INFECTION AND IMMUNITY, Sept. 1990, p. 3109–3115
0019-9567/90/093109-07$02.00/0

Vol. 58, No. 9

Studies of *Clostridium perfringens* Enterotoxin Action at Different Temperatures Demonstrate a Correlation between Complex Formation and Cytotoxicity

BRUCE A. McCLANE* AND ANDREW P. WNEK

Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Received 9 February 1990/Accepted 27 April 1990

The cytotoxicity of *Clostridium perfringens* enterotoxin (CPE) was completely blocked in Vero cells continuously CPE treated at 4°C. [^{125}I]CPE-specific binding to either Vero cells or isolated rabbit intestinal brush border membranes (BBMs) was lower at 4°C than at 24 or 37°C, but reduced enterotoxin binding could not totally explain the loss of cytotoxicity at low temperature. Insertion of enterotoxin into Vero cell membranes or BBMs was temperature independent. However, CPE complex formation (A. P. Wnek and B. A. McClane, Infect. Immun. 57:574–581, 1989) in BBMs and Vero cells was blocked at 4°C. When Vero cells were CPE treated at 4°C, washed to remove unbound toxin, and then shifted to 37°C, complex formation and cytotoxicity were rapidly detected. When CPE binding and complex formation were permitted for 2 min at 37°C, and the Vero cells were then shifted to 4°C, cytotoxicity was detectable at 4°C. These results are consistent with complex formation, rather than complex activity, being the temperature-sensitive step in CPE action which is blocked at 4°C. These studies demonstrate a strong correlation between complex formation and cytotoxicity and are consistent with complex involvement in CPE cytotoxicity. These studies also strongly suggest that CPE insertion precedes both complex formation and induction of small-molecule permeability changes.

Clostridium perfringens enterotoxin (CPE) is a single polypeptide of 35,000 M_r which has a unique mode of action and amino acid sequence (8). This bacterial enterotoxin produces the symptoms associated with *C. perfringens* food poisoning and also may be involved in other gastrointestinal illnesses (8).

Several early (i.e., detectable within 5 min of toxin treatment) events have been described following CPE treatment of mammalian cells. CPE binds to specific proteinaceous receptors on mammalian cells (11, 22). Recent studies (23) indicate that specific binding of enterotoxin to either isolated intestinal brush border membranes (BBMs) or cultured Vero (African green monkey kidney) cells results in rapid formation of a 160,000 M_r membrane complex. This complex apparently contains one CPE molecule, one 50,000 M_r eucaryotic membrane protein, and one 70,000 M_r eucaryotic membrane protein. Coincident with formation of complex at 37°C, [^{125}I]CPE becomes resistant to protease-induced release from either BBMs or Vero cells (11, 12, 22). This phenomenon may represent the insertion of enterotoxin into plasma membranes (11, 12). CPE produces rapid and extensive membrane permeability alterations for small molecules (4, 6, 7, 10). The precise mechanism by which enterotoxin causes these rapid small-molecule permeability alterations remains unknown. No enzymatic activity has yet been detected for this toxin (10, 11), and CPE appears to remain localized on the plasma membrane (15, 20). These primary permeability changes appear to be responsible for CPE cytotoxicity since they then cause secondary enterotoxin effects, including inhibition of macromolecular synthesis and morphologic damage (5).

To fully understand the unique action of this toxin, it is essential to determine which of these recognized early events are required for CPE action. To date, the only early events shown to be involved in CPE action are specific binding of enterotoxin to mammalian receptors (13, 24) and production of small-molecule permeability alterations (5). Currently, there is no experimental support indicating that either insertion or CPE complex is involved in enterotoxin action. Further, the precise order of onset of these early events is unclear because they occur so rapidly at 37°C.

Low-temperature studies are often useful for dissecting the contribution of individual events to the overall cytotoxic process of a bacterial toxin (16). By systematically characterizing the relationship between temperature and each known early event linked to CPE treatment, we now are able in this report to establish a strong correlation between enterotoxin complex formation and cytotoxicity. These results are consistent with the involvement of complex formation in CPE cytotoxicity. Further, our studies also strongly suggest that CPE insertion precedes both complex formation in eucaryotic membranes and production of small-molecule permeability changes.

MATERIALS AND METHODS

Materials. Na^{125}I (17 Ci/mg) and [^{3}H]uridine (35 to 50 Ci/mmol) were purchased from ICN Pharmaceuticals Inc. ^{86}RbCl (1.2 mCi/mg) was purchased from DuPont-NEN Research Products, trypsin was purchased from Difco Laboratories, and pronase was obtained from Calbiochem-Behring.

Enterotoxin. CPE was prepared and purified, and its biologic activity was assayed as described previously (14). Purified enterotoxin was radioiodinated as described previously (11), except that 24 nM potassium iodide was included during the radioiodination with Enzymobeads (Bio-Rad Laboratories). We have observed (A. P. Wnek and B. A.

* Corresponding author.

McClane, submitted for publication) that addition of potassium iodide during radioiodination yields more reproducible and desirable [^{125}I]CPE-binding characteristics (i.e., high specific binding with low nonspecific binding) while retaining biologic and serologic activity. [^{125}I]CPE used in these studies had a specific radioactivity of approximately 3 mCi/mg.

Cells. Vero cells were maintained as monolayer cultures in either roller bottles or 150-cm^2 flasks (Corning Glass Works) as described previously (7).

