Distinct Mechanisms for K⁺ Efflux, Intoxication, and Hemolysis by Bordetella pertussis AC Toxin*

(Received for publication, February 4, 1998, and in revised form, May 6, 1998)

Mary Gray‡, Gabor Szabo§, Angela S. Otero§, Lloyd Gray¶, and Erik Hewlett‡|**
From the Departments of ‡Medicine, §Molecular Physiology and Biological Physics, ¶Pathology, and ||Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Adenylate cyclase (AC) toxin from Bordetella pertussis delivers its catalytic domain to the interior of target cells where it converts host ATP to cAMP in a process referred to as intoxication. This toxin also hemolyzes sheep erythrocytes by a mechanism presumed to include pore formation and osmotic lysis. Intoxication and hemolysis appear at strikingly different toxin concentrations and evolve over different time scales, suggesting that different molecular processes may be involved. The present study was designed to test the hypothesis that intoxication and hemolysis occur by distinct mechanisms.

Although the hemolytic activity of AC toxin has a lag of >1 h, intoxication starts immediately. Because of this difference, we sought a surrogate or precursor lesion that leads to hemolysis, and potassium efflux has been observed from erythrocytes treated with other pore-forming toxins. AC toxin elicits an increase in K⁺ efflux from sheep erythrocytes and Jurkat cells, a human T-cell leukemia line, that begins within minutes of toxin addition. The toxin concentration dependence along with the analysis of the time course suggest that toxin monomers are sufficient to elicit release of K⁺ and to deliver the catalytic domain to the cell interior. Hemolysis, on the other hand, is a highly cooperative event that likely requires a subsequent oligomerization of these individual units. Although induction of K⁺ efflux shares some structural and environmental requirements with both intoxication and hemolysis, it can occur under conditions in which intoxication is reduced or prevented. The data presented here suggest that the transmembrane pathway by which K⁺ is released is separate and distinct from the structure required for intoxication but may be related to, or a precursor of, that which is ultimately responsible for hemolysis.

Adenylate cyclase (AC) toxin is an important virulence factor for Bordetella pertussis and probably Bordetella bronchiseptica and Bordetella parapertussis (1–4). It is an acylated, 177-kDa protein that can deliver its catalytic domain to the interior of intact target cells (5–8). Inside the cell AC toxin binds endogenous calmodulin, resulting in a 1000-fold increase in its enzymatic activity and production of supraphysiologic levels of intracellular cAMP from host ATP (9). This process is referred to as intoxication. Although intoxication is believed to be the primary contribution of AC toxin to Bordetella virulence, this protein is also lytic for sheep erythrocytes and, thus, responsible for the hemolytic phenotype of phase I B. pertussis (10, 11). Removal of the N-terminal catalytic domain (amino acids 1–400) does not affect hemolysis or pore formation in artificial lipid bilayers (12, 13). Both of these activities are attributed to the C-terminal end of AC toxin that is homologous to Escherichia coli hemolysin and other members of the RTX family of bacterial toxins (13–15). Intoxication and hemolysis require post-translational acylation of the toxin at one or two internal lysine residues, which converts the inactive protoxin to the active form (8, 16). Acylation appears to occur within the bacterium and is dependent on the product of a separate gene, cyaC (17, 18). A similar acylation is required for activation of E. coli hemolysin and probably other members of the RTX family (15, 19, 20).

As with other hemolysins, AC toxin has been shown to produce transmembrane ion conductance in artificial lipid bilayers, and the properties of the responsible structure have been investigated (13, 21). We have shown that pore formation is calcium-dependent, as are intoxication and hemolysis, and have demonstrated that this activity has a cubic or higher concentration dependence, suggesting that an oligomer is the active species (21). Benz et al. (13) studied the single channel properties of the pore formed by AC toxin and found it to be cation-selective and considerably smaller than that of E. coli hemolysin. In keeping with that observation, the hemolytic activity of AC toxin is modest, relative to E. coli hemolysin or other RTX hemolysins (13, 14). The time course is prolonged (hours rather than minutes), and the concentration required to elicit >90% hemolysis is high, in the 10 μg/ml range (22). By using the method of osmotic solute exclusion, Ehrmann et al. (22) estimated the pore created by AC toxin to be <0.6 nm, which is significantly smaller than the 2–3 nm estimated for E. coli hemolysin (23).

