Journal of Cellular Biochemistry 52:107–115 (1993)

# Signal Transduction Pathways and Cellular Intoxication With *Clostridium difficile* Toxins

**Maria Caspar Shoshan, Carla Fiorentini, and Monica Thelestam**

Department of Bacteriology, Karolinska Institute, S-104 01 Stockholm, Sweden (M.C.S., M.T.); Department of Ultrastructures, Istituto Superiore di Sanitá, 00161 Rome, Italy (C.F.)

**Abstract** In cultured cells the cytopathic effects (CPE) of *Clostridium difficile* toxins A and B are superficially similar The irreversible CPEs involve a reorganization of the cytoskeleton, but the molecular details of the mechanism(s) of action are unknown As part of the work to elucidate the events leading to the CPE, cultured cells were preincubated with agents known to either stimulate or inhibit some major signal transduction pathways, whereupon toxin was added and the development of the CPE was followed. Both toxin-induced CPEs were enhanced by phorbol esters and mezerein, which stimulate protein kinase C, while they were inhibited by the phospholipase A2 inhibitors quinacrine and 4-bromophenacylbromide Agents affecting certain G-proteins, cGMP and cAMP levels, phosphatases, prostacyclin, lipoxygenase, and phospholipase C did not affect the development of the CPE of either toxin Thus, the cytoskeletal effect induced by toxins A or B appears to require PLA2 activity and involves at least part of a protein kinase C-dependent pathway, but not pertussis toxin-sensitive G-proteins, cyclic nucleotides, eicosanoid metabolites, or phospholipase C activity In addition, both toxins were shown to activate phospholipase A2. 

**Key words:** *Clostridium difficile*, cytotoxicity, phorbol esters, phospholipase A2

*Clostridium difficile* produces at least two protein toxins involved in the pathogenesis of antibiotic-associated diarrhoea and colitis [Lyerly et al., 1988]. Toxin A is a cytotoxic enterotoxin while toxin B is a more potent cytotoxin [Lyerly et al., 1988]. Both toxins are antiproliferative and are internalized into cells by endocytosis [Florin and Thelestam, 1983; Henriques et al., 1987]. The cytopathic effects (CPE) of the toxins are seen as cell retraction and rounding, apparently as a result of rearrangement of the microfilament (MF) system [Mitchell et al., 1987; Fiorentini and Thelestam, 1991]. It has been hypothesized that toxin B, and possibly toxin A, act on the link between the MF and the plasma membrane [Thelestam et al., 1992], but the molecular modes of action are unknown. Many toxins act on defined cellular signal transduction pathways, e.g., cholera and pertussis toxins, which act on different G proteins [Johnson, 1982; Houslay, 1987], and *Clostridium botulinum* exoenzyme C3, which acts on a rho-protein apparently involved in regulation of the MF system [Chardin et al., 1989].

The aim of this work was to examine whether also the *Clostridium difficile* toxins exert their effects through some defined signal transduction pathway(s). The approach was to interfere with these pathways by treatment of cells with substances known to inhibit or stimulate them, and after subsequent toxin treatment examine whether the changed signal transduction activity would alter the development of the CPE. The signal transduction pathways studied in the present work are schematically represented in Figure 1. The results suggest that the modes of action of the two toxins at some point intersect a protein kinase C-dependent pathway. Phospholipase A2 (PLA2) in intoxication with either toxin was shown to be required primarily for the completion of endocytosis of the toxins. However, both toxins were also shown to activate PLA2.

Abbreviations used AA, $^{14}$C-arachidonic acid, 8-bcGMP, 8-bromo cyclic GMP, BPB, 4-bromophenacyl bromide, CPE, cytopathic effect, HBSS, Hanks' balanced salt solution, PGE2, prostaglandin E2, PKC, protein kinase C, PLA2, phospholipase A2, PLC, phospholipase C, QA, quinacrine, $TCD_{50}$, tissue culture dose causing 50% cell rounding, TPA, 12-*O*-tetradecanoylphorbol-13-acetate

Received September 14, 1992, accepted December 6, 1992

Address reprint requests to Maria Caspar Shoshan, Department of Bacteriology, Karolinska Institute, Box 60 400, S-104 01 Stockholm, Sweden

