PART II

GRAM POSITIVE FOODBORNE BACTERIAL PATHOGENS

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CHAPTER 7

The Action, Genetics, and Synthesis of *Clostridium perfringens* Enterotoxin

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1. INTRODUCTION

THE gram-positive anaerobe *Clostridium perfringens* is ideally suited for its role as a major foodborne pathogen (see McClane, 1997, for review). The widespread natural distribution of *C. perfringens* in both soil and the gastrointestinal (GI) tract of humans and other animals provides this bacterium with ample opportunities to contaminate foods. *C. perfringens* has an excellent ability to survive in incompletely cooked foods due to the relative heat tolerance of its vegetative cells (which will grow at temperatures up to at least 50°C) and its ability to form heat-resistant endospores. The exceptionally short doubling time of *C. perfringens* (reportedly <10 minutes in some studies) makes it relatively easy for this bacterium to contaminate foods at levels (~10^6–10^7 vegetative cells/gram of food) necessary for causing *C. perfringens* foodborne disease (see below). Finally, strains of this bacterium associated with food poisoning produce a protein toxin, named *C. perfringens* enterotoxin (CPE), that is highly active on the human GI tract. As will be discussed in detail below, CPE is considered the virulence factor responsible for the GI symptoms of *C. perfringens* type A food poisoning.

2. EPIDEMIOLOGY OF *C. PERFRINGENS* TYPE A FOOD POISONING

Recent statistics from the Centers for Disease Control (see Table 7.1) indicate that *C. perfringens* currently ranks as the second most common cause

TABLE 7.1. Bacterial Foodborne Disease in the USA from 1989–1992.

Bacterium	Outbreaks	Cases	Mean # of Cases per Outbreak
Bacillus cereus	16	382	24
Campylobacter spp.	23	598	26
Clostridium botulinum	40	84	2
Clostridium perfringens	40	3801	95
E. coli	9	135	15
Listeria monocytogenes	1	2	2
Salmonella spp.	455	18,190	64
Shigella spp.	19	1207	64
Staphylococcus aureus	42	1433	34
Vibrio spp.	9	57	6
Group A *streptococci*	2	135	68

Compiled from Bean et al. (1996).

of foodborne disease in the U.S.A. Almost all *C. perfringens* foodborne illness in the U.S. and other industrialized countries involves *C. perfringens* type A food poisoning (McClane, 1997), which is so named because cases of this illness are nearly always caused by type A isolates of *C. perfringens* (McClane, 1997). The designation "type A" refers to a widely used classification scheme for *C. perfringens* that assigns (see Table 7.2) isolates to one of five types (A-E) depending upon their ability to express four "typing" toxins (i.e., α, β, ι and ϵ toxins). Note from Table 7.2 that CPE expression is not a component of this typing scheme; in fact, type A, C, D, and (possibly) B isolates of CPE-producing *C. perfringens* exist in nature (Skjelkvale and Duncan, 1975; Songer and Meer, 1996; Meer and Songer, 1997; Markovic et al., 1993). The overwhelming involvement of type A isolates in *C. perfringens* type A food poisoning may simply reflect the preponderance of CPE-producing *C. perfringens* type A isolates in the environment, as suggested by some recent epidemiological surveys (Songer and Meer, 1996; Meer and Songer, 1997).

TABLE 7.2. Toxin Typing Classification Scheme for *C. perfringens* Isolates.

		Toxins Produced		
Type:	Alpha	Beta	Epsilon	Iota
A	+	–	–	–
B	+	+	(+)	–
C	+	+	–	–
D	+	–	(+)	–
E	+	–	–	+

Adapted from McDonel (1986); (+) indicates that epsilon toxin is initially produced as an inactive prototoxin.

One epidemiologically interesting aspect of *C. perfringens* type A food poisoning is the unusually large size of most outbreaks, e.g., data shown in Table 7.1 indicate that the typical *C. perfringens* type A food poisoning outbreak involves ~100 cases. The fact that recognized *C. perfringens* type A food poisoning type outbreaks are typically of such large size is not surprising considering that most of these confirmed outbreaks occur in institutional settings. Institutions such as nursing homes, prisons, and hospitals represent favorable environments for *C. perfringens* type A food poisoning outbreaks since these establishments depend heavily on serving foods involving large meat items, such as roasts and turkeys, in order to feed many people at peak meal times. Large meat items are the most common food vehicles for *C. perfringens* type A food poisoning (Bean et al., 1996), at least in part, because they are fairly difficult to thoroughly cook. Incomplete cooking increases the probability that contaminating *C. perfringens* cells/spores will survive the cooking process and multiply to pathogenic levels ($>10^6$–10^7 cells/gram of food) before these foods are consumed. It is also relevant that institutional food is frequently prepared in advance and held for long periods before serving. If these prepared foods are improperly held, this could lead to a *C. perfringens* type A food poisoning outbreak (in fact, holding foods under improper conditions is considered the single most common contributing factor to *C. perfringens* food poisoning outbreaks (Bean et al., 1996).

