Hemolytic activity of adenylate cyclase toxin from *Bordetella pertussis*

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Adenylate cyclase (AC) toxin from *B. pertussis* enters eukaryotic cells where it produces supraphysiologic levels of cAMP. Purification of AC toxin activity [(1989) J. Biol. Chem. 264, 19279] results in increasing potency of hemolytic activity and electroelution of the 216-kDa holotoxin yields a single protein with AC enzymatic, toxin and hemolytic activities. AC toxin and *E. coli* hemolysin, which have DNA sequence homology [(1988) EMBO J. 7, 3997] are immunologically cross-reactive. The time courses of hemolysis elicited by the two molecules are strikingly different, however, with AC toxin eliciting cAMP accumulation with rapid onset, but hemolysis with a lag of ~45 min. Finally, osmotic protection experiments indicate that the size of the putative pore produced by AC toxin is 3–5-fold smaller than that of *E. coli* hemolysin.

Bordetella pertussis; Adenylate cyclase toxin; Pore; *E. coli* hemolysin; Calcium

1. INTRODUCTION

B. pertussis organisms are described as hemolytic on the basis of the zone of hemolysis surrounding colonies on blood plates, but the molecule responsible for this activity has not previously been isolated or characterized. AC toxin is a calmodulin-stimulated adenylate cyclase with apparent M_r 216000 which enters eukaryotic cells to catalyze supraphysiologic levels of cAMP [1–3]. Several observations have suggested a possible relationship between AC toxin and hemolysin. First, nonhemolytic mutants prepared by transposon Tn-5 insertion had little or no AC activity [4]. Next, AC toxin was shown to lyse multilamellar liposomes, but was not hemolytic over a short (1 h) time course [5]. Finally, Glaser et al. [6] determined that the AC toxin gene possessed sequence homology with the structural gene (hlyA) of *E. coli* hemolysin and that there were homologous ancillary genes [7]. In this paper, we demonstrate that purified AC holotoxin, including toxin electroeluted following PAGE, is sufficient for hemolysis and characterize the hemolytic activity in comparison to *E. coli* hemolysin.

2. MATERIALS AND METHODS

2.1. *Toxin purification*
The AC toxin was purified as described previously [2] from *Bordetella pertussis* strain BP338. Electroelution of the 216-kDa AC

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holotoxin molecule was performed according to Hunkapiller et al. [8] and as described previously [2]. Bovine serum albumin was electroeluted and precipitated using the same procedure to serve as a control.

E. coli hemolysin from a hemolysin-producing strain 0040 (serotype 04, provided by Dr Tim Pruitt) was prepared as described by Eberspacher et al. [9]. Supernatant medium from a nonhemolytic strain of *E. coli* 10405 was prepared identically and used as a control.

2.2. *Hemolysis assay*
Hemolysis assays were performed according to Clerc et al. [10] with minor modifications. Fifty microliters of washed sheep red cells (Cocalico Biologicals, Reamstown, PA) at a density of 4×10^9/ml were added to each well of a 96-well flat-bottomed microtiter plate followed by addition of 50 μl of sample. After incubation for 5 h at 37°C or the indicated time period the cells were resuspended and 150 μl of cold PBS was added to each well. After 15 min of centrifugation at 4°C and 2200 × g, 100 μl was transferred to another plate. Hemoglobin absorbance was measured at 540 nm in a Multiscan Spectrophotometer. Specific hemolysis was determined by:

$$\frac{\text{hemoglobin release by sample} - \text{hemoglobin release by PBS}}{\text{total hemoglobin released by NH}_4\text{OH}}$$

and was expressed as % of total hemolysis.

2.3. *Adenylate cyclase toxin activity*
AC toxin activity (accumulation of intracellular cAMP) was measured in sheep erythrocytes by incubating 50 μl of sheep cells at 4×10^9/ml with 50 μl of sample. After the indicated incubation times, cells were cooled to 4°C, pelleted, the supernatant was removed, and cells were resuspended in 450 μl PBS and 10% TCA. After TCA precipitation (to remove the hemoglobin), 400 μl of the supernatant was removed and extracted 3 times with H₂O-saturated ether to remove the TCA. HCl was added to yield a final concentration of 0.1 N and cAMP was then measured by automated radioimmunoassay.

