\(_i\) level after a lag period of a few minutes, most likely through activation of ATP-dependent PMCA pumps extruding Ca\(^{2+}\) from the cytosol. These results, however, did not offer an explanation as to why the CyaA-AC\(^-\) toxoid promoted a faster increase of [Ca\(^{2+}\)]\(_i\) than CyaA, and more insight into this issue had to be gained from examination of the [Ca\(^{2+}\)]\(_i\)-increasing capacities of other mutant CyaA constructs (see below).

\(\text{2812} \quad \text{JOURNAL OF BIOLOGICAL CHEMISTRY} \quad \text{VOLUME 282•NUMBER 5•FEBRUARY 2, 2007} \)
Pore Forming Activity of CyaA—Considering that the CyaA molecule was itself mediating influx of calcium ions into cells, the role of individual CyaA domains in this process was examined using a set of specifically mutated and truncated toxin molecules.

As expected for the isolated enzymatic (AC) domain, unable to bind and penetrate cells (50), exposure of J774A.1 monocytes to the AC polypeptide alone did not result in any observable [Ca$^{2+}$]$_i$ increase, as documented in Fig. 6A. Most unexpectedly, however, no [Ca$^{2+}$]$_i$ increase was observed in cells exposed to 3 μg/ml of the truncated CyaA-DAC variant (Fig. 6A), which lacks the AC domain and corresponds to the pore-forming and hemolytically fully active RTX portion of CyaA (59). In agreement with previous reports (6, 59) CyaA-DAC exhibited the same specific hemolytic activity as the intact recombinant CyaA (Table 1). Hence, the inability of CyaA-DAC to promote any [Ca$^{2+}$]$_i$ increase strongly suggested that the small hemolytic pores formed by CyaA in cellular membrane accounted for only a marginal, if any, Ca$^{2+}$ influx into cells. Moreover, this result indicated that the concomitant presence of the AC domain and hemolysin moieties, and possibly their cooperation, was required for the [Ca$^{2+}$]$_i$ increasing activity of CyaA on J774A.1 cells.

To corroborate in more detail the structure-function relationships underlying the contribution of the individual domains of CyaA to Ca$^{2+}$ influx into cells, we examined the [Ca$^{2+}$]$_i$ increasing activities of a previously characterized set of mutant CyaA variants that carried point substitutions of glutamate residues 509, 516, and 570 within the predicted membrane- and pore-forming domain of CyaA. As summarized in Table 1 and reported earlier (36, 39), the various substitutions affected differently the capacities of the respective constructs to translocate the AC domain into cells and to form the hemolytic pores. A particularly strong effect on activity of CyaA resulted from the substitution of Glu$^{570}$ by an α-helix-breaking proline residue in the CyaA-E570P construct. This was still capable of binding tightly to the CD11b/CD18 receptor, while being essentially unable to deliver the AC domain into cell cytosol and to form hemolytic pores (Table 1 and data not shown). Moreover, as documented in Fig. 6B, no [Ca$^{2+}$]$_i$ increasing activity was associated with the CyaA-E570P protein either, showing that the putative transmembrane segment of CyaA, consisting of the Glu$^{570}$ residue, played a pivotal role not only in AC delivery and pore formation by CyaA but also in its capacity to mediate Ca$^{2+}$ influx into J774A.1 cells.

Calcium Influx into Cells Depends on Translocation of the AC Domain Polypeptide across Cellular Membrane and Not on the binding of CyaA to the cellular receptor and penetration of the toxin across plasma membrane of CD11b-expressing monocytes. As shown in Fig. 5A, this could be readily ruled out, because the same intracellular CaM levels were reproducibly formed in cells exposed to CyaA in the presence or absence of 100 μM La$^{3+}$. Hence, the presence of La$^{3+}$ ions had no effect on the capacity of CyaA to bind and penetrate cells. At the same time, however, the presence of 100 μM La$^{3+}$ completely abrogated the capacity of CyaA to promote [Ca$^{2+}$]$_i$ increase, as documented in Fig. 5B. This clearly demonstrated that the [Ca$^{2+}$]$_i$ increase depended on the influx of extracellular Ca$^{2+}$ ions into the cytosol of J774A.1 cells. However, as further documented in Fig. 5B, CyaA induced a full [Ca$^{2+}$]$_i$ increase also in cells that were pretreated with 100 μM La$^{3+}$ and were washed once prior to addition of CyaA (or CyaA-AC$^-$; see supplemental Fig. 6). Most likely, the inhibition of [Ca$^{2+}$]$_i$ increase by La$^{3+}$ ions was not because of inhibition of the calcium channels present in cell membrane prior to addition of CyaA. Such channels would indeed be expected to remain blocked by tightly bound La$^{3+}$ ions even upon washing of the La$^{3+}$-exposed cells in La$^{3+}$-free buffer. Collectively, these results show that the increase of [Ca$^{2+}$]$_i$ in J774A.1 cells exposed to CyaA was because of entry of extracellular calcium into the cytosol of cells by a mechanism not involving voltage-, receptor-, or store-operated channels. Most likely, the influx of Ca$^{2+}$ was because of a novel type of calcium path involving the CyaA molecule itself.