Binding studies. (i) BBMs. BBMs from the small intestine of female New Zealand White rabbits were prepared as described previously (17). Specific binding of [^{125}I]CPE (0.5 μg) to BBMs (100 μg) at 4, 24, or 37°C was determined as described previously (11). Specific binding was calculated by subtracting the counts associated with BBMs cotreated with a 50-fold or greater excess of unlabeled enterotoxin (nonspecific binding) from the counts associated with membranes treated only with [^{125}I]CPE (total binding). Specific binding in BBMs was approximately 80% of total binding.

(ii) Vero cells. Tissue culture dishes (diameter, 60 mm; Falcon) were inoculated with 10^6 cells per dish and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. The resultant monolayers were washed twice with 3 ml of Hanks balanced salt solution (HBSS) at the same temperature as subsequently used for binding determination. [^{125}I]CPE (0.5 μg) in 1.5 ml HBSS at 4, 24, or 37°C (as used for washing) was then added in the presence or absence of at least a 50-fold excess of unlabeled enterotoxin. All subsequent steps were performed at the binding solution temperature. Cells were gently shaken for the specified incubation times, and then the binding solution was removed and microcentrifuged to pellet nonadherent cells. HBSS (1.5 ml) was then added to each culture dish, and adherent cells were removed from the monolayer by scraping with a rubber policeman. This suspension was then added to the corresponding microfuge tube containing the pelleted nonadherent cells from that dish, and the microfuge tubes were again microcentrifuged for 3 min to pellet the cells. HBSS (1 ml) was added to each dish to collect any remaining cells, and this material was also centrifuged in the tube containing the previously collected cells from that dish. Pellets were counted in a Packard gamma counter, and specific binding was determined as for BBMs. After 20 min, specific binding represented approximately 60% of total binding at 37 or 24°C and 30% of total binding at 4°C.

Measurement of CPE insertion into BBMs or Vero cells. All steps in the insertion study were carried out at one temperature (4, 24, or 37°C) for a particular sample. Insertion experiments were performed as described previously (11), with slight modification. [^{125}I]CPE (0.5 μg) in the presence or absence of a 50-fold excess of native CPE was bound to BBMs (100 μg) or a plate (diameter, 60 mm) of Vero cells (10^6 cells) at the desired temperature for the specified time. Samples were washed to remove unbound [^{125}I]CPE, and proteases were added. Samples treated with proteases at 24 or 37°C received either pronase (50 μg) or trypsin (100 μg) for 5 min. Samples at 4°C were treated with 250 μg of pronase or 500 μg of trypsin for 5 min. This increased concentration of proteases was necessary to obtain protease activity for 4°C samples that was comparable to activity for 24 and 37°C samples. This was determined by using Azocoll protease activity assays (21) at 4, 24, and 37°C and confirmed by determining the amount of proteases needed at different temperatures to inhibit enterotoxin binding by 95% when BBMs were pretreated with protease prior to addition of [^{125}I]CPE. After incubation with proteases, BBMs and Vero cells were washed, harvested, and counted for radioactivity as described for the binding experiments.

Identification of BBM and Vero cell [^{125}I]CPE complexes by SDS-PAGE without sample boiling. [^{125}I]CPE binding mixtures containing either BBMs or Vero cells were prepared as described previously (23), except that binding mixtures were incubated at 4, 24, or 37°C. Binding mixtures were microcentrifuged for 3 min at the incubation temperature, and 1% sodium dodecyl sulfate (SDS) in PBS at 4°C was added to the pellets for 15 min to extract membrane proteins for electrophoresis. The SDS extractions were microcentrifuged to remove insoluble debris. Samples (30 μl) of the supernatants were added to an equal volume of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 0.001% bromophenol blue) and were analyzed by SDS-PAGE without sample boiling by using 6% acrylamide gels as described previously (23). After electrophoresis, gels were autoradiographed with intensifying screens (Cronex Hi-Plus) at −70°C for 2 to 4 days for BBMs and 7 to 14 days for Vero cells. Autoradiographs were scanned on a videodensitometer (Bio-Rad model 620) with the 1-D-Analyst program (Bio-Rad).

Measurement of CPE-induced permeability changes. Vero cells were seeded into 16-mm wells in tissue culture dishes (Costar) at a density of 5×10^4 cells per well. After 3 days, fresh medium was added.

(i) $^{86}Rb^+$ efflux experiments. Confluent 6-day-old monolayers were washed with medium 199 without serum and incubated for 2 h at 37°C with 2 ml of medium 199 containing 4 μCi of ^{86}RbCl (7). The cultures were then washed twice with HBSS at 4, 24, or 37°C. CPE (either 5 or 50 μg per well) in HBSS at 4, 24, or 37°C (as used previously for washing) was then added to each culture, and the cells were incubated for the desired time at the same temperature used for washing and toxin treatment. The culture supernatant was then gently removed, Vortex mixed, and centrifuged for 3 min at 4°C in an Eppendorf microcentrifuge. Radioactivity in the supernatant was measured by counting 1.0-ml samples in a Packard gamma counter. Released radioactivity was calculated as described by Thelestam and Möllby (19): percent maximal release = [(toxin-induced release − spontaneous release)/(maximal release − spontaneous release)] × 100.

Spontaneous release (i.e., background label release without toxin) of $^{86}Rb^+$ label after 22.5 min at 37°C was approximately 25% of maximal release. As described previously (11), the maximal release of cytoplasmic label represents total cytoplasmic radioactivity at the end of radiolabeling (i.e., before CPE addition) and was determined after cell membrane rupture by the addition of 1 ml of 1 M citric acid and 1 ml of a 0.5% saponin buffer to each well. Maximal release was approximately 0.5×10^4 to 1×10^4 cpm per culture for $^{86}Rb^+$ label.