A major focus of our work has been to determine the sequence of events involved in AC toxin’s interaction with target cells and the relationships among these activities. The onset of intoxication is rapid and maximal in 30–60 min at toxin concentrations of about 1 μg/ml (22, 24). However, the hemolytic activity of AC toxin demonstrates a lag of >1 h even at toxin concentrations of 10 μg/ml (22). The significant difference in both time course and concentration dependence for intoxication and hemolysis suggested to us that these two processes occur by different mechanisms. In order to address this, we hypothesized that there might be an earlier event in the course of toxin-cell interaction that is an antecedent of hemolysis. Potassium efflux has been used previously to measure pore formation by toxins and other proteins that traverse cell membranes (25–32). This efflux of K⁺ has been shown to be rapid in onset.
and to precede cell swelling and eventual lysis. Potassium is present inside the cell at concentrations greater than 100 mM and can be measured simply and directly in the culture medium by flame photometry. By using this method, we have found that AC toxin-treated sheep erythrocytes release K+ with an onset comparable to that of intoxication. The toxin concentration dependence of K+ efflux is a first order process, suggesting that a monomer is responsible. Whereas intoxication has a similar concentration dependence, hemolysis shows a cubic or higher power dependence, suggesting that an oligomer is necessary. Potassium efflux can be dissociated from intoxication because it occurs under conditions in which intoxication is reduced or prevented. These results suggest that K+ efflux is an early event in toxin-cell interaction that can be dissociated from intoxication. This process may reflect the initial insertion of the toxin monomer into the target cell membrane in a process culminating in toxin oligomerization and hemolysis.

**EXPERIMENTAL PROCEDURES**

**Production and Purification of Adenylate Cyclase Toxin—**Production of recombinant AC toxin was done as described previously (33) with minor modifications. *E. coli* BL21 cells (Stratagene, La Jolla, CA) containing plasmid pT7ACT1 for expression of wild type AC toxin, or plasmid pACT7 for production of CyaC B. pertussis toxin, were grown to an optical density of 0.2 at 600 nm at 37 °C in 2X YT medium (1.6% Bacto-tryptone, 1% Bacto-yeast, 85 mM NaCl) containing 150 μM calmodulin. Isopropyl-β-D-thiogalactopyranoside (1 mM) was added to the cultures which were incubated an additional 4 h. Cultures were centrifuged, and the supernatant (400 ml of erythrocytes at a 40% hematocrit with AC toxin at 37 °C unless indicated. The reaction was initiated with the addition of [3H]cAMP. AC activity was measured by incubating 10⁶ cpm of [3H]cAMP). Cyclic AMP was separated from substrate by a 15-fold dilution of toxin solution into HBSS. The urea concentration present after this dilution was <500 mM and free calcium concentration was 1.1 mM. The difference in onset of intoxication and K+ efflux, and hemolysis shown in Fig. 1 were fitted by Equation 2.

\[
\alpha (1 - \exp(-t/t_\text{r}))
\]

where \(t\) is time, \(t_r\) is the time constant of the rise of effect, \(\alpha\) is the amplitude of the effect, and \(n\) is a parameter related to the stoichiometry of the molecular complex involved in this process. A value of \(n = 1\) corresponds to a simple exponential rise expected for a first order process. Larger values of \(n\) are expected when several molecules must combine to form a functional complex.

Nonlinear curve fitting was carried out using the program Origin (Microcal Software). The resulting fits are shown as solid lines, and the corresponding parameters are given in the figure legends. An analogous procedure was used to fit the Hill equation to the data of Fig. 3.