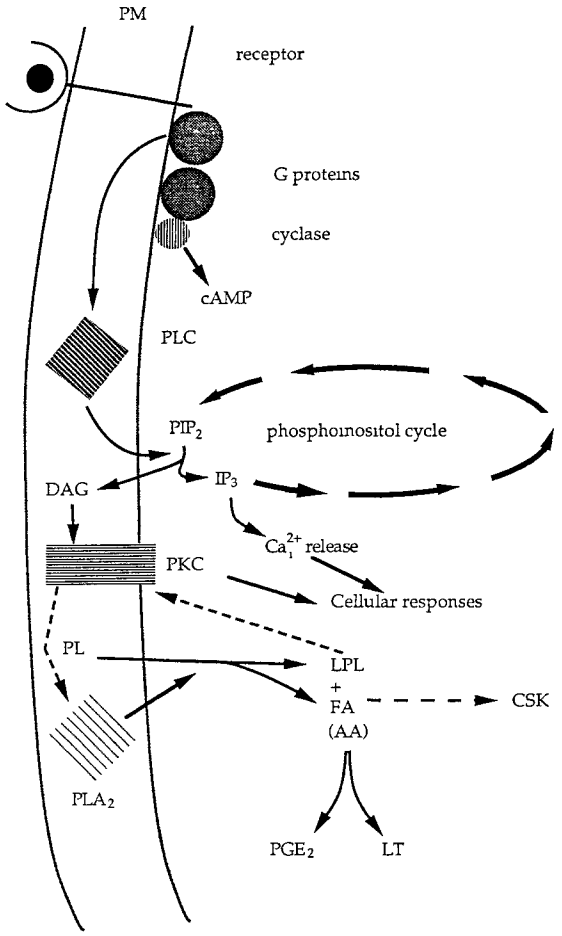


**Fig. 1.** Schematic representation of signal transduction pathways PM = plasma membrane To the right of the PM is the inside of the cell From top to bottom an extracellular ligand bound to its receptor activates a G protein which may either regulate a cyclase or a phospholipase (PLC) The cyclase produces cAMP, while PLC catalyzes hydrolysis of $PIP_2$ to $IP_3$ and diacylglycerol (DAG) $IP_3$ effects release of intracellular calcium and DAG is the physiological stimulator of protein kinase C (PKC) Both calcium and PKC elicit a large number of cellular responses, including stimulation of phospholipase A2 (PLA2) and effects on the cytoskeleton PLA2 catalyzes hydrolysis of membrane phospholipids to lysophospholipids (LPL) and free fatty acid (FA), both of which may interact with the cytoskeleton (CSK) and also with PKC If the FA is arachidonic acid (AA), it may be processed by lipoxygenase and prostacyclin to form prostaglandins (PGE), leukotrienes (LT), and other metabolites Information for this figure was drawn from several sources, including Houslay [1987], Huang [1989], and Takai et al [1981]

## MATERIALS AND METHODS

### Cells

Human embryonic lung fibroblasts (MRC-5) were grown in Eagle's Minimum Essential Medium supplemented with 10% newborn bovine serum, 5 mM *L*-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml. Mouse adrenal cortex tumor (Y1) epithelioid cells were grown in Ham's F-10 with the same supplements, although with 10% fetal bovine serum. Chinese hamster ovary cells (CHO) were grown in Ham's F-12 with the same supplements including fetal bovine serum. All media and sera were from Flow Laboratories, Irvine, Scotland. These supplemented media will be referred to as growth media.

### Toxins

Toxin A prepared according to Sullivan et al. [1982] was a generous gift from Dr. Paola Mastrantonio, Istituto Superiore di Sanitá, Rome, Italy. Toxin A was used at a concentration of 1.5 μg/ml. Toxin B was prepared from culture supernatants of *C. difficile* strain VPI 10463 according to Shoshan et al. [1990]. Briefly, the proteins in culture supernatants were separated on Waters Accell QMA ion exchange resin (Millipore, Gothenburg, Sweden). The cytotoxic fractions were pooled and further purified on MonoQ ion exchange FPLC column. The dose used, unless otherwise stated, was 100 or 200 $TCD_{50}$, determined as described [Caspar et al., 1987]. This corresponds to approximately 0.8 or 1.6 ng, respectively.