(ii) Temperature shift experiments. (a) Shift of Vero cell cultures from 4 to 37°C. Vero cells were labeled with ^{86}RbCl as described above. Following radiolabeling, cultures were washed twice with 2 ml of HBSS at 4°C, and CPE (50 μg per well) in 2 ml of HBSS at 4°C was added for 15 min. Cultures were washed twice with 2 ml of HBSS at 4°C to remove unbound enterotoxin. Cultures then received a final wash of 2 ml of HBSS at either 4 or 37°C. The final wash buffer was removed, 2 ml of HBSS at either 4 or 37°C (as used for the final wash) was added, and cultures were incubated at the temperature of the final wash for 15 min. Culture supernatant then was removed and processed for released radioactivity as described above.

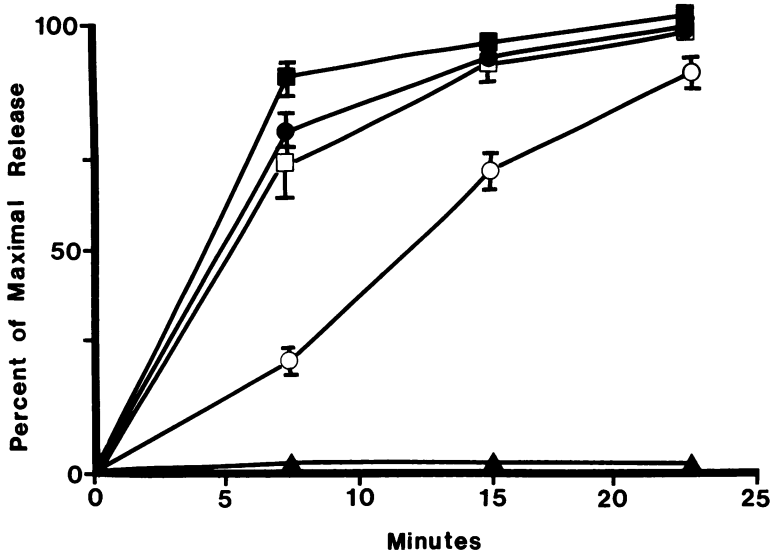

FIG. 1. Effects of temperature on CPE-induced ^{86}Rb release from Vero cells. ^{86}Rb-labeled Vero cells were treated continuously with 25 (■, ●, and ▲) or 2.5 (○ and □) μg of enterotoxin per ml at 4 (▲), 24 (● and ○), or 37°C (■ and □) for the indicated times. Culture supernatants were then removed and processed for radioactivity determination as described in Materials and Methods. Error bars represent standard errors of the mean. Points without error bars had standard errors too small to depict. Results shown are from duplicate samples in three experiments.

(b) Shift of Vero cell cultures from 37 to 4°C. Vero cells were labeled with $^{86}RbCl$ as described above. Following radiolabeling, cultures were washed twice with 2 ml of HBSS at 37°C, and CPE (1.0 μg per well) in 2 ml of HBSS at 37°C was added for 2 min. Cultures were rapidly washed twice with HBSS at 37°C to remove unbound enterotoxin and then received a final wash with 2 ml of HBSS at either 4 or 37°C. After the final wash buffer was removed, 2 ml of HBSS at either 4 or 37°C (as used for the final wash) was added for 15 min. Culture supernatant then was removed and processed for released radioactivity as described above.

Determination of CPE-induced morphologic damage. Vero cells were seeded into 35-mm tissue culture dishes (Corning Glass Works) at a density of 2.5×10^5 cells per well. After an overnight incubation at 37°C in a humidified atmosphere containing 5% CO_2, the culture medium was removed. Cultures were washed twice with washing buffer (HBSS at 4, 24, or 37°C). CPE (either 5 or 50 μg per well) in 2 ml of HBSS at either 4, 24, or 37°C (corresponding to washing buffer temperature) was added for up to 60 min.

Viability determination. Viability determination was performed as described previously (9) using erythrosine B viability dye.

RESULTS

The strategy used in these studies was first to determine whether overall CPE cytotoxicity is inhibited by continuous incubation at 4°C and, second, if temperature sensitivity was observed, to identify which specific enterotoxin event(s) is temperature sensitive. If successful, this approach would establish an experimental correlation between the temperature-sensitive event and cytotoxicity. To help ensure the validity of conclusions from these studies, both BBMs (the physiologic site of enterotoxin action) and Vero cells (useful in vitro models for CPE cytotoxicity) were used wherever possible in these studies.

CPE cytotoxicity does not occur at low temperature. CPE is considered to be a cytotoxic bacterial toxin (8) on the basis of three commonly used criteria for cytotoxicity. Specifically, enterotoxin causes (i) plasma membrane permeability alterations, (ii) permeability to viability dyes, and (iii) morphologic damage in mammalian cells (5, 9, 11). To examine whether membrane permeability changes caused by toxin are temperature sensitive, CPE-induced ^{86}Rb release was studied at several temperatures. As reported previously (7, 11), ^{86}Rb release at 37°C was very rapid and was essentially complete within 10 min under the specified experimental conditions (Fig. 1). At 24°C, this effect occurred to a significant extent but was slowed at lower toxin doses. The most striking result shown in Fig. 1 is that no ^{86}Rb release was detectable when cells were treated continuously with CPE at 4°C, even when high toxin doses were given for long incubation times. ^3H-nucleotide release (11) was also temperature sensitive and completely blocked during continuous toxin treatment at 4°C (data not shown). This indicates that both early (e.g., ^{86}Rb release) and late (e.g., ^3H-nucleotide release) CPE-induced membrane permeability alterations are similarly affected by temperature.