2.4. *Western blot*
Western blotting was performed as described previously [11].

2.5. *Determination of free calcium concentration*
Free calcium concentrations in the presence of varying amounts of
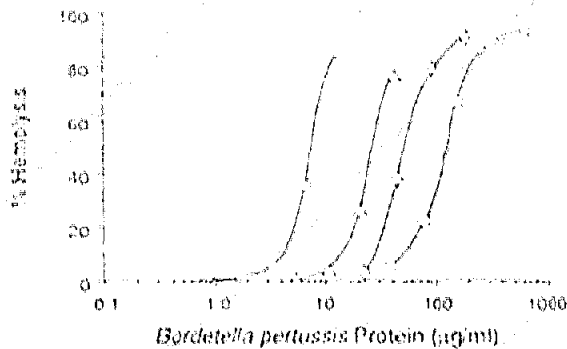
Fig. 1. Hemolytic activity of fractions from AC toxin purification as described previously [2]. Sheep erythrocytes (2 × 10⁸) were incubated with urea extract of *B. pertussis* (□—□), phenyl-Sepharose purified AC toxin (○—○), sucrose gradient purified AC toxin (△—△), or calmodulin-Sepharose-purified AC toxin (▬▬) for 5 h at 37°C. The adenylate cyclase enzymatic activities of these fractions were as follows: urea extract (0.111 μmol cAMP/min/μg), phenyl-Sepharose purified AC toxin (0.634 μmol/cAMP/min/μg), sucrose gradient purified AC toxin (1.24 μmol cAMP/min/μg) and calmodulin-Sepharose purified AC toxin (14.7 μmol cAMP/min/μg).

magnesium, EDTA and calcium were calculated using the EGTA computer program [12] and equilibrium constants found in Critical Stability Constants [13]. These have been previously validated experimentally.

3. RESULTS AND DISCUSSION

3.1. *Hemolytic activity of AC toxin*

Each step in the purification of AC toxin which results in an increase in toxin specific activity [2], results in an increase in hemolytic activity (Fig. 1). The calmodulin-Sepharose-purified AC toxin, however, contains some small molecular weight protein degradation fragments, in addition to principal moiety, the M_r 216000 holotoxin. To be certain that the observed hemolysis reflects activity of the holotoxin molecule alone, the M_r 216000 band was electroeluted following SDS-PAGE, precipitated to remove residual SDS, and tested for hemolytic activity. Electroeluted AC toxin at a concentration of 24 μg/ml with enzymatic activity of 10.8 μmol/min/mg and toxin activity of 0.38 μmol cAMP/mg J774 cell protein/mg toxin, elicited 18.7% hemolysis of 2 × 10⁸ sheep erythrocytes in 5 h. Electroeluted BSA, used as a control at equivalent protein concentration, yielded only 2% hemolysis. The demonstration of enzyme, toxin and hemolytic activities after such treatments as urea extraction or electroelution indicate that AC toxin is very stable to denaturation and renaturation. The toxin and hemolytic activities are relatively less stable, however, in that their specific activities are reduced after electroelution. Nevertheless, these data confirm that the M_r 216000 holotoxin is sufficient for hemolysis.

3.2. *Comparison with* E. coli *hemolysin*
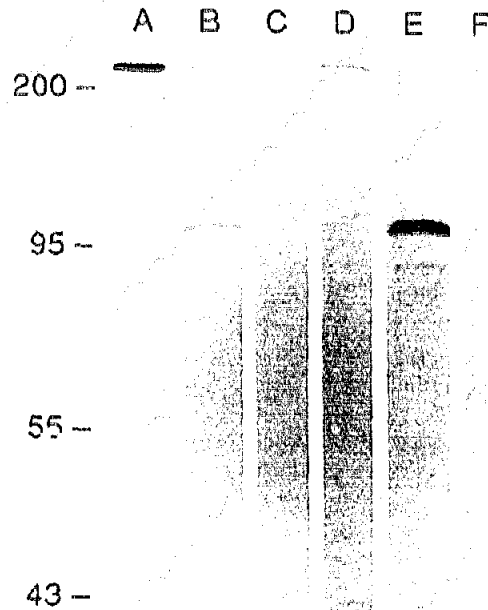
In light of the hemolytic activity of AC toxin and the