The large size of most recognized *C. perfringens* type A food poisoning outbreaks is probably somewhat artificial. Because victims of *C. perfringens* food poisoning usually suffer relatively mild symptoms (see below), this illness is much more likely to receive the full attention of public health authorities when large numbers of people, in a common setting (such as an institution), simultaneously become sick, i.e., smaller outbreaks of *C. perfringens* type A food poisoning often go unrecognized. Supporting the view that the ~1000 cases/year of *C. perfringens* type A food poisoning indicated in Table 7.1 significantly underestimate the prevalence of this illness, Todd has estimated (Todd, 1989a, 1989b) that *C. perfringens* type A food poisoning actually affects >650,000 North Americans each year, resulting in ~8 deaths/year and annual costs of >$120 million.

3. THE PATHOGENESIS OF *C. PERFRINGENS* TYPE A FOOD POISONING

In contrast to many cases of foodborne botulism, *C. perfringens* type A food poisoning is rarely, or never, an intoxication resulting from consumption of foods containing preformed CPE (McClane, 1997). Instead, as shown in Figure 7.1, the pathogenesis of *C. perfringens* type A food poisoning involves the in vivo production of CPE (McClane, 1997).

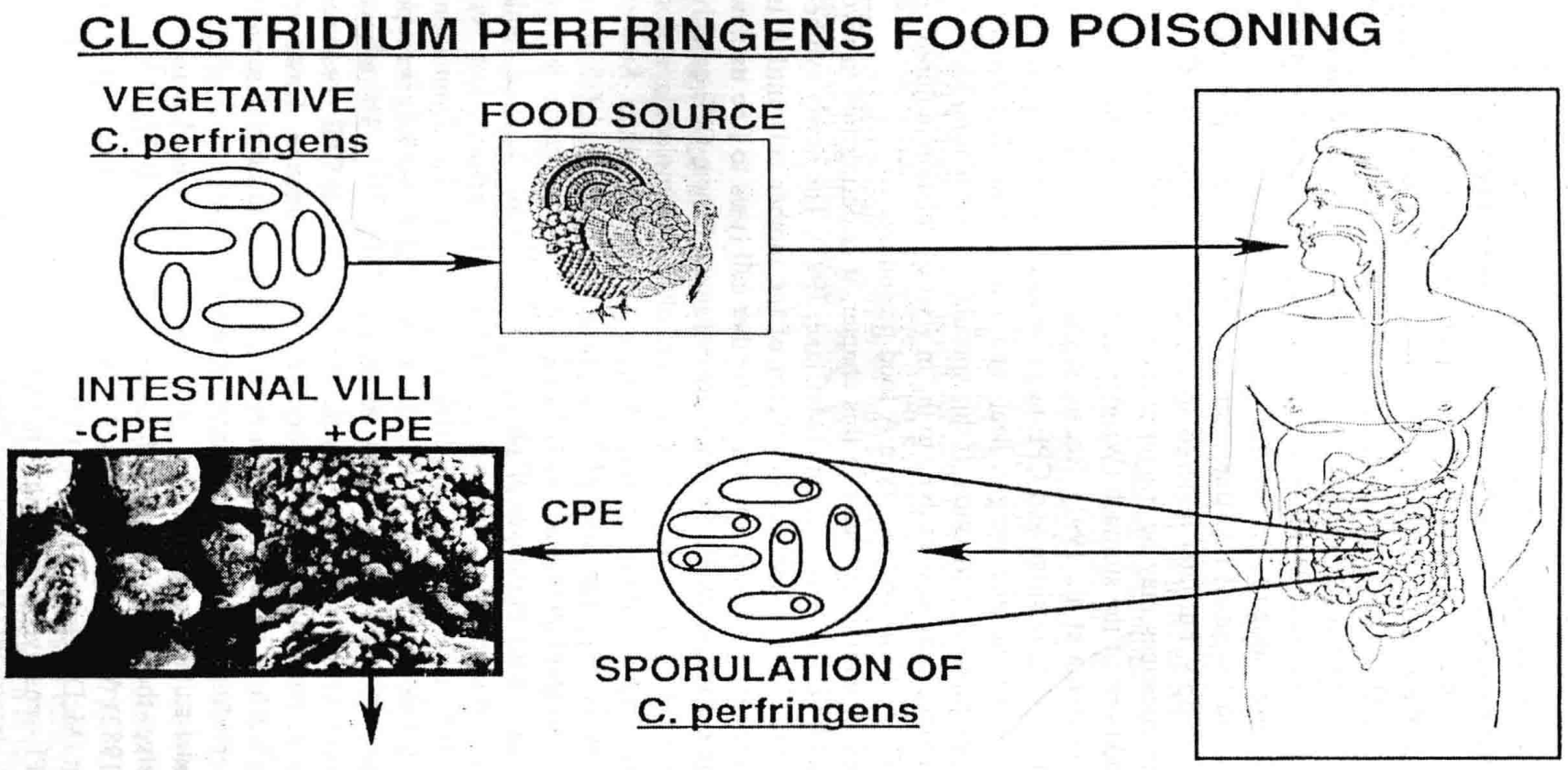

Figure 7.1 Pathogenesis of *Clostridium perfringens* type A food poisoning. Vegetative cells of a CPE-producing strain of *C. perfringens* multiply rapidly in contaminated food (typically a meat- or poultry-containing product). After ingestion, these bacteria pass into the small intestine, where they sporulate and produce CPE. CPE causes intestinal tissue damage, which culminates in the diarrheal and cramping symptoms associated with *C. perfringens* type A food poisoning. Reproduced with publishers' permission from McClane (1992).