Two additional assays for CPE cytotoxicity were also performed (data not shown). CPE-induced morphologic damage to Vero cells is also temperature sensitive. When Vero cells were treated with moderate doses (5 μg per culture) of enterotoxin, blebbing and cell rounding typical of CPE treatment (9) occurred rapidly at 37°C but developed more slowly at 24°C. Morphologic damage was absent at 4°C, even for cultures continuously treated at 4°C for 60 min with high (50 μg per culture) toxin concentrations. Viability (i.e., the ability to exclude erythrosine B viability dye) was also rapidly lost in toxin-treated cells at 37°C and was more slowly lost at 24°C. However, cells continuously treated with CPE at 4°C remained viable for at least 1 h. Therefore, four different cytotoxicity assays indicate that CPE cytotoxicity is temperature sensitive and is blocked completely during continuous 4°C incubations.

CPE binding is reduced at low temperature. Since cytotoxicity is blocked during continuous CPE treatment at 4°C,

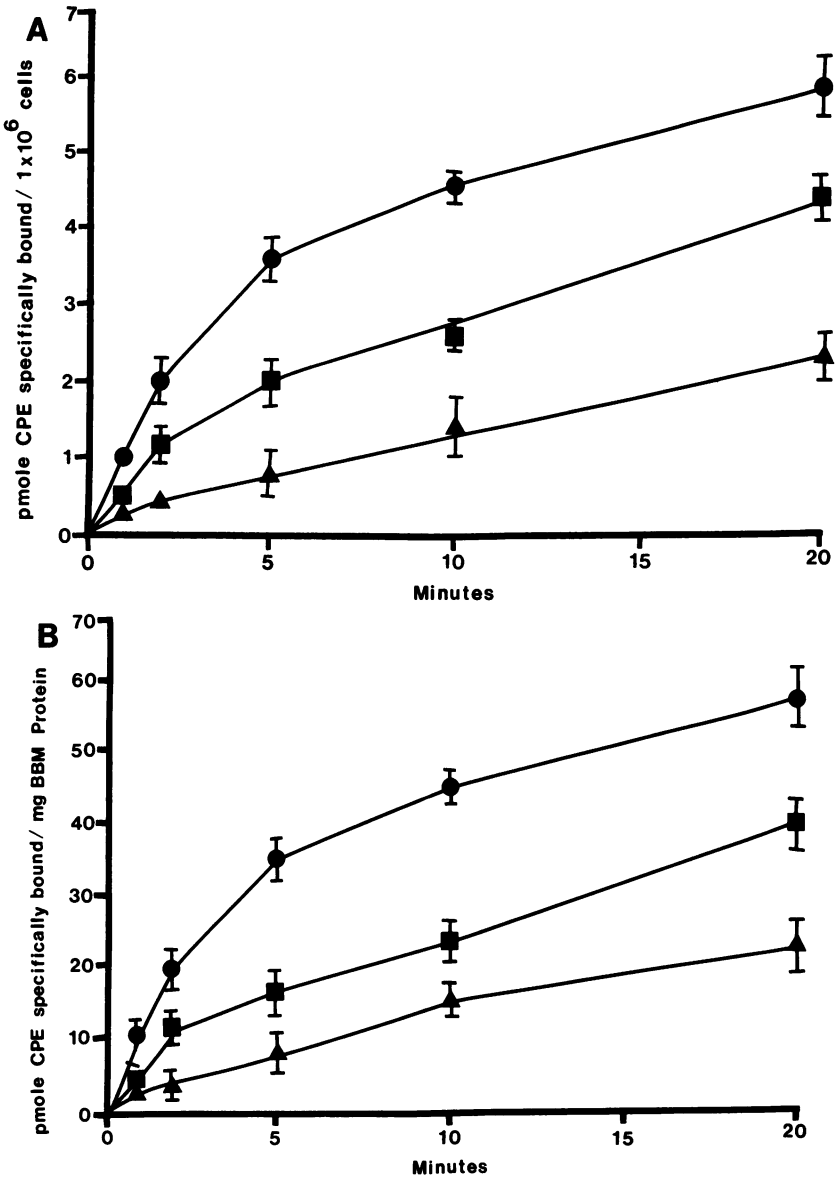

FIG. 2. Effects of temperature on [^{125}I]CPE-specific binding to (A) Vero cells and (B) intestinal BBMs. Vero cells or BBMs were incubated with [^{125}I]CPE (0.5 μg) at 4 (▲), 24 (■), or 37°C (●). Specific binding was determined as described in Materials and Methods. Values represent means ± standard errors of the mean. Error bars not shown were too small to depict. Values shown are from duplicate samples from three experiments.

studies were initiated to determine the event(s) responsible for this effect. The essential first step in CPE action is specific binding of enterotoxin to a membrane receptor(s) (13). Therefore, [^{125}I]CPE-specific binding was measured at 4, 24, or 37°C. Specific binding of enterotoxin to either Vero cells (Fig. 2A) or BBMs (Fig. 2B) was temperature dependent and lower at 4°C. However, significant CPE-specific binding was detectable at 4°C. For example, after 15 to 20 min (equivalent to the time required in Fig. 1 for complete ^{86}Rb release at 24 or 37°C), there was about one-third the amount of enterotoxin-specific binding to Vero cells or BBMs at 4°C compared with 37°C.