Fig. 2. Immunological cross-reactivity between AC toxin and *E. coli* hemolysin. Sucrose gradient-purified AC toxin (9 μg) (lanes A and D), *E. coli* hemolysin (14.6 μg) (lanes B and E), and PEG precipitate from nonhemolytic *E. coli* (22.2 μg) (lanes C and F) were separated by SDS-PAGE and electroblotted onto PVDF membranes. Lanes A, B and C were probed with 1:1000 dilution of 9D4 (monoclonal antibody raised against AC toxin), and lanes D, E and F were probed with a 1:500 dilution of a rabbit polyclonal antibody against *E. coli* hemolysin (kindly provided by Dr Rod Welch). Preimmune rabbit serum at 1:500 dilution was used as a control and did not recognize any bands on the blots.

known sequence homology with *E. coli* hemolysin [6,7], we conducted an immunological and functional comparison of the two proteins. Fig. 2 demonstrates that a monoclonal antibody (9D4) raised against *Bordetella pertussis* AC toxin [2] reacts with AC toxin (lane A) and with *E. coli* hemolysin (lane B), but not with proteins in the culture medium from a non-hemolytic *E. coli* (lane C). Similarly, polyclonal antiserum against *E. coli* hemolysin (kindly provided by Dr R. Welch) reacts with both the AC toxin (lane D) and *E. coli* hemolysin (lane E). 9D4 does not, however, neutralize AC toxin activity or the hemolytic activities of AC toxin or *E. coli* hemolysin. These data do indicate that at least one antigenic domain is conserved between the two proteins.

Previous analysis of the nucleotide sequences of AC toxin and *E. coli* hemolysin has revealed a nonameric repeat GGDGDDTLX that is present in multiple copies in both genes [6,14]. The predicted tertiary structure of the repeat region resembles a calcium-binding domain present in proteins from other organisms and eukaryotic cells [14,15]. *B. pertussis* AC toxin requires calcium to intoxicate eukaryotic cells and to disrupt liposomes [5]. However, Wolff et al. [17] and Greenlee et al. [18] have shown that calmodulin activation of the
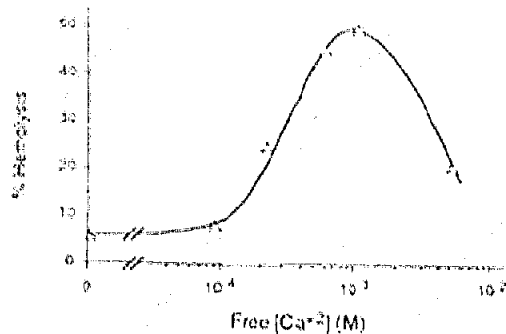
Fig. 3. Calcium dependence of AC toxin hemolytic reaction. Calcium and magnesium were added to sucrose gradient-purified AC toxin in 10 mM tricine, 0.15 M NaCl, 0.4 mM EDTA to yield the free calcium concentrations indicated. AC toxin (70 μg/ml) was then incubated with 2 × 10⁸ sheep erythrocytes for 5 h at 37°C and hemolysis was measured as described in Section 2.

which are responsible for increasing intracellular cAMP levels in eukaryotic cells. The time courses of the biological effects of these two toxins on erythrocytes are substantially different. The hemolytic activity of *E. coli* hemolysin begins within the first 15 min, is complete by 3.5 h and, as expected, is not associated with an increase in cAMP levels (Fig. 4A). In contrast, cAMP accumulation has an immediate onset in cells treated with AC toxin, yet hemolysis exhibits a lag of 45 min–1 h (Fig. 4B). The lag time of the onset of hemolysis is not less than 45 min regardless of the concentration of toxin employed. Although both *E. coli* hemolysin [21] and AC toxin [5] interact with the membranes of target cells within seconds of exposure, it is clear that hemolysis is not an immediate consequence of that interaction in the case of *B. pertussis* AC toxin.