**RESULTS**

**Temporal Relationships among Intoxication, Hemolysis, and K+ Efflux—**The difference in onset of intoxication and hemolysis has made it difficult to determine the relationship between these two functional activities of AC toxin and suggests that they occur by different mechanisms. In studies using erythrocytes, addition of other pore-forming toxins has been shown to cause a rapid release of K+ which was followed by cell swelling and eventual lysis. That is to say, efflux of K+ can be used as a diagnostic tool to measure increases in membrane permeability and is thought to represent the initial manifestation of the transmembrane pore that is responsible for ultimate osmotic lysis (25–32). In the present study, we hypothesized that K+ efflux would occur in erythrocytes treated with AC toxin and that it would precede hemolysis. The timing of K+ efflux from AC toxin-treated sheep erythrocytes in relation to intoxication or hemolysis is illustrated in Fig. 1, A and B, respectively. Both intoxication and K+ efflux (Fig. 1A) exhibit virtually immediate onset with first order kinetics. In comparison, hemolysis is delayed and negligible in magnitude for periods as long as 90 min even at a high toxin concentration (20 μg/ml) (Fig. 1B). These data clearly suggest that K+ efflux is an indicator of toxin-mediated membrane perturbation, which precedes hemolysis and is associated temporally with intoxication. As shown in Fig. 2, AC toxin also elicits K+ release from Jurkat cells, a human T-cell leukemia line that is not lysed by AC toxin. This observation indicates that the process resulting in K+ efflux is not restricted to non-nucleated cells or cells such as erythrocytes that are lysed by AC toxin. In light of these observations, it was important to characterize in depth K+ efflux as a consequence of AC toxin interaction with target cells. In order to determine relevance of this phenomenon to hemolysis, sheep erythrocytes were chosen for use in subsequent experiments as the model system.

**Concentration Dependences of Intoxication, Hemolysis, and...
To examine more closely the relationship between K⁺ release and hemolysis, we evaluated the concentrations of toxin required for these two activities and compared them to that necessary for intoxication. The concentration dependences of intoxication and K⁺ efflux at 30 min and hemolysis at 4.5 h are shown in Fig. 3. The differences between intoxication or K⁺ efflux and hemolysis are striking. A quantitative assessment of these differences was obtained by fitting the data to the Hill equation, shown as solid lines. Intoxication and K⁺ efflux demonstrate Hill coefficients near unity (\(n = 1.39 \pm 0.10\) and \(0.82 \pm 0.04\) respectively), whereas that for hemolysis is markedly higher (\(n = 4.99 \pm 0.71\)), suggesting cooperativity as observed previously (21). These data along with the analysis of the time course strongly suggest that toxin monomers are sufficient to elicit release of K⁺ and the delivery of the catalytic domain to the cell interior. Hemolysis, on the other hand, is a highly cooperative event that likely requires a subsequent oligomerization of these individual units.

Structural and Functional Requirements for Intoxication, Hemolysis, and K⁺ Efflux—In light of the discrepancy between the stoichiometry for K⁺ efflux and hemolysis, processes that were expected to be closely linked, we began an investigation to compare and contrast K⁺ efflux with intoxication and hemolysis.

**Fig. 1.** Time course of intoxication, K⁺ efflux, and hemolysis in sheep erythrocytes. AC toxin was incubated with erythrocytes at 37 °C. At the time indicated, cells were spun, and the supernatant was removed to measure K⁺ efflux and hemolysis. The pellet was washed and intracellular cAMP measured as described under “Experimental Procedures.” Solid lines show the fit of Equation 2 to the data. A, AC toxin, 10 μg/ml. Parameters for the fit of the intoxication data were \(a = 331 \pm 30, \tau = 17 \pm 5\) min, \(n = 1.4 \pm 0.3\) and for the K⁺ efflux data were \(a = 6.2 \pm 0.5, \tau = 106 \pm 16\) min, \(n = 0.8 \pm 0.02\). B, AC toxin, 20 μg/ml. Parameters for the fit of the hemolysis data were \(a = 66 \pm 0.5, \tau = 1.2 \pm 0.02\) h, \(n = 14 \pm 0.4\) and for K⁺ efflux data were \(a = 97 \pm 6, \tau = 1.9 \pm 0.5\) h, \(n = 1.0 \pm 0.1\). Each point is the mean of duplicate determinations. This graph is representative of three separate experiments.

**Fig. 2.** AC toxin causes an increase in K⁺ efflux in Jurkat cells. AC toxin at indicated concentrations was added to \(2 \times 10^7\) Jurkat cells in 500 μl and incubated for 30 min at 37 °C. Cells were spun, and the supernatant was used to measure K⁺ efflux. Each point is the mean of duplicate determinations. This graph is representative of three separate experiments.
Both intoxication and hemolysis require acylation of an internal lysine residue in a process mediated by CyaC (8, 16, 17). The data in Fig. 4 demonstrate that non-acylated protoxin (CyaC·) does not eliciting K⁺ efflux even at toxin concentrations of 20 μg/ml. Thus, acylation is necessary for all three activities.