The first step in acquiring *C. perfringens* type A food poisoning is consuming food, usually a meat or poultry product (see above), that has become contaminated with large numbers of vegetative cells of a CPE-positive *C. perfringens* isolate. Most of these ingested bacteria are killed in the stomach by gastric acid (McClane, 1997). However, if the ingested food contained sufficiently high numbers (as mentioned previously, $>10^6$–10^7 cells/gram of food) of vegetative *C. perfringens* cells, some of these bacteria may survive exposure to gastric acid long enough to escape into the small intestine.

Once present in the small intestines, surviving vegetative *C. perfringens* cells initially multiply, but later undergo sporulation. Sporulation of *C. perfringens* in the intestines may be triggered by exposure of these bacteria to the acidic conditions of the stomach (Wrigley et al., 1995) or to bile salts in the intestines (Heredia et al., 1991). Recent studies (Shih and Labbe, 1996) indicate that both CPE-positive and CPE-negative isolates of *C. perfringens* can produce a low-molecular weight, heat- and acid-resistant factor(s) that stimulates *C. perfringens* sporulation. If this sporulation-stimulating factor(s) is produced in vivo, it could contribute to the in vivo sporulation required for the development of *C. perfringens* type A food poisoning.

As discussed in more detail later in this chapter, it is during this in vivo sporulation that CPE expression occurs (McClane, 1997). The newly synthesized CPE accumulates inside the cytoplasm of the mother cell until it is released into the intestinal lumen when the mother cell lyses to free its now mature endospore (McClane, 1997). Once present in the lumen, CPE quickly binds to receptors on the intestinal epithelium and (through it molecular action discussed below) induces desquamation of the intestinal epithelium. Studies with animal models (Sherman et al., 1994; McDonel and Duncan, 1975) have demonstrated that development of this CPE-induced intestinal tissue damage strongly correlates with the onset of physiologic symptoms such as fluid/electrolyte loss (an effect that corresponds clinically to diarrhea). However, it is possible that other CPE-induced effects, e.g., intestinal inflammation, could also contribute to the gastrointestinal symptoms of *C. perfringens* type A food poisoning (Sherman et al., 1994; Krakauer et al., 1997). There have been reports (Bowness et al., 1992; Nagata et al., 1997) that CPE possesses superantigenic activity, which could contribute to inflammation. However, this hypothesis has been called into question by another recent study (Krakauer et al., 1997) reporting that CPE lacks superantigenic activity.

Animal model studies suggest that *C. perfringens* type A food poisoning primarily involves the small intestine (McDonel and Duncan, 1977; McDonel and Demers, 1982). While all regions of the rabbit small intestine respond to CPE treatment (McDonel and Duncan, 1977), the rabbit ileum appears to be particularly CPE-sensitive. Interestingly, the rabbit colon does not significantly respond to CPE treatment (McDonel and Demers, 1982), which could indicate that *C. perfringens* type A food poisoning of humans does not involve the

large intestine (however, this hypothesis still needs to be tested with human colonic tissue).

C. perfringens type A food poisoning is clinically characterized by diarrhea and abdominal cramps that develop about 8–16 hours after ingestion of contaminated foods (McClane, 1997). This incubation period stems primarily from the time required for *C. perfringens* to complete its in vivo sporulation. As mentioned, no significant release of CPE into the intestinal lumen occurs until sporulation is completed (this typically takes ~12 hours). Animal model studies indicate that, once CPE has been released into the intestinal lumen, it exerts its effects very rapidly, i.e., CPE-induced intestinal tissue damage can develop within 15–30 minutes (Sherman et al., 1994). In most affected people, symptoms of *C. perfringens* type A food poisoning continue for ~12–24 hours before self-resolving. Fatalities from this illness are relatively rare, but do occur in some elderly or debilitated individuals. Treatment for *C. perfringens* type A food poisoning is primarily symptomatic and no vaccine is currently available (further discussion later). Although *C. perfringens* type A food poisoning victims often develop substantial levels of serum IgG against CPE (Birkhead et al., 1988), there is no evidence that prior exposure to *C. perfringens* food poisoning provides any significant long-term protection against future bouts of this illness (McClane, 1997).

4. EVIDENCE OF CPE INVOLVEMENT IN *C. PERFRINGENS* TYPE A FOOD POISONING

A considerable amount of epidemiologic evidence now implicates CPE as the major (if not only) virulence factor responsible for the diarrheal and cramping symptoms of *C. perfringens* type A food poisoning. Some of this evidence includes;

(1) A strong correlation exists between illness and the presence of CPE in the feces of *C. perfringens* type A food poisoning victims (Batholomew et al., 1985; Birkhead et al., 1988).

(2) CPE is present in the feces of food poisoning victims at levels (Batholomew et al., 1985; Birkhead et al., 1988) shown to cause significant intestinal effects in animal models (McDonel and Duncan, 1975).

(3) Human volunteers who ingested highly purified CPE developed the same diarrheal and cramping symptoms that are characteristic of *C. perfringens* type A food poisoning (Skjelkvale and Uemura, 1977).

(4) CPE-positive *C. perfringens* strains are much more effective than CPE-negative strains at producing either fluid accumulation in rabbit ileal loops or diarrhea in human volunteers (McClane, 1997).