We hypothesized that *E. coli* hemolysin and AC toxin might use different mechanisms to lyse red cells since the kinetics of hemolysis are not the same. It has been shown that *E. coli* hemolysin forms transmembrane pores in erythrocytes [21], so we asked whether AC toxin also forms pores in red cells. Bhakdi et al. [21] showed that addition of dextran 4, but not smaller sugars, provided osmotic protection to rabbit erythrocytes exposed to *E. coli* hemolysin. On the basis of these experiments, the pore created by *E. coli* hemolysin was estimated to have a functional diameter of 2–3 nm. In contrast, addition of sucrose, mannitol or arabinose protects erythrocytes from lysis by AC toxin (Table I). The only sugar that allows lysis to occur in AC toxin-treated cells is erythritol. Erythritol may be transported across red cell membranes of some species, however, so that it may not be possible to establish the lower limit of the pore size with this sugar. As expected, these sugars do not protect the cells from *E. coli* hemolysin-mediated lysis. There is no effect of the sugars on AC toxin-induced cAMP accumulation (data not shown)
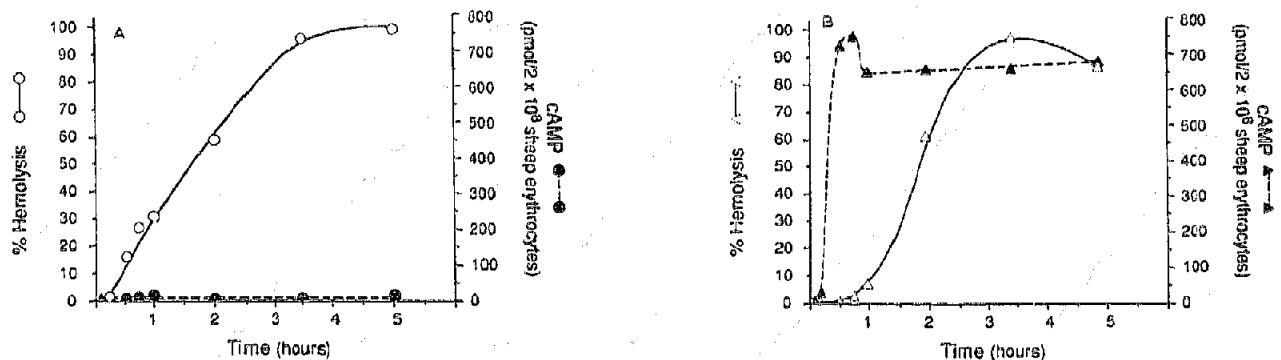
B. pertussis AC enzymatic activity can occur in the absence of calcium. These data suggest that the binding and/or entry of the toxin rather than the AC enzymatic reaction is the major calcium-requiring step. Fig. 3 demonstrates that the AC toxin hemolytic reaction also requires calcium, as has been reported for hemolysis caused by *E. coli* hemolysin [19,20]. The hemolytic reaction and the liposome-disrupting action of the AC toxin [5] are both half-maximal at a calcium concentration of 0.3 mM. The decrease in hemolysis observed at higher calcium concentrations is not seen in the liposome or intoxication assays and may reflect some interaction between calcium and the erythrocyte membrane which inhibits toxin insertion.

Despite the similarities noted above, AC toxin has a molecular mass nearly twice that of *E. coli* hemolysin with part of the size difference due to the amino-terminal catalytic and calmodulin-binding domains [13]