Calcium influx into cells was also monitored before CyaA, was added and the influx of calcium ions into cells was monitored over time. The J774A.1 cells were loaded by Fura-2/AM and preincubated with the indicated inhibitors of cellular calcium channels before CyaA, was added and the influx of calcium ions into cells was monitored over time. The respective concentrations and preincubation times for the different inhibitors were as follows. A, 1 μM thapsigargin, 40 min; B, 10 μM nifedipine, 20 min; C, 30 μM flunarizine, 20 min; D, 10 μM SKF 96365, 20 min; E, 100 μM 2-APB, 20 min; and F, no inhibitor control.
To ascertain whether loss of $[\text{Ca}^{2+}]_i$ increasing activity was associated with the loss of capacity to translocate the AC domain across cellular the membrane, or whether it correlated with the loss of the capacity to form pores, or both, the $[\text{Ca}^{2+}]_i$ increasing activity of the CyaA-E509P and CyaA-E509K/E516K proteins was analyzed. These constructs were shown previously to be selectively affected in the capacity to deliver the AC domain into cells, while still possessing a full or a severalfold enhanced specific pore forming activity, respectively (36). Nevertheless, as shown in Fig. 6B, the two proteins exhibited an importantly reduced (CyaA-E509P) or essentially nil $[\text{Ca}^{2+}]_i$-increasing capacity (CyaA-E509K/E516K), clearly showing that the loss of capacity to mediate calcium influx into cells was selectively associated with the loss of AC translocation capacity of CyaA. Moreover, this defect could not be compensated for by even a much enhanced pore-forming capacity of the hyperhemolytic CyaA-E509K/E516K construct (cf. Table 1). This goes well with the conclusion reached above for CyaA-ΔAC that the hemolytic pores formed by CyaA were not mediating any substantial influx of $\text{Ca}^{2+}$ into cells.

These results suggested that the influx of $\text{Ca}^{2+}$ ions into cells was directly linked to translocation of the AC domain polypeptide across the cellular membrane. Moreover, as in the case of the E570P substitution, the structural integrity and charge of the predicted transmembrane segment consisting of residues Glu$^{509}$ and Glu$^{516}$ also appeared to be required for both the capacity of CyaA to deliver AC and to mediate influx of $\text{Ca}^{2+}$ into cells. The Glu$^{509}$ residue appeared, however, to be more specifically involved in the $\text{Ca}^{2+}$ passage across the plasma membrane of cells. The CyaA-E509P exhibited almost a half-maximal (~50%) specific capacity to deliver AC into cells and to dissipate cytosolic ATP to cAMP, as compared with intact CyaA (Table 1), whereas its capacity to promote $\text{Ca}^{2+}$ influx into cells was selectively impaired to a much greater extent. CyaA-E509P induced a rather slow calcium influx and promoted only a
ion-exchange chromatography on DEAE-Sepharose, followed by hydrophobic chromatography on phenyl-Sepharose as specified under "Experimental Procedures" and according to methods described previously (51).

Adenylate cyclase enzyme activities of mutant proteins were determined as described previously (52).

Specific hemolytic activity of the proteins was determined by incubation of 5 × 108 red blood cells with 5 μg/ml of the toxin variants for 5 h at 37°C in the presence of 2 mM Ca2+. The amount of released hemoglobin was measured as A490, and the activity of intact CyaA was taken as 100%. The results represent the average of three independent experiments performed in duplicate ± S.D. (n = 5).

Activities of the toxins were compared on the basis of CyaA concentrations required to yield accumulation of 10 pmol of intracellular cAMP in 105 J774A.1 cells during 30 min of incubation.

Because of very low affinity for calmodulin, resulting in very low specific intracellular AC enzyme activity of the CyaA-247LQ (pACM247) construct (47), its cell invasiveness could not be assessed by determination of the formed intracellular AC enzyme activity of the CyaA-247LQ construct in the cytosol of sheep erythrocytes, where it becomes inaccessible to digestion (inactivation) by externally added trypsin. The internalized AC activity was then determined at a 100 μM calmodulin concentration upon lysis of washed erythrocytes, as described previously (47). Activity of intact CyaA in this assay was taken as 100%.

Because of loss of enzymatic activity resulting from the mutations in the AC domain, the capacity to deliver the AC domain into cytosol of cells could not be determined.