### Chemicals

Okadaic acid was a generous gift from Prof. Lars Edebo, University of Gothenburg, Sweden. Freeze-dried cholera toxin was kindly donated by Prof. J.P. Craig, State University of New York, New York, NY. Pertussis toxin was from the National Bacteriological Laboratory, Stockholm, Sweden. All other test compounds were from Sigma Chemical Co., St. Louis, MO. Stock solutions of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 4β-phorbol-12,13-dibutyrate (PDBu), mezerein, 4-bromophenacyl bromide (BPB), and 1-(5-isoquinoline sulfonyl)-2-methyl piperazine dihydrochloride (H-7) were in dimethylsulfoxide. Indomethacin, esculetin, and quinacrine were dissolved in ethanol, caffeine in methanol, and the rest in growth media. The solvent concentration on cells never exceeded 0.1%.

$^{14}$C-arachidonic acid (AA) was from Amersham Sweden AB, Solna, Sweden. Phospholipids and lipid standards were from Sigma Chemical Co. Aquasol scintillation cocktail was from DuPont NEN Products Division, Dreieich, Germany. Organic solvents were from Merck, Darmstadt, Germany.

**TABLE I. Summary of Effects of Tested Compounds on Toxin-Induced CPEs***

| Tested compound | Concentration | Preincubation time | Effect on CPE of: Toxin A (Y1 cells) | Toxin B (MRC-5 cells) | References[a] |
|---|---|---|---|---|---|
| Compounds affecting cAMP, cGMP, and G proteins | | | | | |
| Caffeine | 10 μg/ml | 18 h | None | None | Zollner [1990] |
| Cholera toxin | 0.37 mg/ml | 18 h | ND | None | Johnson [1982] |
| | | 48 h[b] | None | None | |
| 8-bcGMP | 4.5 mM | 130 min | None | None | Zollner [1990] |
| Pertussis toxin | 320 μg/ml | 18 h | None | None | Hewlett et al. [1983] |
| | | 48 h[b] | None | None | |
| Compounds affecting PKC | | | | | |
| TPA | 50 ng/ml | (see Fig. 2) | Stim[c] | stim | Nishizuka [1984] |
| | 5 ng/ml | 15 min | None[c] | None | |
| PDBu | 200 ng/ml | 15 min | stim[c] | stim | Nishizuka [1984] |
| | | 15 h | None[c] | None | |
| Mezerein | 1 μM | 15 min | stim[c] | Stim | Nishizuka [1984] |
| Staurosporine | 10 nM | 60 min | None | None | Hunter [1987] |
| H-7 | 50 μM | 60 min | None | None | Hunter [1987] |
| Phosphatase inhibitors | | | | | |
| Okadaic acid | 0.12 μM | 30 min | None | None | Hannigan and Williams [1991] |
| LiCl | 20 mM | 20 min | ND | None | Berridge and Irvine [1989] |
| Compounds affecting eicosanoid metabolism and phospholipases | | | | | |
| Quinacrine | 10 μM | 60 min | Delay | Delay | Zollner [1990] |
| BPB | 100 μM | 25 min | ND | Delay | Zollner [1990] |
| Indomethacin | 200 μM | 30 min | None | None | Haystead et al. [1989] |
| Esculetin | 5 μM | 5 min | None | None | Kimura et al. [1985] |
| Neomycin | 15 mM | 45 min | None | None | Lassing and Lindberg [1985] |

*The development of the CPEs was followed at 20 min intervals until 100% CPE was reached in samples containing toxin only. If the CPE reached 100% before this time point in samples containing toxin and drug, this was noted as stimulation. See also Figure 2. No CPE had developed in samples containing toxin and quinacrine or BPB at the time point when toxin controls were 100% round. This was noted as delay. See also Figure 3. ND = no data; stim = stimulation.

[a]References to a similar use of each drug.

[b]48 h preincubations were used in experiments with Chinese hamster ovary cells, in which cholera toxin induces cell elongation and pertussis toxin the formation of "rosettes" [Hewlett et al., 1983]. No morphological effect of either toxin was seen in MRC-5 cells.

[c]On MRC-5 cells.