Fig. 4. Time course of hemolysis and cAMP accumulation by *E. coli* hemolysin (A) and AC toxin (B). *E. coli* hemolysin (47.5 μg/ml) (A) or calmodulin-Sepharose-purified AC toxin (47.5 μg/ml) (B) was incubated with 2 × 10⁸ sheep erythrocytes at 37°C and hemolysis (O—O, △—△) was calculated at each point as described in Section 2. Aliquots of the same preparations of *E. coli* hemolysin (A) and AC toxin at the same concentrations (B) were incubated with 2 × 10⁸ sheep erythrocytes and cAMP concentrations (●—●, ▲—▲) were determined as described in Section 2. *B. pertussis* adenylate cyclase toxin activity and cAMP levels for *E. coli* hemolysin-treated erythrocytes represent the sum of intracellular cAMP and extracellular cAMP (from the supernate of lysed cells) at each time point. Asterisk indicates the derived values for hemolysis and the background value for intracellular cAMP (1.6 pmol/2 × 10⁸ sheep erythrocytes) at time zero.

Table I

Osmotic protection of sheep erythrocytes from AC toxin-mediated lysis

Added toxin:	% Hemolysis[a]		
	None	AC toxin	E. coli hemolysin
Added sugar (75 mM)			
None - PBS	3.0 ± 0.19[b]	32.7 ± 3.6	95.2 ± 1.2
Sucrose (0.92)[*]	2.9 ± 0.18	4.2 ± 0.55	95.0 ± 1.7
Mannitol (0.72)	3.4 ± 0.37	5.1 ± 0.33	100 ± 4.9
Arabinose (0.62)	3.5 ± 0.39	8.0 ± 1.5	90.5 ± 5.8
Erythritol (0.56)	5.3 ± 0.80	42.9 ± 4.6	97.9 ± 1.5

[*] Estimated diameter of sugar (nm)

[a] AC toxin (40–125 μg) or E. coli hemolysin (at equal concentrations with AC toxin in each individual experiment) were added to the sheep red cells for 15 min at 37°C prior to the addition of the sugar or buffer control. The assay was incubated for 5 h. The resultant values for hemolysis are expressed as the mean ± SE. As described by Bhakdi et al. [21], Dextran 4 (30 mM) completely inhibited AC toxin and E. coli hemolysin mediated hemolysis measured after an hour.

indicating that the prevention of hemolysis does, in fact, represent osmotic protection and not blockade of toxin binding or entry. Therefore, the osmotic protection data do suggest that AC toxin is forming a pore of less than 0.62 nm in diameter, at least 3–5-fold smaller than that of E. coli hemolysin.

The hemolysis observed with AC toxin is useful to study toxin/membrane interactions, but there is reason to question whether hemolytic activity is relevant to the pathophysiology of whooping cough. The organism is not invasive and thus it is unlikely that hemolysis is an essential feature of the clinical disease. Studies done in our laboratory, however, suggest that AC toxin can be lethal for phagocytic leukocytes. Geber et al. [22] showed that after 2 h of treatment with AC toxin, J774 cells (a macrophage-like cell line), had accumulated >5000 pmol cAMP/mg cell protein and had cell death of 64% as determined by release of ^{51}Cr. After 6–8 h, 86% of the cells had lysed. These data suggest that lysis of nucleated cells may also occur after intoxication by AC toxin. Whether cAMP accumulation and ATP depletion play a role in the lysis of nucleated cells is unknown, but the demonstration that AC toxin disrupts artificial membranes indicates that enzymatic activity may not be required for lysis. Furthermore, we have evidence in red cells to suggest that very high levels of cAMP or loss of ATP is not responsible for AC toxin hemolysis. Calmodulin, which has been shown to inhibit intoxication of red cells by AC toxin [23,24] does not inhibit hemolysis (manuscript in preparation), suggesting that AC enzymatic activity is not required for lysis of red cells.

It appears that AC toxin and E. coli hemolysin do not have identical mechanisms for cell disruption even though they have sequence similarity and immunological cross-reactivity. AC toxin forms a smaller pore and lyses the cells with a different time course. Since at least a portion of the toxin is entering the cells, it may be that the catalytic domain of the toxin is impeding free flow of water and ions through the pore and that lysis begins only after delivery and processing of the toxin in the membrane. Further study of the mechanism of AC toxin hemolysis is necessary to determine which domains of the protein are necessary for toxin membrane interaction and which regions of the protein are involved in the mechanism of AC toxin hemolysis.

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