Reduced CPE binding at low temperature does not fully explain the complete blockage of cytotoxicity at 4°C. A reduction in CPE-specific binding at 4°C could theoretically explain why cytotoxicity is blocked at low temperatures, since there is an excellent positive correlation between enterotoxin-binding levels and cytotoxicity (13). However, while reduced binding at 4°C contributes to the loss of cytotoxicity, another CPE event(s) may also be reduced or completely blocked at 4°C. To address this possibility, temperature shift experiments were performed in which a high concentration (25 μg/ml) of CPE was bound at 4°C, and the treated cultures were then washed free of unbound toxin and subsequently tested for cytotoxicity by measuring ^{86}Rb release after 15 min at either 4 or 37°C. If an additional CPE event besides specific binding is also inhibited or blocked at low temperature, then warming these cultures to 37°C should at least partially restore cytotoxicity.

Consistent with Fig. 1, enterotoxin caused virtually no ^{86}Rb release (i.e., 0.1% ± 0.1% of maximal release) when cultures were both CPE treated and subsequently incubated

at 4°C. However, when CPE was bound to Vero cells at 4°C, and these cultures were then warmed to 37°C, substantial ^{86}Rb release occurred (i.e., 65.2% ± 7.5% of maximal release). This effect could not be due to increased binding of residual unbound CPE after shifting of cultures to 37°C since unbound CPE was removed at 4°C and cultures were extensively washed at 4°C prior to warming. These results are also not explainable by the possibility that CPE binds to one receptor at 4°C which has no biologic activity and, after shifting to 37°C, dissociates to bind to the cytotoxicity-mediating receptor, since no [^{125}I]CPE dissociation is detectable for at least 4 h at 4 or 37°C (data not shown). Therefore, these results strongly suggest that an additional postbinding CPE event(s) is also temperature sensitive.

CPE insertion occurs at all temperatures. Previous studies have shown that at 24°C, specifically bound [^{125}I]CPE becomes progressively resistant to release from Vero cells and BBMs by proteases (11, 12, 22). This phenomenon may represent the insertion of enterotoxin into membranes following binding (12). When the possible effects of temperature on CPE insertion were studied, the previous 24°C insertion results were confirmed with both Vero cells and BBMs. In both models, toxin insertion at 24°C was progressive and rapid (i.e., insertion was essentially complete within 2 min of CPE treatment). When insertion studies were performed at 37 or 4°C with Vero cells or BBMs, significant and rapid enterotoxin insertion also occurred. At 37°C, insertion was complete within 1 min of CPE treatment, while at 4°C this effect was complete within 2 to 5 min of toxin treatment. These results indicate that insertion of CPE, like several other bacterial toxins (2, 25), is largely temperature independent since insertion is only slightly slowed at 4°C.

CPE complex formation is blocked at 4°C. Recent studies (23) indicate that soon after binding, CPE forms an identical large (160,000 M_r) membrane complex at 24°C in both Vero cells and BBMs. The possible effects of low temperature on complex formation were studied in BBMs (Fig. 3) and Vero cells (Fig. 4). The kinetics of complex formation was highly temperature sensitive as determined by densitometry of gel autoradiographs. After 20 min of toxin treatment of either BBMs (Fig. 3) or Vero cells (data not shown), approximately 70% of specifically bound CPE was associated with complex at 37°C, compared with 60% of specifically bound enterotoxin in complex at 24°C. With continuous 4°C incubation, no complex formation was detectable after 20 min in BBMs (Fig. 3) or Vero cells (data not shown). Even after 60 min of continuous toxin treatment at 4°C, less than 5% of specifically bound CPE was associated with complex in either BBMs (Fig. 3) or Vero cells (Fig. 4).

CPE complex formation is rapidly detected after Vero cells and BBMs are shifted from 4 to 37°C. Collectively, the above results suggest that blockage of CPE complex formation may be involved in the loss of cytotoxicity at 4°C. If this is true, then it would be predicted that shifting either Vero cells or BBMs containing enterotoxin bound at 4°C to higher temperatures should cause a significant increase in complex formation since our results show that cytotoxicity is increased by this experimental manipulation.

Results shown in Fig. 4 are totally consistent with this prediction. Even after 60 min of continuous binding (and 5 additional min of incubation after removal of unbound toxin) at 4°C, little or no complex formation was detectable in Vero cells. However, for Vero cells with CPE prebound at 4°C which are then warmed to either 24 or 37°C for as little as 5 min, there was a rapid increase in complex formation. By densitometry, this increase is approximately 3.5-fold at 24°C and 5-fold at 37°C after warming for 5 min. Similar results were observed with BBMs (data not shown).