For CyaA-336OVA-AC− and CyaA-108OVA-AC− variants, the cell invasive capacities could not be quantified and can only be inferred as being present (+) based on the demonstrated capacity of these constructs to deliver the inserted OVA epitope into the cytosol of antigen-presenting cells for processing and presentation in complex with major histocompatibility complex I molecules, as described previously (32, 46).

transient [Ca2+]i increase, which at its maximum reached only about 10% of the final [Ca2+]i levels attained in cells exposed to identical amounts of intact CyaA (cf. correspondence of the A340/380 ratio to [Ca2+]i, in Fig. 6B). Thus, the correlation between translocation of the AC domain and Ca2+ influx into cells was not direct, although both processes depended on the integrity of the same predicted transmembrane segments of CyaA. Nevertheless, translocation of the AC domain appeared to be a prerequisite for Ca2+ influx into cells, the latter being compromised in the absence of the AC domain as such (CyaA-DAC) or in the presence of substitutions that block translocation of the AC domain into cells. In turn, calcium influx as such did not appear to be required for AC translocation to occur, as exemplified by the relatively high capacity of the CyaA-E509P construct to deliver the AC domain despite its relatively modest capacity to promote influx of Ca2+ into cells (cf. Table 1 and Fig. 6B).

**The Translocating AC Domain Itself Takes Part in a Path Allowing Calcium Influx**—The observation that the AC domain translocation into cells was a prerequisite for Ca2+ influx into cells indicated that the AC domain itself might be playing a role in transport of Ca2+ across the cellular membrane. To test this hypothesis, we analyzed the impact of conformational alterations within the AC domain on this process, examining the [Ca2+]i-increasing capacities of a variety of protein variants that carry substitutions and oligopeptide inserts within the AC domain.

These constructs were previously derived from CyaA by several types of mutations that ablate the enzymatic activity of AC (by either disrupting its catalytic site by Asn substitution of Lys in CyaA-K58Q or its ATP-binding site by a Cys-Thr insert placed between residues 188 and 189 of CyaA-AC−) or its calmodulin-binding site (a Leu-Gln insert placed between residues 247 and 248 of CyaA-247LQ), respectively (25, 46–48). Further enrolled in the examination of the respective capacities to mediate Ca2+ influx were the CyaA-336OVA-AC− and CyaA-108OVA-AC− toxoids, which carried on the top of the dipeptide insert disrupting the ATP-binding site (CyaA-AC−) also an additional SIINEFKL oligopeptide insert derived from the sequence of ovalbumin (OVA). This CD8+ T cell epitope was inserted at the so-called "permissive" positions within AC, next to residues 108 or 336, respectively, and it has repeatedly been shown earlier that such OVA-tagged constructs were able to deliver their modified AC domains into the cytosol of CD11b-expressing antigen-presenting cells (32, 46). Furthermore, in agreement with previous work showing that mutations within the AC domain do not affect cell binding and pore forming activities of CyaA, all of the used toxoids exhibited an intact hemolytic and cell-binding capacity, as documented in Table 1.

Quite surprisingly then, the capacity of these mutant AC− constructs to promote calcium influx into cells varied substantially, as documented in Fig. 7. The [Ca2+]i increasing activity of the various toxoids ranges from a rather low activity exhibited by the CyaA-247LQ, CyaA-K58Q, and CyaA-108OVA-AC− constructs up to an enhanced or at least intact [Ca2+]i increasing activity exhibited by the CyaA-336OVA-AC− construct, when compared with its corresponding nonmutated counterpart CyaA-AC−. Most striking was the difference in the respective capacities of promoting Ca2+ influx between the CyaA-336OVA-AC− and CyaA-108OVA-AC− toxoids, where both

---

**TABLE 1**

| Protein|^a^ | Adenylate cyclase activityb | Hemolytic activityc | AC domain delivery into cells |
|---------|-----------------|-------------------------|----------------------|
| CyaA    | 100 ± 10        | 100 ± 20                | 100 ± 14^e            |
| CyaA-AC | 110 ± 11        | 110 ± 11                | 110 ± 11^d            |
| CyaA-DAC| 112 ± 11        | 112 ± 11                | 112 ± 11^d            |
| CyaA-E509P | 95 ± 16       | 95 ± 16                 | 95 ± 16^d            |
| CyaA-E509K/E516K | 500 ± 50  | 500 ± 50                 | 500 ± 50^d^c   |
| CyaA-E570P | 15 ± 1       | 15 ± 1                  | 15 ± 1^d^c   |
| CyaA-247LQ | 11 ± 1        | 11 ± 1                  | 11 ± 1^d^c   |
| CyaA-K58Q | 112 ± 11      | 112 ± 11                | 112 ± 11^d^c |
| CyaA-336OVA-AC− | 114 ± 15   | 114 ± 15                 | 114 ± 15^d^c |
| CyaA-108OVA-AC− | 105 ± 10    | 105 ± 10                 | 105 ± 10^d^c |

^a^ The proteins were expressed in E. coli XLI-Blue and purified by urea extracts by ion-exchange chromatography on DEAE-Sepharose, followed by hydrophobic chromatography on phenyl-Sepharose as specified under "Experimental Procedures" and according to methods described previously (51).

^b^ Adenylate cyclase enzyme activities of mutant proteins were determined as described previously (52).