### Screening of Drugs for Effect on the CPE

Cells were preincubated with each compound listed in Table I for different time intervals at 37°C. Toxin A or B was then added and the incubation continued. The development of the cytopathic effect (CPE) was scored by light microscopy as the fraction actinomorphically altered cells, as described [Caspar et al., 1987]. The CPE in duplicate samples was scored at various time points and compared to the CPE in duplicate controls containing only toxin. Duplicate controls containing only the test compound were also included. Each experiment was performed at least twice.

Because Y1 cells are more sensitive than fibroblasts to the weak cytotoxic effect of toxin A, these were mainly used for toxin A experiments, while MRC-5 fibroblasts were used for toxin B, unless otherwise indicated.

### Release of $^{14}$C-Arachidonic Acid (AA)

MRC-5 fibroblasts grown to near confluency in 96-well microtiter plates were incubated with AA (0.1 μCi/ml) for 16–18 h for optimal incorporation into the sn-2 position of membrane phospholipids [Furstenberger et al., 1987]. The medium was then removed and the cells washed 3× with HBSS. New medium with toxin or other additions was added to the cells (200 μl/well) and the incubation continued at 37°C. After 3.5 h, 180 μl of the medium of each sample was removed and added to 1.5 ml Aquasol for liquid

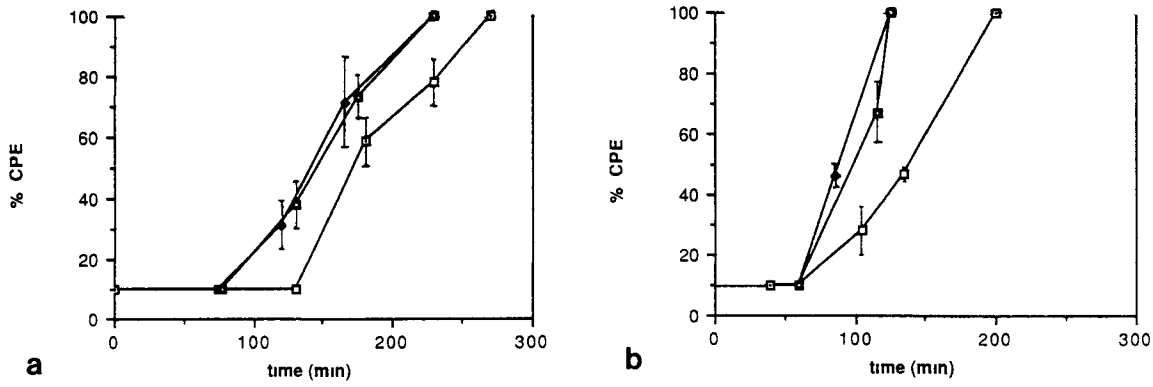


Fig. 2. Effect of TPA on development of CPEs MRC-5 cells were treated with toxin A (1 5 μg/ml) or toxin B (100 $TCD_{50}$) as described in Materials and Methods Cells were either preincubated 15 min with TPA (50 ng/ml) or TPA was added 75 min after toxin addition The CPE was scored by light microscopy as described [Caspar et al , 1987] **a:** With toxin A □, toxin only, ◆, preincubation 15 min with TPA before toxin addition, ■, TPA added to toxin-treated cells after 75 min **b:** With toxin B □, toxin only, ◆, preincubation 15 min with TPA before toxin addition, ■, TPA added to toxin-treated cells after 75 min

scintillation counting. Each assay was done in quadruplicate at each time point, and each experiment was performed at least twice.

### Thin-Layer Chromatography of Sample Media

Sample medium (6 ml) was collected after 3.5 h of toxin treatment and analysed for presence of free AA by thin-layer chromatography according to Flower and Blackwell [1976]. Medium from untreated control cells was similarly treated. Samples and authentic standards were run in parallel lanes on silica gel plates (DC-60, Merck). Then 2- or 3-mm wide sections of each lane were scraped off, and the radiolabel in each was quantified by scintillation counting.