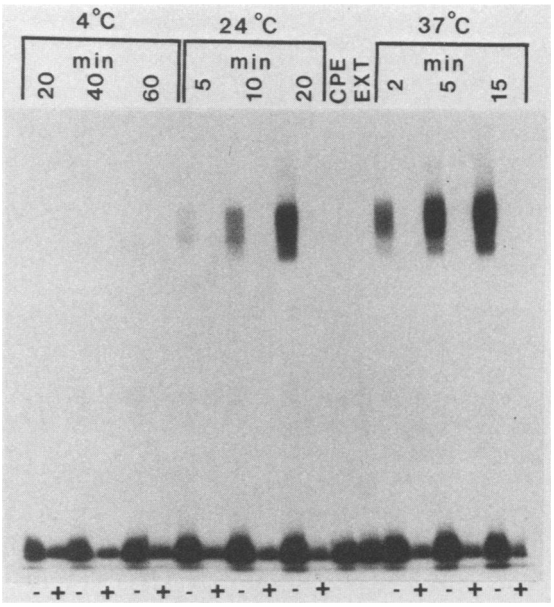

FIG. 3. Effect of temperature on the kinetics of CPE complex formation. [^{125}I]CPE (0.5 μg) was added to BBMs in the presence (+) or absence (−) of a 50-fold excess of unlabeled enterotoxin as described previously (23) for the indicated times at 4, 24, or 37°C. After unbound [^{125}I]CPE was removed, enterotoxin complex was extracted from BBMs with SDS (final concentration, 1%) at 4°C. Samples were analyzed by SDS-PAGE without sample boiling, as described previously (23). After electrophoresis, gels were autoradiographed and scanned with a video densitometer. CPE, [^{125}I]CPE (0.5 μg) in solution (i.e., no membranes) run in this gel system; EXT, sample of [^{125}I]CPE (0.5 μg) added to supernatants from SDS-extracted BBMs (previous studies [23] show that complex does not form under these conditions). Identical temperature kinetics were observed with complex formation in Vero cells (data not shown).

CPE cytotoxicity continues at 4°C when cells are first briefly CPE treated at 37°C. The results presented above demonstrate that when cells are continuously CPE treated at 4°C, both complex formation and cytotoxicity are almost totally inhibited. However, when Vero cells were briefly CPE treated with low toxin doses (0.5 μg/ml) at 37°C prior to washing and shifting to 4°C, significant ^{86}Rb release occurred at 4°C. After 15 minutes, ^{86}Rb release from these temperature-shifted cultures was 20.5% ± 2.2% of maximal release. This result indicates that once ^{86}Rb release starts at 37°C, it cannot be shut off by shifting to 4°C. Collectively, our results demonstrate that there is an additional temperature-sensitive step in CPE action besides specific binding, but this step is not ^{86}Rb release. Complex amounts in samples from the above experiments were analyzed by densitometry of electrophoresis autoradiographs, and it was determined that the amount of complex remained constant after shifting cultures from 37 to 4°C. However, there was an approximately twofold increase in complex in cultures incubated at 37°C after [^{125}I]CPE removal. This suggests that at 37°C there is a slight lag in complex formation after toxin binding. This could explain why after cells were briefly CPE treated at 37°C and then washed there was an approximately twofold increase (44.0% ± 2.1% of maximal release compared with 20.5% ± 2.2% of maximal release) in ^{86}Rb release after 15 min from cells continuously incubated at 37°C compared

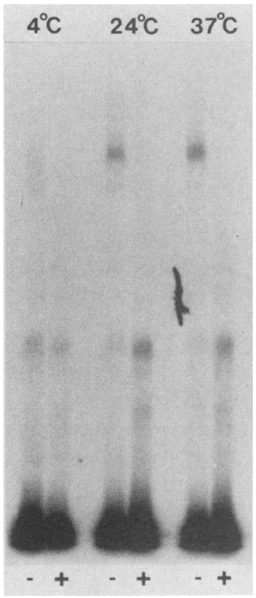

FIG. 4. CPE complex formation occurs rapidly after Vero cells are shifted from 4 to 37°C. [^{125}I]CPE (0.5 μg) was bound to Vero cells at 4°C for 30 min in the presence (+) or absence (−) of a 50-fold excess of unlabeled toxin. After removal of unbound [^{125}I]CPE, cells were treated with HBSS at 4, 24, or 37°C for 5 min, and then enterotoxin complex was analyzed as described in the Fig. 3 legend. Identical results were observed with BBMs (data not shown).

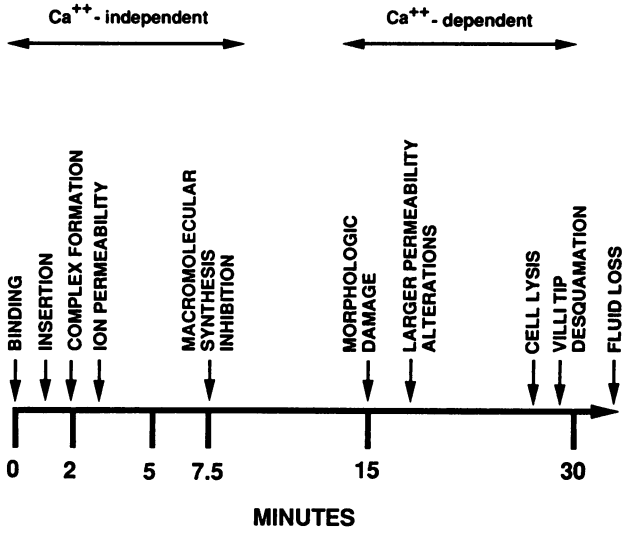

FIG. 5. Revised time line for CPE events incorporating results from this research to revise a similar figure published previously (8).

with cells shifted to 4°C after CPE binding at 37°C. Collectively, these results further support a correlation between the amounts of CPE complex and cytotoxicity and also indicate that complex formation but not complex activity is blocked at 4°C.

DISCUSSION

Recent studies (5–7, 11, 23) have identified several events which occur early in CPE treatment of mammalian cells or isolated membranes. However, the precise order and the importance of each of these early events in enterotoxin cytotoxicity are unclear. Information on the sequence and the role of these events in cytotoxicity is essential to establish an accurate model for CPE action.