**Calcium Requirement**—The amino acid sequence of AC toxin includes approximately 41 copies of a nonameric, glycine, and aspartic acid-containing motif that is involved in calcium binding in other bacterial proteins (6, 14, 15, 39). The functional importance of this domain is illustrated by the observation that extracellular calcium is necessary for intoxication and hemolysis (34, 35, 40–43). Fig. 4 demonstrates that K⁺ efflux is similarly calcium-dependent. Furthermore, Rose et al. (43) and Rogel et al. (10) have observed that toxin prepared in the presence of calcium, but assayed in the presence of the calcium chelator EGTA, is able to induce hemolysis but not intoxication. Rose et al. (43) suggested that AC toxin contains approximately 45 low affinity binding sites for calcium and another 3–5 high affinity sites (43). Calcium bound to these few high affinity sites has been postulated to be responsible for the hemolytic activity of AC toxin when assayed in the presence of calcium chelators. As shown in Fig. 4, when AC toxin is exposed to calcium before addition to sheep RBC in the presence of EDTA, K⁺ efflux was 71% that induced by AC toxin assayed with sheep erythrocytes in the presence of calcium. In contrast, AC toxin exposed to calcium but incubated with RBC in EDTA, increased intracellular cAMP <1% that observed in RBC assayed in the presence of calcium (data not shown). These latter observations are comparable with those reported previously (10, 43) and suggest that calcium occupancy of both high and low affinity sites is necessary for intoxication, whereas occupancy of the high affinity sites alone is sufficient for both K⁺ release and hemolysis.

**Calmodulin Effect**—Differences in calcium requirement notwithstanding, similarities in time course and concentration dependence of intoxication and K⁺ efflux raised the question of how these two activities are related. It has been shown previously that exposure of AC toxin to calmodulin prior to its addition to erythrocytes markedly inhibits intoxication (10, 44, 45) but does not influence hemolysis (10). The effects of calmodulin on the ability of AC toxin to produce K⁺ efflux and intoxication are shown in Fig. 5. Extracellular calmodulin did inhibit intoxication by >90%, consistent with previous data (10, 44, 45). In contrast, K⁺ efflux was not affected by the presence of calmodulin (Fig. 5). This observation not only dissociates intoxication from K⁺ efflux but suggests that intox-
Fig. 5. Calmodulin inhibits the intoxication of sheep erythrocytes by AC toxin, without affecting its ability to increase K⁺ efflux. AC toxin (10 μg/ml) was incubated with calmodulin (CaM, 6 μM) for 20 min at room temperature. Sheep erythrocytes were added and incubated for 1 h at 37 °C. Cyclic AMP accumulation and K⁺ efflux were measured as described under "Experimental Procedures." Each point is the mean of duplicate determinations. This graph is representative of two separate experiments.

Fig. 6. Intoxication, but not K⁺ efflux, is markedly reduced at 0–2 °C. Sheep erythrocytes at 0–2 or 37 °C were incubated with AC toxin (10 μg/ml) for the indicated times. Cyclic AMP accumulation and K⁺ efflux were measured as described under "Experimental Procedures." Each point is the mean of duplicate determinations. This graph is representative of three separate experiments. ●, intoxication, 37 °C; ○, K⁺ efflux, 37 °C; □, K⁺ efflux, 0 °C. The temperature dependence of intoxication and K⁺ efflux—Translocation of the catalytic domain across the cell membrane is highly temperature-dependent, occurring only above 20 °C (47). The temperature dependences of K⁺ efflux and intoxication at 0–2 and 37 °C are illustrated in Fig. 6. Incubation of target erythrocytes at 0–2 °C has only a modest effect on the initial rate of K⁺ efflux and results in no cumulative difference from incubation at 37 °C by 1.5 h. On the other hand, delivery of the catalytic domain, as measured by accumulation of intracellular cAMP, is markedly impaired at the reduced temperature (Fig. 6 and Table I). The reduction in cAMP accumulation at 0–2 °C far exceeds the expected temperature-dependent decrease in enzymatic activity (Table I). As shown in Table I, in vitro enzymatic activity at 0–2 °C is 6.4–6.6% that at 37 °C, whereas intracellular cAMP accumulation ranges from only 0.14 to 0.48% that observed at 37 °C over 30–90 min. Thus, reduced temperature impairs delivery of the catalytic domain to the cell’s interior, thereby inhibiting intoxication, with only a small effect on K⁺ efflux.