^c^ Specific hemolytic activity of the proteins was determined by incubation of 5 × 108 red blood cells with 5 μg/ml of the toxin variants for 5 h at 37°C in the presence of 2 mM Ca2+. The amount of released hemoglobin was measured as A490, and the activity of intact CyaA was taken as 100%. The results represent the average of three independent experiments performed in duplicate ± S.D. (n = 5).

^d^ Activities of the toxins were compared on the basis of CyaA concentrations required to yield accumulation of 10 pmol of intracellular cAMP in 105 J774A.1 cells during 30 min of incubation.

^e^ Because of very low affinity for calmodulin, resulting in very low specific intracellular AC enzyme activity of the CyaA-247LQ (pACM247) construct (47), its cell invasiveness could not be assessed by determination of the formed intracellular cAMP level. Therefore, the relative AC domain delivery capacity was measured as the capacity to translocate the AC-247LQ domain into the cytosol of sheep erythrocytes, where it becomes inaccessible to digestion (inactivation) by externally added trypsin. The internalized AC activity was then determined at a 100 μM calmodulin concentration upon lysis of washed erythrocytes, as described previously (47). Activity of intact CyaA in this assay was taken as 100%.

^f^ Because of loss of enzymatic activity resulting from the mutations in the AC domain, the capacity to deliver the AC domain into cytosol of cells could not be determined.

^g^ For CyaA-336OVA-AC− and CyaA-108OVA-AC− variants, the cell invasive capacities could not be quantified and can only be inferred as being present (+) based on the demonstrated capacity of these constructs to deliver the inserted OVA epitope into the cytosol of antigen-presenting cells for processing and presentation in complex with major histocompatibility complex I molecules, as described previously (32, 46).

---

**FIGURE 7. Structural alterations within the AC domain differentially affect the capacity of CyaA to mediate calcium influx into J774A.1 cells.** Fura-2/AM-loaded J774A.1 cells were exposed to 3 μg/ml of the indicated proteins and a [Ca2+]i increase followed as described under "Experimental Procedures." The shown curves are representative of at least three independent experiments.
of these proteins were reproducibly shown to deliver their AC domains into the cytosol of cells (32, 46). The most plausible explanation of the observed difference in their [Ca$^{2+}$], increasing activity would hence be that insertion of the same SIINFEKL peptide into two different sites within the AC had a different impact on the conformation of the AC domain of the two constructs and that this is translated into the respective difference in the capacities of the toxoids to mediate Ca$^{2+}$ influx into cells. Similarly, a CyaA-247LQ construct, having the calmodulin-binding site disrupted but exhibiting a full capacity to deliver the mutated AC domain into cytosol of erythrocytes (47), was unable to promote Ca$^{2+}$ influx. Altogether the results shown in Fig. 7 corroborate the observation made with CyaA-E509P (see above) that translocation of the AC domain into the cytosol as such must not necessarily be tightly coupled to, or followed by, the transport of Ca$^{2+}$ ions across the cellular membrane. The translocating AC domain polypeptide appears, however, to participate itself in this process, given the effects of structural alterations in the AC domain on transport of calcium ions into cells.

**Newly Inserting Toxin Molecules Form a Transiently Opened Calcium Influx Path in Cellular Membrane**—It was important to determine whether the toxin formed in the cellular membrane a pore-like calcium entry path that would remain open for a prolonged period, or whether calcium entry into cells occurred only transiently, accompanying insertion and membrane translocation solely of the newly arriving toxin molecules. The results shown in Fig. 8 suggest that the latter mechanism applies. In contrast to the typical continued increase of intracellular calcium level in the continued presence of excess unbound toxin (+CyaA), the increase of [Ca$^{2+}$], level was abrogated, and a progressive drop of cytosolic calcium level was observed (Fig. 8A) when J774A.1 cells exposed to CyaA for 5 min were transferred for subsequent incubation into fresh medium not containing the toxin (−CyaA). Moreover, as further shown in Fig. 8B, disruption of cellular actin cytoskeleton and endocytosis mechanisms upon preincubation of cells with 10 μg/ml of cytochalasin D did not abrogate or reverse the drop of [Ca$^{2+}$], observed following transfer of toxin-treated cells into fresh medium without toxin (−CyaA). Hence, it can be concluded that this abrogation of calcium entry and subsequent [Ca$^{2+}$], decrease were not because of removal of the toxin-formed calcium channels from the cell surface by membrane trafficking mechanisms. Rather, these results suggest that only the newly inserting toxin molecules could promote translocation of Ca$^{2+}$ into cells and that the calcium path in the membrane was open quite transiently.

**Toxin-mediated Calcium Influx Does Not Contribute to Residual Cytotoxicity of Enzymatically Inactive CyaA-AC**—Another important question to address was whether the observed capacity to elevate [Ca$^{2+}$], contributes to cytotoxic activity of CyaA. However, as documented in Fig. 9, there was no correlation between the residual cytotoxicity of the various mutant toxoids and their capacity to promote calcium influx into cells (cf. Fig. 6A and Fig. 7 versus Fig. 9). For example, the CyaA-AC and CyaA 108OVA-AC proteins exhibited the same residual cytolytic activity on J774A.1 cells, despite one construct being able to promote calcium influx into cells and the other having an essentially nil capacity to elevate [Ca$^{2+}$].