(5) CPE-specific antibodies can neutralize the intestinal effects of culture lysates from CPE-positive *C. perfringens* strains (Hauschild et al., 1971).

5. THE MECHANISM OF ACTION OF CPE

5.1. INTRODUCTION

The first major insight into the molecular mechanism by which CPE induces intestinal tissue damage was provided by electron microscopy studies of CPE-treated rabbit intestinal epithelial cells (McDonel et al., 1978). Those studies showed that CPE-treated intestinal cells rapidly develop extensive damage to their brush border membranes (BBMs). Since this damage precedes detectable damage to internal organelles, McDonel et al., (1978) suggested that CPE may kill sensitive mammalian cells by damaging their plasma membranes.

A series of studies from several laboratories (McDonel and McClane, 1979; Matsuda and Sugimoto, 1979; McClane and McDonel, 1980; McClane and McDonel, 1981; McClane, 1984; Matsuda et al., 1986; McClane et al., 1988) confirmed that CPE is a membrane-active toxin by demonstrating that this toxin induces alterations in the normal permeability properties of sensitive mammalian cells. Within 5 minutes of treatment, CPE damages plasma membranes of sensitive mammalian cells so they become highly permeable to small molecules (<200 Daltons in size); this CPE-induced membrane "lesion" is nonselective, as CPE-treated cells show increased permeability to cations, anions and small organic molecules such as amino acids.

These small molecule permeability changes contribute to CPE-induced cytotoxicity in at least two ways. First, CPE-induced plasma membrane permeability alterations profoundly disturb cytoplasmic pools of small molecules, which causes a rapid shutdown in vital metabolic processes such as macromolecular synthesis (Hulkower et al., 1989). Second, these permeability alterations disrupt the cellular osmotic equilibrium, which causes a significant water influx into the CPE-treated cell. This water influx can "stretch" the plasma membrane of CPE-sensitive cells to the point of lysis (McClane and McDonel, 1981; McClane, 1984).

Experiments conducted during the past 10 years have shed light on how CPE induces these small molecule permeability alterations. The initial event in CPE action clearly involves the binding of CPE to its receptor(s); for example, mammalian cells lacking CPE receptor(s) are totally nonresponsive to CPE (Horiguchi et al., 1985; Wieckowski et al., 1994). CPE receptor(s) are not only expressed by intestinal cells, i.e., many, but not all (see above), cell types from most, if not all, mammalian species are able to bind CPE at high levels (McDonel, 1980; Horiguchi et al., 1985; Wieckowski et al., 1994).

While the CPE receptor(s) is clearly proteinaceous (McDonel, 1980;

McClane et al., 1988), the precise number and identity of CPE receptor(s) remain unclear. Biochemical studies (Wieckowski et al., 1994) suggested that CPE associates with a mammalian membrane protein of ~40–50 kDa after binding to membranes, forming a "small complex" of ~90 kDa (see Figure 7.2). This small complex was detected following CPE-treatment of all enterotoxin-sensitive cells examined by Wieckowski et al. No small complex was detected in CPE-treated cells that do not bind this toxin (or in detergent extracts of these cells), as would be expected if the 40–50 kDa membrane protein in this small complex was a/the functional CPE receptor.