The temperature dependence of delivery of the catalytic domain was confirmed by additional studies illustrated in Table II. These experiments were based on the concept that if the catalytic domain of AC toxin has not entered the cytoplasm at 0–2 °C, it will remain susceptible to degradation by trypsin added to the extracellular medium. On the other hand, if the catalytic domain has been delivered to the cytoplasm it will be protected from trypsin digestion (18, 42, 47). Erythrocytes exposed to AC toxin at 37 °C for 30 min accumulate 464 nmol of cAMP/10¹⁰ cells, as compared with 1 nmol of cAMP/10¹⁰ cells at 0–2 °C. Cells exposed to AC toxin at 0–2 °C for 30 min, then treated with trypsin and allowed an additional incubation at 37 °C, accumulated 6.5 nmol of cAMP/10¹⁰ RBC. This value represents just 1.2% of the intracellular cAMP produced in cells incubated at 37 °C for the initial 30 min, treated with trypsin, and incubated an additional 30 min at 37 °C (544 nmol cAMP/10¹⁰ RBC). This indicates that at least 98.8% of the AC toxin had remained on the surface of cells held at 0–2 °C and was removed by trypsin treatment of those cells. These data suggest that the catalytic domain is not inserted in the membrane of the target cell at 0–2 °C, a condition under which K⁺ efflux is occurring (Fig. 6), and provide further evidence for K⁺ efflux under conditions that do not support intoxication. From the data presented thus far we conclude the following: 1) the ability of wild type toxin to elicit K⁺ efflux is independent of its ability to increase intracellular cAMP levels; 2) K⁺ efflux is not mediated by a defect in the cell membrane resulting from delivery of the catalytic domain, and; 3) an endogenous, cAMP-activated channel is not responsible for K⁺ efflux.

Temperature Dependences of Intoxication and K⁺ Efflux—To explore further the relationship between K⁺ efflux and release of hemoglobin, we investigated the temperature dependence of hemolysis as compared with K⁺ release. As shown in Fig. 7, hemolysis was markedly delayed at 0–2 °C as compared with 37 °C. As noted earlier, K⁺ efflux was only modestly affected by a reduction in temperature, indicating that K⁺ efflux can be dissociated from hemolysis by a change in temperature. This effect of temperature might be expected if K⁺ efflux was the result of insertion of a toxin monomer, whereas hemoglobin release required oligomerization of toxin molecules resulting from lateral diffusion within the plasma membrane. This interpretation is consistent with the stoichiometric data showing...
different concentration dependences for the two activities. The data suggest that additional steps, including oligomerization, are required for progression from the initial event responsible for $K_1^{(e)}$ efflux to hemoglobin release.

**Effects on Human Erythrocytes**—For reasons that are unknown, human erythrocytes are resistant to hemolysis by AC toxin but can become intoxicated, albeit at a comparatively reduced level (10). The data in Fig. 8 show that treatment of human RBC with AC toxin causes cAMP accumulation to levels approximately 30% those achieved in sheep RBC. In marked contrast, no $K_1^{(e)}$ efflux occurred in human RBC treated with AC toxin for 1 h at 37 °C, even at a concentration of toxin that was 3-fold greater than that typically used for study of $K_1^{(e)}$ efflux (Fig. 8). This observation further demonstrates that $K_1^{(e)}$ efflux and intoxication are independent events, each able to occur in the absence of the other. Furthermore, these data suggest that the transmembrane pathway by which $K_1^{(e)}$ is released is separate from the structure required for intoxication but may be related to, or a precursor of, that which is ultimately responsible for hemolysis.