This research utilized low-temperature studies to address these unresolved questions. This research provides at least two major findings. First, CPE complex formation is coincident with cytotoxicity. No other CPE event studied could explain the complete blockage of cytotoxicity observed with continuous toxin treatment at 4°C. Unless there are unrecognized enterotoxin events which are also blocked completely at low temperature, the strong correlation between complex formation and cytotoxicity suggests that CPE complex formation is required for cytotoxicity. If this suggestion is accurate, then complex formation is only the third early enterotoxin event implicated in cytotoxicity. Specific binding and small-molecule permeability alterations are the only other CPE effects shown to be required for cytotoxicity by experimental evidence (5, 13, 24).

The second major implication of this study concerns the sequence of early events in CPE bioactivity. Previous CPE studies at 24 and 37°C (11, 23) were unable to resolve the order of insertion, complex formation, and small-molecule permeability alterations since the kinetics of these rapid events are indistinguishable at these temperatures. However, this study clearly indicates that at 4°C insertion occurs but both complex formation and cytotoxicity are blocked. This observation strongly suggests that insertion precedes both complex formation and small-molecule permeability alterations. In a recent review of CPE action (8), we arbitrarily listed complex formation as preceding enterotoxin insertion. Figure 5 shows an updated time line which is revised to incorporate the new conclusions from this study.

This revised sequence suggests several implications and directions for future experiments. First, these data raise questions about what CPE complex represents. The simplest interpretation of complex is that it represents enterotoxin bound to a two-subunit protein receptor containing one 50,000 M_r and one 70,000 M_r polypeptide, with each polypeptide capable of binding CPE (23). However, if insertion of enterotoxin precedes complex formation, as suggested by our data, then complex may represent a more complicated structure. For example, either the 50,000 or 70,000 M_r polypeptide may be the CPE receptor. After enterotoxin binds to this receptor and these molecules insert into membranes, there could be a subsequent binding of a second, nonreceptor polypeptide to receptor-bound CPE to form complex. This hypothesis must be experimentally tested. Second, establishment of a strong correlation between complex formation and cytotoxicity suggests that further studies of complex should help clarify CPE action. For example, it remains to be determined whether complex formation might affect membrane function by causing generalized changes in membrane fluidity or by affecting function of a specific membrane protein such as an ion pump. Lastly, our current studies do not support or disprove a role for insertion in CPE cytotoxicity. Experiments to examine this relationship are necessary and are ongoing in our laboratory.

This study is the first to systematically characterize all known CPE events for temperature sensitivity. However, our results are consistent with several previous studies which have examined the effect of low temperature on only one or two enterotoxin events. Several previous studies support our findings that CPE binding is reduced at 4°C (12, 18, 20), although one study (3) has reported that enterotoxin binding is temperature independent. Matsuda et al. have reported (6) that cells treated with toxin at 4°C do not show

morphologic damage. Horiguchi et al. (3) suggested that CPE-induced permeability alterations for larger molecules are blocked at 4°C. Several previous studies (11, 12, 22) support our conclusion that [^{125}I]CPE does not dissociate at either 4 or 37°C after binding. Matsuda et al. (6) have reported that CPE-induced small-molecule permeability alterations occur at 4°C. However, conclusions from their study are difficult to interpret because of flawed methodology. Their cells were first treated with high doses of enterotoxin for 2 min at 37°C prior to being shifted to 4°C. We repeated their experiment with equivalent high CPE doses and then measured ^{86}Rb release immediately after the 2-min incubation at 37°C (Matsuda et al. measured ion flux changes only at the endpoint of their experiment, i.e., after both their 37 and 4°C incubations). We found almost complete (i.e., >95%) ^{86}Rb release had already occurred in the first 2 min of CPE treatment at 37°C. Therefore, this previous experiment does not address enterotoxin activity at 4°C in a meaningful way.

Lastly, it is of interest to compare CPE with other well-characterized membrane-active bacterial toxins, including streptolysin O (SLO), staphylococcal α-toxin, and *Escherichia coli* α-hemolysin HlyA (for a review of other toxins, see reference 1). CPE produces a significantly smaller membrane lesion than these other toxins (0.5- to 1.0-nm lesions for CPE [6, 10] compared with 1.5- to 3.0-nm lesions for HlyA and α-toxin and ≈35-nm lesions for SLO). Only CPE requires specific binding to a protein receptor for toxicity. The toxic activities of SLO, α-toxin, and HlyA are partially temperature sensitive, but this effect can be overcome by the addition of higher toxin concentrations. Like CPE, both SLO and α-toxin (but not HlyA) form high-molecular-weight complexes in membranes at 37°C, and the formation of the SLO and α-toxin complexes is at least partially temperature sensitive. However, unlike CPE complex, which apparently contains both enterotoxin and eucaryotic proteins, the SLO and α-toxin membrane complexes are thought to contain only oligomerized toxin molecules. These results clearly indicate that significant differences exist between the interaction of membranes and CPE compared with these other toxins and also strongly suggest that enterotoxin action differs from the actions of these other toxins. CPE appears to represent a novel class of membrane-active bacterial toxins.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI 19844-07 from the National Institute of Allergy and Infectious Diseases.

We thank Teresa A. Poloka for typing the manuscript.