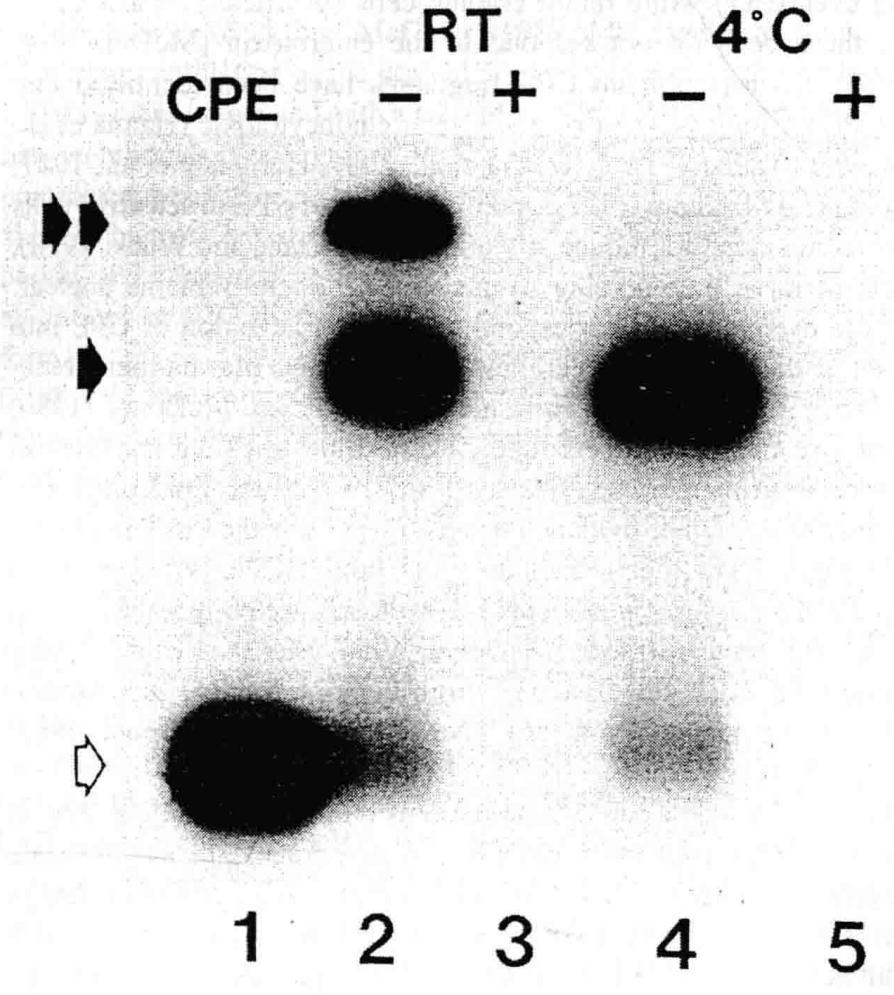

Figure 7.2 Visualization of CPE small and large complexes. Samples include: free ^{125}I-CPE (lane 1) and ^{125}I-CPE incubated with intact BBMs in the presence (+) or absence (–) of 50-fold excess unlabeled CPE at either room temperature (RT: lanes 2 and 3) or 4°C (lanes 4 and 5) prior to extraction with Triton X-100, electrophoresis under nondenaturing conditions, and autoradiography. The migration of small complex and large complex are indicated by single- and double-closed arrows, respectively. Note that similar large complex formation occurs at 37°C and 22°C (data not shown). Reproduced with the publisher's permission from Wieckowski et al. (1994).

However, recent studies (Katahira et al., 1997a,b) using expression cloning techniques now implicate members of the Claudin family (Morita et al., 1999) as functional CPE receptors. Expression of Claudin-3 or -4 in Mouse L cells (which cannot naturally bind CPE and, therefore, are naturally CPE-insensitive) produced transfectants capable of binding CPE at high levels. These transfectants were also highly sensitive to CPE, confirming that these Claudins can serve as ''functional'' receptors capable of mediating CPE cytotoxicity.

Although essential for CPE action, binding of CPE to its receptor(s) is not, by itself, sufficient to induce membrane permeability alterations. Several lines of experimental evidence indicate that CPE has a multistep action requiring post-binding events; (1) while rabbit colonic cells specifically bind CPE at high levels, these cells do not respond to the enterotoxin (McDonel and Demers, 1982), (2) recombinant CPE fragments have been identified that occupy the CPE receptor of sensitive cells yet are noncytotoxic (Hanna et al., 1991; Hanna and McClane, 1991; Hanna et al., 1989; Horiguchi et al., 1987; and Kokai-Kun and McClane, 1997), and (3) binding of CPE to sensitive cells at low temperatures does not induce any toxicity (McClane and Wnek, 1990).

The nature of these post-binding events in CPE action remains unclear. However, these events cannot correspond to the internalization of CPE into the cytoplasm of the mammalian cell since CPE remains plasma membrane-associated throughout its action (Tolleshaug et al., 1982). McDonel (1980) proposed that, because membrane-bound CPE becomes resistant to protease-induced release from membranes, this toxin inserts into the lipid bilayer of the plasma membrane. Other evidence has also been obtained that indirectly supports McDonel's hypothesis, including (1) bound CPE is not released from membranes by chemical treatments known to release peripherally bound proteins from membrane surfaces (McDonel, 1980; McClane et al., 1988), and (2) bound CPE does not dissociate from cells or isolated membranes, whether this binding occurs at 4°C or higher temperatures (McDonel, 1980; McClane et al., 1988).

While the evidence cited above is consistent with a post-binding step in CPE action involving the insertion of CPE into membranes, the existing data also appear fully compatible with the hypothesis that a conformational change to CPE occurs after this toxin becomes sequestered in small complex, with the end-result being that the CPE present in small complex becomes ''locked'' onto the membrane surface (further discussion later).

To better understand the nature of the post-binding steps in CPE action, studies have explored the membrane topology of CPE at 4°C, a temperature where CPE binding and small complex formation occur, but subsequent steps in CPE action are inhibited (McClane and Wnek, 1990, further discussion below). Kokai-Kun and McClane (1996) showed that CPE antibodies still specifically recognize membranes containing CPE allowed to complete the post-binding physical change. While this result, by itself, does not rule out

the possibility that a portion(s) of CPE might be inserted into membranes when the toxin becomes localized in small complex, it does indicate that at least some region(s) of the enterotoxin remains exposed on membrane surfaces during this step in CPE action. Recent followup experiments (Wieckowski et al., 1999) demonstrated that Pronase treatment of membranes containing bound CPE results in substantial degradation of this toxin when it is present in small complex. This new result provides further evidence that most, if not all, of the CPE molecule remains surface-exposed following small complex formation and thereby raises additional doubts about the hypothesis that CPE in small complex becomes inserted into the lipid bilayer of the plasma membrane.

Perhaps the single most important insight into CPE's molecular action was the discovery (Wnek and McClane, 1989; McClane and Wnek, 1990) that, following completion of the post-binding physical change step in CPE action, CPE becomes associated with a second, larger (~160 kDa) complex in mammalian membranes (see Figure 7.3). Formation of this "large" CPE-containing complex appears to be directly responsible for CPE-induced membrane permeability changes, based upon the following observations (1) the inability of bound and "physically changed" CPE to induce membrane permeability alterations at 4°C directly correlates with the inhibition of large complex formation that also occurs at this low temperature (McClane and Wnek, 1990), (2) if cells containing CPE bound at 4°C are shifted to warmer temperatures, the subsequent onset of membrane permeability alterations closely coincides with large complex formation (McClane and Wnek, 1990), and (3) recent studies (Kokai-Kun and McClane, 1997) using recombinantly derived CPE fragments have established a strong correlation between the amount of large complex present in mammalian cells and the extent of CPE-induced membrane permeability alterations occurring in these cells (further discussion later).