**DISCUSSION**

CyaA belongs to the so-called pore-forming toxins able to permeabilize cellular membranes, and many of these toxins were found previously to elicit calcium influx into cells by various mechanisms. Elevation and modulation of free cytosolic calcium concentrations by toxins indeed appear to belong to basic strategies of host cell manipulation by pathogens (40). Nevertheless, there persists a controversy about the capacity and mechanisms by which specifically the RTX family of toxins would promote calcium influx into cells. Uhlen et al. (60) reported that sublytic doses of α-hemolysin (HlyA) produced by uropathogenic *E. coli* stimulated oscillatory calcium responses in renal epithelial cells through activation of L-type
CyaA-dependent Entry of Ca\(^{2+}\) into J774A.1 Monocytes

...calcium channels. Working with different concentrations of HlyA on different cell lines, however, other authors reported that HlyA induces elevation of cytoplasmic calcium by allowing passive influx of calcium ions into cells through the toxin pores (61, 62). Although CyaA also belongs to the RTX pore-forming toxin family like HlyA, the pores formed by CyaA appear to be much smaller than pores formed by HlyA (6), and this may explain why CyaA has to rely on yet another mechanism than HlyA in promoting calcium influx into cells.

We show here that CyaA is endowed with the capacity to cause elevation of [Ca\(^{2+}\)], in CD11b-expressing myeloid cells by a novel mechanism that does not involve cellular calcium transport systems, signaling of cAMP resulting from enzymatic activity of the toxin, nor the pore forming activity of CyaA, respectively. Instead, the capacity of the toxin to cause entry of Ca\(^{2+}\) into cytosol of target cells required the structural integrity of the predicted transmembrane segments of the CyaA molecule itself, such as the presence of glutamate residues 509, 516, and 570. It also required a certain conformation of and a capacity to translocate the AC domain moiety of CyaA across the target cell membrane. Given the impact of the various structural alterations in the AC domain on the capacity of the toxins to promote Ca\(^{2+}\) influx into J774A.1 cells, the results would suggest that translocation of the AC domain of the toxin itself takes part in formation of a novel type of calcium path in target membranes that allow Ca\(^{2+}\) ions to permeate from the external medium directly into the cytosol of cells. The AC polypeptide may then be cooperating in formation of the calcium path with the segments of the pore-forming domain of CyaA.

It is tempting to speculate that the calcium transport might occur by a mechanism of “piggy-backing” of Ca\(^{2+}\) ions bound to the AC domain, yielding co-translocation of Ca\(^{2+}\) into cells in the course of AC polypeptide penetration across the membrane. Alternatively, the requirement for AC domain translocation, in promoting entry of Ca\(^{2+}\) into cells, might reflect the involvement of the AC polypeptide in formation of a novel type of transmembrane calcium path. Co-translocation of Ca\(^{2+}\) ions with the AC polypeptide itself appears, however, to be a less likely option, because translocation of the AC domain could be uncoupled from calcium influx by the proline substitution of glutamate residue 509 in CyaA-E509P or by an OVA epitope insertion in CyaA-108OVA-AC\(^{-}\). Indeed, the E509P mutant exhibited about a half-maximal (~50%) specific capacity to deliver the AC domain into J774A.1 cells, whereas it displayed only a residual (~10%) capacity to elicit [Ca\(^{2+}\)], in cells. Moreover, the CyaA-108OVA-AC\(^{-}\)- or CyaA247LQ proteins did not elicit any elevation of [Ca\(^{2+}\)], despite their previously shown capacity to translocate the AC domain across the cellular membrane (46, 47). More likely, however, the translocating AC polypeptide would transiently take part in formation of a lesion or a calcium conduit in the cellular membrane (cf. results shown in Fig. 8), allowing calcium entry into cells. This would then also involve the transmembrane segments of the pore-forming domain of CyaA, but not the hemolytic pore of CyaA as such. This is witnessed here by the inhibitory effects on calcium influx of the point mutations that affect translocation of AC across membrane (i.e. E509P and E570P) and/or exacerbate at the same time the pore-forming capacity of CyaA, such as the double substitution in CyaA-E509K/E516K, respectively.

However, it remains to be determined whether any as yet unidentified cellular membrane proteins take part in formation of the novel and transient calcium path in cellular membrane by CyaA. The heterodimeric \(\alpha_M\beta_2\) integrin CD11b/CD18 used by CyaA as cellular receptor would obviously appear to be a first choice candidate for such an additional component cooperating with CyaA in formation of the calcium path. In fact, CD11b/CD18 is itself known to play a prominent role in calcium signaling in myeloid cells (63). Moreover, being the type I membrane proteins, CD11b and CD18 subunits of the integrin offer two transmembrane segments that might potentially interact with the transmembrane segments of CyaA and contribute to formation of a calcium conduit across the membrane. However, intriguingly enough, CyaA appears to bind CD11b/CD18 in a way that by itself does not promote any calcium entry into cells. This is best documented by the inability of the CyaA-AC or CyaA-247LQ mutants to elicit calcium entry into J774A.1 cells, despite their fully conserved capacity to tightly bind CD11b/CD18, to penetrate cellular membranes, deliver the AC domain (CyaA-247LQ), and form the hemolytic pores (6, 47, 59, 64).