**DISCUSSION**

Although efflux of potassium from target cells has been described previously as a marker of pore formation in cell membranes by a variety of toxins (25–32), its demonstration here in response to AC toxin has special significance in the investigation of the complex sequence of events by which this toxin acts. In the past, the delayed onset and variability of the hemolytic response precluded its direct comparison with intoxication, which is rapid and robust, suggesting that different mechanisms may be responsible for these two activities. We show here that $K_1^{(e)}$ efflux occurs at a rate similar to intoxication and at toxin concentrations similar to those necessary to increase intracellular cAMP levels. Intoxication and hemolysis are both acylation- and calcium-dependent (8, 16, 34, 35, 40–43); $K_1^{(e)}$ efflux is similarly dependent on both the acylation and calcium

### Table I

| Incubation time (min) | In vitro Enzymatic activity$^a$ | Intoxication of sheep RBC$^b$ |
|-----------------------|-------------------------------|-------------------------------|
|                       | 0–2 °C | 37 °C | 0–2 °C | 37 °C |
| 10                    | 1.17 ± 0.03 (6.6%)$^c$ | 17.7 ± 0.45 | —d | — |
| 20                    | 2.19 ± 0.04 (6.4%) | 34.3 ± 1.09 | — | — |
| 30                    | 3.03 ± 0.12 (6.4%) | 47.6 ± 2.00 | 0.49 ± 0.02 (0.14%) | 338 ± 4.88 |
| 60                    | — | — | 0.94 ± 0.15 (0.27%) | 352 ± 33.5 |
| 90                    | — | — | 1.47 ± 0.06 (0.48%) | 308 ± 5.09 |

$^a$ Enzymatic activity expressed as micromoles of cAMP/μg of AC toxin.

$^b$ Intracellular cAMP expressed as nanomoles of cAMP/10$^{10}$ RBC, AC toxin concentration, 10 μg/ml.

$^c$ Represents percent of activity at 37 °C.

$^d$ —, not determined.

### Table II

The catalytic domain of AC toxin is susceptible to trypsin treatment when incubated with sheep erythrocytes at 0–2 °C

| Intoxication | 0–2 °C | 37 °C |
|--------------|-------|-------|
| AC toxin$^a$ (30 min incubation only at temperature indicated) | 1.05 ± 0.07 (0.2%)$^b$ | 464 ± 29.8 |
| AC toxin (30 min incubation at temperature indicated, then trypsin at 0 °C for 20 min, followed by, trypsin inhibitor, at 37 °C, 30 min) | 6.56 ± 0.47 (1.2%) | 544 ± 31.9 |
| AC toxin (30 min incubation at temperature indicated, then trypsin inhibitor at 0 °C for 20 min, followed by trypsin, at 37 °C, 30 min) | 363 ± 15.7 (70.2%) | 517 ± 6.8 |

$^a$ AC toxin at 10 μg/ml.

$^b$ Represents percent of activity at 37 °C.

$^c$ The background cAMP concentration was 0.007 nmol of cAMP/10$^{10}$ RBC.
concentration. AC toxin produced in *B. pertussis* is acylated exclusively at lysine 983, whereas recombinant toxin produced in *E. coli* is acylated at lysine 860 and lysine 983. This double acylation of recombinant toxin has been shown to result in reduced hemolytic potency compared with toxin produced in *B. pertussis* (16). We found that the same is also true for K⁺ efflux, namely AC toxin produced in *B. pertussis* is more potent than the recombinant toxin (data not shown). Whereas the previous report suggested that the additional acylation at lysine 860 might impair oligomerization (16), the power dependence derived from both the time course and the concentration response reveals that monomers are likely responsible for K⁺ efflux and intoxication. Since the stoichiometry strongly suggests hemolysis being elicited by an oligomer, the mechanistic basis for the difference between native and recombinant toxin remains to be determined.

To confirm that K⁺ efflux, thought to be a precursor of hemolysis, is not somehow dependent on intoxication, we used several methods to determine that K⁺ efflux and intoxication are separate events. Both K⁺ efflux and hemolysis can occur when AC toxin is exposed to calcium, then added to erythrocytes in the presence of a molar excess of EDTA. There is, however, no intoxication under these conditions, indicating the requirement for extracellular calcium at the time of toxin interaction with the erythrocyte. This suggests that while the calcium requirements for K⁺ efflux and hemolysis are similar, that for intoxication is different.