Initial studies (Wnek and McClane, 1989) suggested that the large CPE-containing complex contains, at a minimum, one CPE molecule, one 50 kDa membrane protein, and one 70 kDa protein. More recent results from CPE receptor cloning studies (Katahira et al., 1997a) indicate that, at least in some CPE-sensitive mammalian cells, a Claudin may also be present in this large complex. Possible steps leading to the formation of the large CPE-containing complex will be discussed in the following section.

One interesting feature of the large CPE-containing complex is its unusual stability (Wnek and McClane, 1989; Wieckowski et al., 1994). For example, the large CPE complex is considerably more stable in SDS than is the small CPE complex (Wieckowski et al., 1994). Further, the large complex only partially dissociates upon boiling, while the small complex can be easily dissociated by boiling. There is not yet any biochemical explanation for this stability of the large CPE complex.

Finally, the same antibody and protease-challenge techniques that were used to probe the membrane topology of "physically changed" CPE have

also been applied to study the membrane topology of CPE sequestered in large complex. CPE antibodies were shown (Kokai-Kun and McClane, 1996) to specifically recognize membranes containing CPE sequestered in the large complex. This implies that at least a portion of many CPE molecules remains exposed on the surface of membranes after this toxin becomes sequestered in the large complex. This conclusion is supported by recent studies indicating that the CPE molecules in the large complex become slightly smaller after Pronase treatment of large complex-containing membranes (Wieckowski et al., 1998). However, the fact that most of the CPE molecule sequestered in the large complex remains intact in these Pronase-treated membranes indicates that membranes do offer CPE sequestered in the large complex substantial protection against Pronase, which is consistent with these CPE molecules being closely associated with, or possibly even inserting into, membranes.

5.2. A WORKING MODEL FOR CPE ACTION

The recent findings regarding CPE binding and membrane topology demand some changes in our thinking about CPE action. In response, a new four-step working model for CPE action is presented in Figure 7.3 and discussed below.

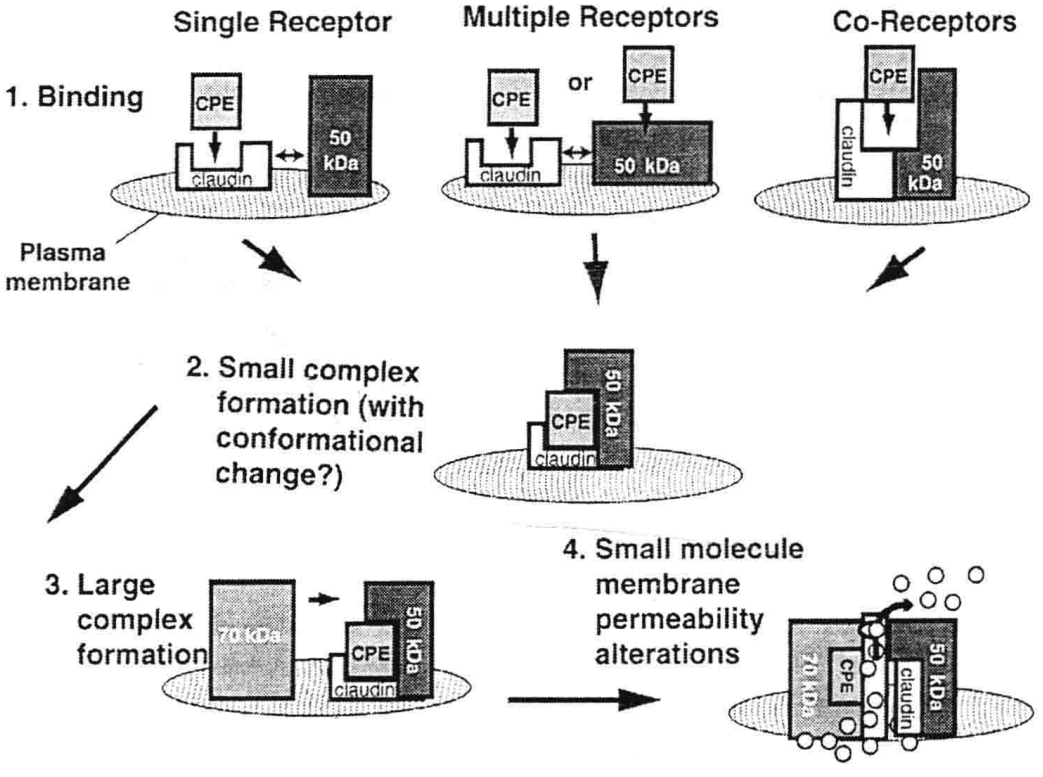

Figure 7.3 Working model for early events in CPE action. See text for a discussion of each proposed step in CPE action. Steps 1–4 in this model can occur in as little as 5 minutes at 37° and that steps 3–4 are inhibited at 4°C.