In contrast to results obtained with CyaA on excitatory cells harboring cAMP-regulated L-type calcium channels (33, 45), no [Ca\(^{2+}\)], mobilization in response to cAMP elevation was detected here for CyaA interaction with myeloid J774A.1 cells. Moreover, the CyaA-AC\(^{-}\) construct unable to produce any cAMP induced a more rapid calcium influx than the enzymatically active CyaA. An attractive interpretation of this intriguing observation would have been that CAMP produced by CyaA triggers activation of PMCA by the cAMP-dependent protein kinase, and this allows efficient extrusion of the incoming Ca\(^{2+}\) ions in the early stages of cell exposure to the active toxin (65, 66). However, no slowing down of [Ca\(^{2+}\)], increase in cells...
treated with the CyaA-AC^−_ construct was observed in the presence of a high concentration of the membrane-permeable cAMP, showing that cAMP signaling was not accounting for the slower influx of calcium into cells treated by intact CyaA. More likely, as discussed below, it was the altered conformation of the AC enzyme domain, perturbed by a dipeptide insert at the ATP-binding site, that allowed for the faster calcium influx kinetics observed with CyaA-AC^−_. Upon a faster elevation of [Ca^{2+}], by CyaA-AC^−, however, once a certain threshold level of [Ca^{2+}], is reached, activation of calcium pumps may account for the subsequent decrease of [Ca^{2+}], in cells treated by CyaA-AC^−. In contrast, in cells treated by the enzymatically active CyaA, the massive and ongoing dissipation of ATP into cAMP would progressively yield depletion of the intracellular ATP pool, eventually preventing compensation of the calcium influx by Ca^{2+} extrusion through PMCA and SERCA pumps, yielding a progressive increase of [Ca^{2+}], to a persistently high level. This interpretation of differences in kinetics of influx and final [Ca^{2+}], levels resulting from the treatment of cells with intact CyaA and CyaA-AC^− is, indeed, supported by the observation that upon depletion of cellular ATP by treatment with 2DG and FCCP, the CyaA-AC^− toxoid also caused a persistent elevation of cellular [Ca^{2+}], levels, as did CyaA (cf. Fig. 3).

Intriguingly, under conditions of ATP depletion, the kinetics of calcium influx in the early phases of cell exposure to CyaA-AC^− was slowed down and resembled the kinetics of calcium influx promoted by CyaA. This raises the hypothesis that the slow entry of calcium upon treatment by CyaA might be due to rapid local ATP depletion at the cellular membrane, caused by the incoming active AC enzyme and resulting in shortage of ATP supply for maintenance of the membrane potential by membrane pumps. Incapacity of the membrane pumps to restore the potential after membrane injury (permeabilization) by the toxin could then be expected to yield a drop of electrical potential and thus a slower calcium flux into cells along the electrical gradient. In turn, in the case of the CyaA-AC^−, no ATP depletion would occur, and short-circuiting of the membrane potential by lesions resulting from toxoid penetration across the membrane would likely be rapidly overcome by membrane pumps restoring the membrane potential, thus allowing for a faster initial calcium influx into cells.

Cytosolic calcium levels are, indeed, tightly controlled, and their modulation belongs to the most prominent mechanisms of cellular signaling that regulate many cellular pathways. These include pathways leading to cell death through apoptosis or necrosis, of which both types of cell death can indeed result from CyaA action. However, there was no correlation between the residual cytotoxicity of the various mutant toxoids and their capacity to promote calcium influx into cells (cf. Fig. 6A and Fig. 7 versus Fig. 9). In this respect, CyaA appears to differ somewhat from the RTX leukotoxin of Actinobacillus actinomycetemcomitans, and the capacity of CyaA to promote calcium influx into cells does not appear to account for toxin-induced cell death, whereas elevation of [Ca^{2+}], by leukotoxin still appears to be a prelude for downstream events involved in toxin-induced cytolysis (67).