LITERATURE CITED

1. **Bhakdi, S., G. Menestrina, F. Hugo, W. Seeger, and J. Tranum-Jensen.** 1988. Pore-forming bacterial cytolysins, p. 71–77. *In* F. J. Fehrenbach et al. (ed.), Bacterial protein toxins. Gustav Fischer Verlag, New York.
2. **Farahbakhsh, Z. T., and B. J. Wisnieski.** 1989. The acid-triggered entry pathway of *Pseudomonas* exotoxin A. Biochemistry **28**:580–583.
3. **Horiguchi, Y., T. Uemura, S. Kozaki, and G. Sakaguchi.** 1985. The relationship between cytotoxic effect and binding to mammalian cultured cells of *Clostridium perfringens* enterotoxin. FEMS Microbiol. Lett. **28**:131–135.
4. **Horiguchi, Y., T. Uemura, S. Kozaki, and G. Sakaguchi.** 1986. Effects of Ca^{+2} and other cations on the action of *Clostridium perfringens* enterotoxin. Biochim. Biophys. Acta **889**:67–71.
5. **Hulkower, K. I., A. P. Wnek, and B. A. McClane.** 1989. Evidence that alterations in small molecule permeability are involved in the *Clostridium perfringens* type A enterotoxin-induced inhibition of macromolecular synthesis in Vero cells. J. Cell. Physiol. **140**:498–504.
6. **Matsuda, M., K. Ozutsumi, H. Iwahashi, and N. Sugimoto.** 1986. Primary action of *Clostridium perfringens* type A enterotoxin on HeLa and Vero cells in the absence of extracellular calcium: rapid and characteristic changes in membrane permeability. Biochem. Biophys. Res. Commun. **141**:704–710.
7. **McClane, B. A.** 1984. Osmotic stabilizers differentially inhibit permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. Biochim. Biophys. Acta **777**:99–106.
8. **McClane, B. A., P. C. Hanna, and A. P. Wnek.** 1988. *Clostridium perfringens* enterotoxin. Microb. Pathog. **4**:317–323.
9. **McClane, B. A., and J. L. McDonel.** 1979. The effects of *Clostridium perfringens* enterotoxin on morphology, viability and macromolecular synthesis in Vero cells. J. Cell. Physiol. **99**:191–200.
10. **McClane, B. A., and J. L. McDonel.** 1980. Characterization of membrane permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. Biochim. Biophys. Acta **600**:974–985.
11. **McClane, B. A., A. P. Wnek, K. I. Hulkower, and P. C. Hanna.** 1988. Divalent cation involvement in the action of *Clostridium perfringens* type A enterotoxin: early events in enterotoxin action are divalent cation independent. J. Biol. Chem. **263**:2423–2435.
12. **McDonel, J. L.** 1980. Binding of *Clostridium perfringens* [^{125}I]enterotoxin to rabbit intestinal cells. Biochemistry **19**: 4801–4807.
13. **McDonel, J. L., and B. A. McClane.** 1979. Binding versus biological activity of *Clostridium perfringens* enterotoxin in Vero cells. Biochem. Biophys. Res. Commun. **87**:497–504.
14. **McDonel, J. L., and B. A. McClane.** 1988. Production, purification and assay of *Clostridium perfringens* enterotoxin. Methods Enzymol. **165**:94–103.
15. **Petrie, H. T., R. A. Schlegel, and J. L. McDonel.** 1982. Intracellular antibody against *Clostridium perfringens* enterotoxin fails to counteract toxin-induced damage. Cell Biol. Int. Rep. **6**:705–711.
16. **Sandvig, K., S. Olsnes, J. E. Brown, O. W. Petersen, and B. van Deurs.** 1989. Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from *Shigella dysenteriae* 1. J. Cell Biol. **108**:1331–1343.
17. **Sigrist, H., P. Ronner, and G. Semenza.** 1975. A hydrophobic form of the small-intestinal sucrase-isomaltase complex. Biochim. Biophys. Acta **406**:433–446.
18. **Sugimoto, N., K. Ozutsumi, and M. Matsuda.** 1985. Morphological alterations and changes in cellular cations induced by *Clostridium perfringens* type A enterotoxin in tissue culture cells. Eur. J. Epidemiol. **1**:264–273.
19. **Thelestam, M., and R. Möllby.** 1975. Sensitive assay for detection of toxin-induced damage to the cytoplasmic membrane of human diploid fibroblasts. Infect. Immun. **12**:225–232.
20. **Tolleshaug, H., R. Skjelkvåle, and T. Berg.** 1982. Quantitation of binding and subcellular distribution of *Clostridium perfringens* enterotoxin in rat liver cells. Infect. Immun. **37**:486–491.
21. **Wnek, A. P., and B. A. McClane.** 1983. Identification of a 50,000 M_r protein from rabbit brush border membranes that binds *Clostridium perfringens* enterotoxin. Biochem. Biophys. Res. Commun. **112**:1099–1105.
22. **Wnek, A. P., and B. A. McClane.** 1986. Comparison of receptors for *Clostridium perfringens* type A and cholera enterotoxins in rabbit intestinal brush border membranes. Microb. Pathog. **1**:89–100.
23. **Wnek, A. P., and B. A. McClane.** 1989. Preliminary evidence that *Clostridium perfringens* type A enterotoxin is present in a 160,000-M_r complex in mammalian membranes. Infect. Immun. **57**:574–581.
24. **Wnek, A. P., R. J. Strouse, and B. A. McClane.** 1985. Production and characterization of monoclonal antibodies against *Clostridium perfringens* type A enterotoxin. Infect. Immun. **50**:442–448.
25. **Zalman, L. S., and B. J. Wisnieski.** 1985. Characterization of the insertion of *Pseudomonas* exotoxin A into membranes. Infect. Immun. **50**:630–635.