5.2.1. Binding of CPE to Its Receptor(s)

While the recent studies by Katahira et al. (1997) have clearly demonstrated that Claudins can serve as functional CPE receptors, many questions about CPE binding/receptors remain unresolved. In fact, at least three possible scenarios for CPE binding, as shown in Figure 7.3, appear equally compatible with existing information about CPE binding/receptors. The first scenario envisions Claudins as the only functional CPE receptor(s) used by all CPE-binding cells. The second scenario hypothesizes the existence of multiple types of CPE receptors. In this view, which is consistent with some kinetic studies of CPE binding suggesting that some mammalian cells express two classes of CPE receptor with very different affinities (McDonel, 1980; McDonel and McClane 1979), both Claudins and the 40–50 kDa membrane protein described by Wieckowski et al. (1994) may serve as "functional" CPE receptors, i.e., binding of CPE to either of these proteins would initiate a cytotoxic response. If there are two different types of CPE receptors, it should be resolved whether both of these CPE receptors can be expressed by a single CPE-sensitive mammalian cell. A third plausible scenario for CPE binding is that a Claudin and the 40–50 kDa protein serve as co-receptors for CPE binding, i.e., a cytotoxic response is initiated when CPE binds to both a Claudin and the 40–50 kDa membrane protein.

5.2.2. A Post-Binding Physical Change Occurs to CPE

As discussed above, results from recent antibody probe and Pronase-challenge studies cast increasing doubt on previous proposals that the second step in CPE action involves insertion of CPE into lipid bilayers. However, some sort of post-binding physical change does appear to occur immediately after binding since, as mentioned, CPE binding at 4°C (where large complex formation and membrane permeability alterations are inhibited) is irreversible (McDonel, 1980; McClane et al., 1988).

An appealing hypothesis is that the post-binding physical change step in CPE action corresponds to small complex formation, or to a conformational change immediately following small complex formation. For example, if CPE uses only a Claudin receptor, the resultant CPE:Claudin complex might subsequently interact with the 40–50 kDa membrane protein to form small complex (this would be consistent with immunoprecipitation studies indicating that a 40–50 kDa eucaryotic protein is present in the small complex (Wieckowski et al., 1994)). Formation of this small complex (perhaps coupled with a conformational change to the small complex) could effectively "lock" CPE onto the surface of the plasma membrane, explaining why CPE does not dissociate from membranes (even under conditions inhibiting large complex formation) and is not released from membranes by the addition of chemicals

known to release peripherally bound membrane proteins (McClane, 1997). The possibility that small complex formation, and/or a conformational change to the newly formed small complex, is responsible for the post-binding physical change step in CPE action also appears to be similarly compatible with the multiple receptor scenario in Figure 7.3. If multiple types of CPE receptor do exist, then CPE bound to one type of receptor might subsequently interact with the second receptor to form small complex. Lastly, if CPE binds simultaneously to co-receptors, the post-binding physical change step in CPE action could be envisioned as involving a post-binding conformational change to the CPE: co-receptor complex (i.e., small complex), which effectively locks CPE onto the membrane surface.

5.2.3. Formation of CPE Large Complex

Since evidence indicates that CPE-sensitive cells form both small complex and large complex (see Figure 7.2) and that small complex formation precedes large complex formation (Wieckowski et al., 1994), it can be hypothesized that large complex formation results from an interaction between the "physically changed" small complex (which, at least in some cell types, apparently consists of CPE, a Claudin and/or a 40–50 kDa protein, see above) and a 70 kDa membrane protein previously linked to large complex (Wnek and McClane, 1989). The strong inhibition of large complex formation observed at low temperatures (McClane and Wnek, 1990) suggests that the interaction between physically changed small complex and the 70 kDa protein requires diffusion of membrane proteins through the lipid bilayer of membranes.

5.2.4. Onset of Small Molecule Permeability Alterations

While there is now considerable evidence implicating large complex formation in the onset of CPE-induced small molecule membrane permeability alterations, the direct mechanism by which large complex formation causes these membrane permeability effects remains unknown.

One appealing and simple mechanistic explanation would be that large complex corresponds to a pore-like structure, which allows free passage of small molecules across the plasma membranes of mammalian cells. If true, then large complex would represent an unusual pore structure that is comprised of a heterogeneous mixture of both eucaryotic and procaryotic proteins. Such a putative pore structure might result from CPE inserting into the membrane as part of the pore structure, which would be consistent with Pronase challenge studies indicating that most of the CPE molecule sequestered in large complex is inaccessible to Pronase challenge. Other evidence supporting CPE forming part or all of a pore, includes observations (Sugimoto et al., 1988) indicating that sonication of purified CPE into artificial membranes induces channel-

like permeability alterations (however, note that no membrane proteins were present in this model system).

6. THE CPE PROTEIN

6.1. BIOCHEMISTRY

CPE is a 35,317 M_r, protein with an isoelectric point of 4.3 (McClane, 1997). This 319 amino acid, single polypeptide has a unique primary sequence (Czeczulin et al., 1993), except for some limited homology with the Antp70/C1 protein of *Clostridium botulinum* (Melville et al., 1997). The significance, if any, of the limited homology between CPE and Antp70/C1 is unclear. The primary sequence of CPE appears to be highly conserved among CPE-positive *C. perfringens* isolates, based upon DNA sequencing studies (Collie et al., 1998) demonstrating that identical *cpe* open reading frame (ORF) sequences are present in seven different *cpe*-positive *C. perfringens* isolates. Circular dichroism studies (Granum and Stewart, 1993) indicated that the secondary structure of CPE contains ~80% β-sheet and ~20% random coil. Due to difficulties in obtaining diffraction-grade crystals for x-ray analysis, no information is available regarding the 3-D structure of the enterotoxin.