We and others have recently shown that it is primarily the devastating capacity of CyaA to dissipate cellular ATP into cAMP that accounts for cytotoxicity of CyaA and that this is assisted by the cytolytic activity of CyaA resulting from permeabilization of cellular membrane by the “hemolytic” CyaA pores (38, 39). The latter activity was recently proposed to account for the residual cytotoxic activity (~10%) of the enzymatically inactive recombinant CyaA-AC^− toxoid (39). In this respect it is noteworthy that despite a fully conserved hemolytic activity, the CyaA-ΔAC and CyaA-247LQ constructs exhibited a significantly lower (p < 0.001), about five times reduced, specific residual cytotoxic (cytolytic) activity than CyaA-AC^− (Fig. 9). This suggests that other factors, synergizing with the hemolytic (pore-forming) activity, might be involved in the residual cytolytic activity of the toxoid. Indeed, a shared feature of CyaA-ΔAC and CyaA-247LQ proteins, which makes them different from CyaA-AC^+, is their incapacity to bind calmodulin, because CyaA-ΔAC has the calmodulin site deleted with the entire AC domain, and the CyaA-247LQ has this site disrupted by the Leu-Gln insert. An intriguing possibility would therefore be that rather than the capacity to promote calcium influx into cells, the residual cytotoxicity of CyaA-AC^− might depend on its capacity to outcompete cytosolic calmodulin from complexes with its cellular ligands.

We have previously proposed a model of interaction of CyaA with the membrane, which postulates that CyaA can interact with target membranes in the form of two different conformers, one leading to a monomeric CyaA inserted into the membrane with its AC domain translocated into cell cytosol and the other conformer forming oligomeric CyaA pores (36). As depicted in the proposed model in Fig. 10, the present results tend to indicate that calcium influx into cells is associated with the presumably monomeric CyaA species translocating the AC domain into cells, rather than with the oligomerizing CyaA species forming the hemolytic pores. Furthermore, the results obtained here with several AC−_toxoids carrying substitutions and peptide inserts in their AC domains (i.e. CyaA-AC^−, CyaA-247LQ, CyaA-K58Q, and CyaA-1080VA-AC^−) indicate that there is a relation between the conformation and/or sequence of the translocating AC domain and its capacity to promote calcium influx. It indeed appears difficult to conceive that peptide insertions within the AC would exert any effects on the calcium influx associated with translocation of the AC polypeptide, if the AC was crossing the membrane in a fully unfolded form. The effects of peptide insertions within the AC on the passage of Ca^{2+} across the membrane would rather imply that the AC domain of CyaA is translocating across the membrane in at least a partially folded state, such as a molten globule having secondary structures preserved. These could then be differently perturbed by various peptide inserts. Alternatively, the effects on calcium influx of the inserts within AC might indicate that even when translocated to the cytosolic site of the cellular membrane, the folded and calmodulin-bound form of AC would remain part of the formed calcium conduit, and the various peptide inserts within the AC domain would thus differently affect the function/conformation of the calcium entry path.
It should further be noted that in this study relatively high concentrations of CyaA (>0.3 µg/ml) had to be employed in order to observe any substantial increase of cytosolic calcium level in J774A.1 cells. It is nevertheless likely that the capacity of CyaA to elicit [Ca^{2+}]_{i} elevation in cells still supports synergistically its main cytotoxic activity that consists of dissipation of cytosolic calcium and a biphasic calcium level in time. In turn, in cells exposed to the used high concentration of the enzymatically active toxin, conversion of ATP to cAMP would result in depletion of cellular ATP and a persistent increase of [Ca^{2+}]_{i} level.

Acknowledgments—We gratefully acknowledge the excellent technical help of H. Kubinova and S. Charvatova and the gift of the pACTM247 construct by D. Ladant.

REFERENCES

1. Goodwin, M. S., and Weiss, A. A. (1990) Infect. Immun. 58, 3445–3447
2. Harvill, E. T., Cotter, P. A., Yuk, M. H., and Miller, J. F. (1999) Infect. Immun. 67, 1493–1500
3. Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D., and Leclerc, C. (2001) J. Exp. Med. 193, 1035–1044
4. Bellalou, J., Sakamoto, H., Ladant, D., Geoffroy, C., and Ullmann, A. (1990) Infect. Immun. 58, 3242–3247
5. Szabo, G., Gray, M. C., and Hewlett, E. L. (1994) J. Biol. Chem. 269, 22496–22499
6. Benz, R., Maier, E., Ladant, D., Ullmann, A., and Sebo, P. (1994) J. Biol. Chem. 269, 27231–27239
7. Confer, D. L., and Eaton, J. W. (1982) Science 217, 948–950
8. Hanks, E., and Farrel, Z. (1985) J. Biol. Chem. 260, 5526–5532
9. Hewlett, E., and Wolff, J. (1976) J. Bacteriol. 127, 890–898
10. Friedman, R. L., Fiederlein, R. L., Glasser, L., and Galgiani, J. N. (1983) Infect. Immun. 39, 139–140
11. Hewlett, E. L., Smith, D. L., Myers, G. A., Pearson, R. D., and Kay, H. D. (1983) Clin. Res. 31, 365A
12. Khelef, N., and Guiso, N. (1995) FEMS Microbiol. Lett. 134, 27–32
13. Khelef, N., Zychlinsky, A., and Guiso, N. (1993) Infect. Immun. 61, 4064–4071
14. Njamkepo, E., Pinot, F., Francois, D., Guiso, N., Polla, B. S., and Bachelet, M. (2000) J. Cell. Physiol. 183, 91–99
15. Pearson, R. D., Symes, P., Conboy, M., Weiss, A. A., and Hewlett, E. L. (1987) J. Immunol. 139, 2749–2754