Inhibition of intoxication by exogenous calmodulin is a peculiar phenomenon because it is target cell-specific (44, 45, 48). In several cell types, addition of calmodulin to AC toxin prior to addition to cells has little or no effect on intoxication, whereas in others an inhibitory effect is striking. The working hypothesis is that calmodulin bound to the calmodulin-binding site in the catalytic domain can interfere with its delivery to the cell interior. Why this would be cell-specific remains unknown, although membrane composition and transmembrane potential may be involved (21, 24). Nevertheless, previous data showed that exposure of AC toxin to calmodulin prior to its addition to sheep RBC inhibited intoxication, whereas it had no effect on hemolysis (10). This observation served as a way to determine if K⁺ efflux was associated with intoxication or hemolysis. In fact, we observed that K⁺ efflux from sheep RBC is not affected by prior addition of calmodulin to AC toxin, indicating again that K⁺ efflux is dissociable from intoxication.

Previous studies showed that toxin insertion, measured by the presence of AC toxin tightly bound to sheep erythrocytes, occurs at temperatures between 4 and 36 °C, but that translocation of the catalytic domain across the red cell membrane required temperatures above 20 °C (47). Our results confirm those reported earlier that delivery of the catalytic domain does not occur at 0–2 °C. Surprisingly, we found that K⁺ efflux was only slightly affected at the reduced temperature when compared with that at 37 °C. This observation suggests that K⁺ efflux may represent the initial interaction of a part of the toxin molecule with the cell membrane and establishes that K⁺ efflux is not dependent on the delivery of the catalytic domain and/or the resultant cAMP accumulation. In addition, a mutant AC toxin containing a single amino acid substitution, which inhibits its enzymatic activity at least 1000-fold, elicited an efflux in K⁺ similar to that of wild type toxin.

Whereas these observations support the suggestion that intoxication and K⁺ efflux are fully dissociable, they do link K⁺ efflux and hemolysis. Although the concentration dependence of K⁺ efflux suggests that a monomer is responsible for efflux, data presented here and elsewhere (21) indicate that an oligomer is necessary for hemolysis. In addition, temperature had only a modest effect on K⁺ efflux, although its effect on hemolysis was dramatic. This suggests that the insertion of monomeric AC toxin allows K⁺ release, which is followed by a time-, temperature-, and concentration-dependent oligomerization leading to the formation of a larger transmembrane pore required for hemolysis. A diagrammatic representation of these data is presented in Fig. 9.

Human erythrocytes are resistant to hemolysis by AC toxin even though the toxin does bind and they do become intoxicated (10). In the context of evaluating K⁺ efflux, we have confirmed these earlier results, and we demonstrate that AC toxin does not elicit K⁺ efflux from human RBC. In sheep erythrocytes, we have shown by several different approaches that we could inhibit the intoxication by AC toxin without affecting its capability to elicit K⁺ efflux. The ability of AC toxin to intoxicate human red blood cells without causing an increase in K⁺ efflux shows that the converse is also true, namely that the delivery of the catalytic domain is not dependent upon the toxin-cell interaction.
interaction by which K⁺ efflux occurs. Although it is possible that the lack of K⁺ efflux from human red blood cells is due to a difference in membrane composition from sheep erythrocytes, these data establish that K⁺ efflux and intoxication can be mutually exclusive events. Given these observations, one must consider that concurrent intoxication and K⁺ efflux might even reflect a mixed population of AC toxin molecules exhibiting one or the other activity.

Taken together these data strongly support the concept that AC toxin binds to the erythrocyte membrane as a monomer. This interaction with the membrane can result in K⁺ efflux and/or the insertion of the catalytic domain resulting in increases in intracellular cAMP. These two pathways are distinct and separable, each able to occur in the absence of the other. Insertion of the monomeric AC toxin allowing K⁺ efflux is a precursor of toxin oligomerization which is necessary for hemolysis. Because of its relative temperature insensitivity, K⁺ efflux may result from partial insertion of the toxin molecule in the membrane. This step may be followed by a temperature-sensitive, more complete insertion of AC toxin that results in delivery of the catalytic domain to the interior of the cell. On the other hand, K⁺ efflux and intoxication could be the result of a mixed population of AC toxin molecules, each able to achieve only a single function. Nevertheless, the monomers involved in K⁺ efflux then coalesce to form a larger, oligomeric pore responsible for hemolysis. These observations provide a new perspective on the functions of this novel toxin, and studies are underway to characterize further its mechanisms of action.

Acknowledgments—We thank Peter Sebo for the constructs used for expression of AC toxin; Dede Haverstock for assistance with the Jurkat cells; Dr. Robert Carey and Nancy Howell for the use and assistance with their flame photometer; and Starr Palmore for help with the manuscript.

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