Unlike several enterotoxins produced by other gram-positive bacteria (e.g., the staphylococcal enterotoxins), CPE has a heat-labile biologic activity. Heating CPE to 56°C for 5 min destroys its biologic activity (McClane, 1997). CPE's toxicity is also sensitive to pH extremes, i.e., biologic activity is lost when the enterotoxin is exposed to pH <5 or >10 (McClane, 1997). Interestingly, while some proteases (e.g., Pronase and subtilisin) inactivate the biologic activity of CPE (McClane, 1997), there is a 2–3 fold increase in biologic activity when CPE is digested, in vitro with intestinal proteases such as trypsin and chymotrypsin (Granum et al., 1981; Granum and Richardson, 1991). It is possible that, during food poisoning, intestinal proteases such as trypsin and chymotrypsin (Granum et al., 1981; Granum and Richardson, 1991) similarly activate CPE after this toxin has been released into the intestinal lumen upon the completion of sporulation (McClane, 1997).

6.2. CPE STRUCTURE/FUNCTION RELATIONSHIPS

Although the 3-D structure of CPE has not yet been solved, steady progress has nonetheless been achieved towards understanding how the CPE molecule exerts its action. As shown in Figure 7.4 and discussed below, CPE's binding and toxic activity domains appear to be segregated on discrete regions of the toxin.

FUNCTIONAL REGIONS OF CPE

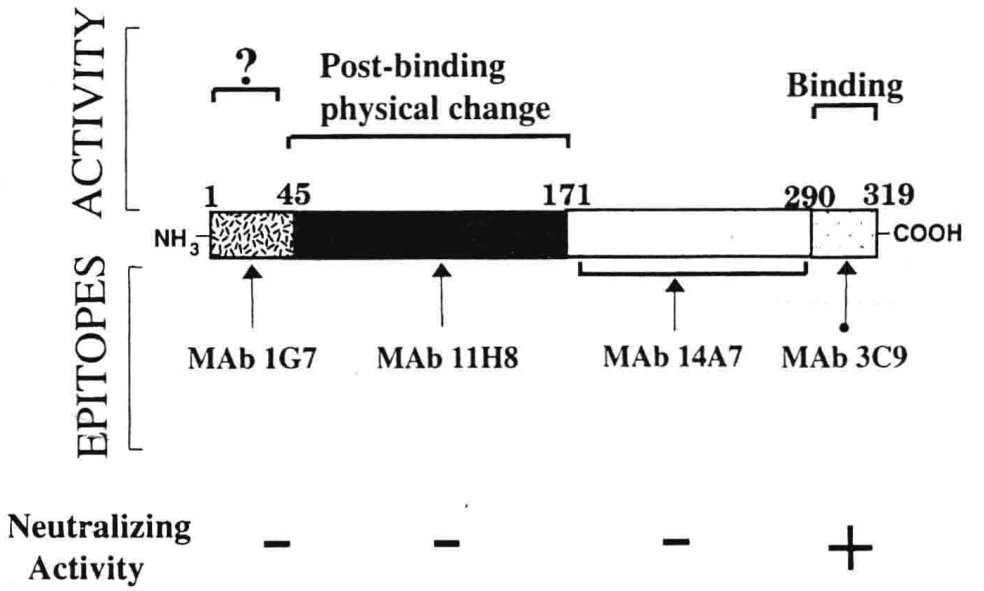

Figure 7.4 Map of the CPE structure/function relationship. CPE regions that appear to be required for the post-binding physical change, binding and various epitopes are noted. Of the four MAbs shown, only MAb 3C9 neutralizes CPE cytotoxicity.

Receptor binding activity was initially mapped to the C-terminal half of the CPE molecule in a series of studies using CPE fragments produced by either chemical cleavage or recombinant DNA approaches (Horiguchi et al., 1987; Hanna et al., 1989). CPE's receptor binding activity was then further localized to the extreme C-terminus of the toxin in studies demonstrating (1) a synthetic peptide possessing the same sequence present in the 30 C-terminal amino acids of CPE exhibits similar, if not identical, binding properties as native CPE (Hanna et al., 1991), and (2) deletion of the last five C-terminal amino acids from the CPE molecule is sufficient to abolish binding of this toxin to mammalian cells (Kokai-Kun and McClane, 1997).

CPE fragments have also proven invaluable for probing function(s) present on the N-terminal half of the enterotoxin protein. For example, it has been demonstrated (Kokai-Kun and McClane, 1997) that removing up to the first 44 N-terminal amino acids from native CPE causes a 2–3 fold increase in CPE's ability to induce membrane permeability alterations. This increased biologic activity was shown (Kokai-Kun and McClane, 1997) to result from these N-terminal CPE fragments being able to form, on a molar basis, approximately 2–3 fold more large complex than native CPE (this effect also explains the 2–3 fold increase in CPE activity induced by trypsin or chymotrypsin treatment, since these two proteases remove the first 25 and 36 amino acids,