CyaA-dependent Entry of Ca^{2+} into J774A.1 Monocytes

Fig. 10. Proposed model of the mechanism of calcium influx into cells mediated by translocating AC domain polypeptide. The current model (36) predicts that CyaA can interact with target membranes in a form of two different conformers, one leading to a monomeric translocation precursor inserted into the membrane with its AC domain that is translocated into cell cytosol and the other conformer forming oligomeric CyaA pores. The results indicate that calcium influx into cells does not occur through the oligomeric hemolytic CyaA pore and is associated with the CyaA species translocating the AC domain into cells. In this process the predicted transmembrane segments of the oligomeric CyaA are involved in the calcium path allowing Ca^{2+} influx into cell cytosol. This is supported by results showing that mutations reverting the charge or disrupting the α-helical character of the predicted transmembrane segments, as well as peptide insertions and point mutations affecting structure of the AC domain, affect the capacity of CyaA to promote calcium influx. In cells exposed to CyaA-AC, the activation of calcium pumps at certain [Ca^{2+}]_{i} threshold levels would allow compensation for the calcium influx by Ca^{2+} extrusion, yielding subsequent decrease of [Ca^{2+}]_{i} and a biphasic calcium level in time. In turn, in cells exposed to the used high concentration of the enzymatically active toxin, conversion of ATP to cAMP would result in depletion of cellular ATP and a persistent increase of [Ca^{2+}]_{i} level.

face of the cytoplasmic membrane, where the tetravalent calmodulin occurs itself at often higher than 1 µM concentrations (69, 70). Although already 1 µM free Ca^{2+} concentrations can yield inhibition of the AC enzyme in vitro (71), the situation can be quite different in vivo where such high calcium concentrations are unlikely to occur at physiologically low toxin concentrations. Nevertheless, the enhancement of the affinity of AC for binding of the calcium-loaded calmodulin at lower than 1 µM free Ca^{2+} concentrations can be expected to enhance the capacity of the AC to compete with other cellular proteins for binding of calmodulin (72). Even a rather local increase of [Ca^{2+}]_{i}, at the inner surface of the cell membrane, from the basal level (~100 nM) to only a several times higher concentration, may hence importantly enhance the extent of activation of the translocated AC enzyme by calmodulin (71). Therefore, the limited calcium influx accompanying translocation of the AC domain across the cellular membrane might reflect an optimized strategy of target cell intoxication by CyaA.
CyaA-dependent Entry of Ca^{2+} into J774A.1 Monocytes

16. Weingart, C. L., and Weiss, A. A. (2000) Infect. Immun. 68, 1735–1739
17. Wolff, J., Cook, G. H., Goldhammer, A. R., and Berkowitz, S. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3841–3844
18. Bagley, K. C., Abdelwahab, S. F., Tuskan, R. G., Fouts, T. R., and Lewis, G. K. (2002) J. Leukocyte Biol. 72, 962–969
19. Ross, P. J., Lavelle, E. C., Mills, K. H., and Boyd, A. P. (2004) Infect. Immun. 72, 1568–1579
20. Boyd, A. P., Ross, P. J., Conroy, H., Mahon, N., Lavelle, E. C., and Mills, K. H. (2005) J. Immunol. 175, 730–738
21. Vojtova, J., Kamanova, J., and Sebo, P. (2006) Curr. Opin. Microbiol. 9, 69–75
22. Skinner, L. J., Enee, V., Beurg, M., Jung, H. H., Ryan, A. F., Hafidi, A., Aran, J. M., and Dulon, D. (2003) J. Neurophysiol. 90, 320–332
23. Bassinet, L., Fitting, C., Housset, B., Cavaillon, J. M., and Guiso, N. (2004) Infect. Immun. 72, 5530–5533
24. Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullmann, A., and Danchin, A. (1988) Mol. Microbiol. 2, 19–30
25. Iwaki, M., Ullmann, A., and Sebo, P. (1995) Mol. Microbiol. 17, 1015–1024
26. Hackett, M., Walker, C. B., Guo, L., Gray, M. C., Van, C. S., Ullmann, A., Shabanowitz, J., Hunt, D. F., Hewlett, E. L., and Sebo, P. (1995) J. Biol. Chem. 270, 20250–20253
27. Hackett, M., Guo, L., Shabanowitz, J., Hunt, D. F., and Hewlett, E. L. (1994) Science 266, 433–435
28. Rhodes, C. R., Gray, M. C., Watson, I. M., Muratore, T. L., Kim, S. B., Hewlett, E. L., and Grisham, K. H. (2005) J. Leukocyte Biol. 72, 962–969
29. Breed, W. E., Klemm, W. R., Lamb, W. J., Blanken, K., Grabarek, Z., Mrksich, M., and Tang, W. J. (2002) EMBO J. 21, 6721–6732
30. Perschini, A., and Stemmer, P. M. (2002) Trends Cardiovasc. Med. 12, 32–37