## RESULTS

### Involvement of Signal Transduction Pathways in the CPE

**Cyclic nucleotides and G proteins.** It was earlier shown that the *C. difficile* toxins do not affect intracellular levels of cyclic nucleotides [Thelestam and Florin, 1984]. Here we asked whether raising the levels of cAMP or cGMP would either inhibit or stimulate the toxin-induced CPE development, e.g., via protein kinase A or G. The results indicated that this was not the case, as neither caffeine, cholera toxin or 8-bcGMP affected the cellular intoxications induced by toxins A and B (Table I). Neither did extended incubations of CHO cells with pertussis toxin (in order to inhibit a pertussis toxin-sensitive class of G proteins [Houslay, 1987]) alter the cellular response to subsequently added toxins A or B. With toxin B, this result was confirmed also in MRC-5 cells.

**Cellular phosphorylation.** The CPEs of toxins A and B were not affected by pretreatment of the cells with staurosporine or H-7, substances which inhibit protein kinase C (PKC), or by prolonged treatment with PDBu leading to downregulation of PKC (Table I). Okadaic acid inhibits protein phosphatases 1 and 2A, and thereby supports high levels of ser/thr phosphorylation [Haystead et al., 1989]. Okadaic acid at concentrations down to 0.2 μM rounded up MRC-5 cells, while 0.12 μM did not. This concentration known to be active in other cells [Rivedal et al., 1990] did not affect the CPE induced by either toxin in either cell line. Interestingly, the morphological effect of phosphatase inhibitor Na-orthovanadate (200 μM) per se resembled that of both toxins: within 20–30 min fibroblasts showed a cytoskeletal effect only partly reversible after removal of the drug. Because of this effect, the drug was not used together with toxins.

On the other hand, preincubation of cells with either of three tumor promoters known to activate PKC (TPA, PDBu, and mezerein) had a stimulating effect on both intoxications seen as a considerable reduction of the dose-dependent latency period before the onset of the CPE (Table I). These substances had no cell rounding effects per se. The stimulation was further investigated with TPA (50 ng/ml). We found that i) TPA could be added as late as 75 min after toxin addition and significantly increase the rate of CPE development (Fig. 2); ii) the effect of TPA

was especially dramatic on the CPE of toxin B (Fig 2); iii) the overnight, endpoint titers of the toxins were not altered by TPA treatment of cells (data not shown). The CPE of toxin B doses $< 10$ $TCD_{50}$ was not stimulated by preincubating cells with TPA; i.e., the development was normal.

Presumably, the latency periods of these low doses are so long as to allow the continuous presence of TPA to downregulate PKC before intoxication has reached the point at which PKC can participate. Pretreatment of the toxins with TPA before addition to the cells did not alter the CPEs.

We conclude that while the toxins may share part of a pathway involving PKC, active PKC is not required for the development of the CPEs. Neither do inhibited ser/thr dephosphorylation or inhibited dephosphorylation of $IP_3$, resulting in depletion of inositol, affect intoxication (see below).

**Phospholipase C and phosphatidyl inositol cycle.** Neomycin inhibits PLC-mediated hydrolysis of phosphatidyl inositol, thereby interfering with the phosphatidyl inositol cycle (Fig. 1). Neomycin affected neither of the toxin-induced CPEs (Table I), suggesting that this signalling pathway is not involved in the cytoskeletal effects. LiCl reduces the amounts of inositol since it perturbs the inositol phosphate cycle by inhibiting enzymes which hydrolyse the inositol phosphates [Berridge and Irvine, 1989]. LiCl, however, did not affect the toxin B CPE (Table I). Pertussis toxin inhibits certain G proteins including one which regulates phosphatidylinositol-specific PLC and one which regulates adenylate cyclase (Fig. 1) [Houslay, 1987]. In conclusion, we believe that neither inositol regulation nor pertussis toxin-sensitive PLC regulation are involved in the modes of actions of toxins A and B.

**Phospholipase A2.** Phospholipase A2 (PLA2) converts membrane phospholipids to lysophospholipids and free fatty acid, which both may stimulate PKC [Huang, 1989]. Cellular PLA2s specific for arachidonic acid in the sn-2 position of phospholipids are in addition the rate-limiting enzymes of eicosanoid metabolism whereby they mediate production of prostaglandins via prostacyclin activity and of leukotrienes via lipoxygenase. The two latter enzymes are inhibited by indomethacin and esculetin, respectively [Hannigan and Williams, 1991; Kimura et al., 1985]. PLA2 activity can be inhibited by low amounts of quinacrine (QA) or bromophenacyl bromide (BPB) [Zollner, 1990]. Quinacrine (QA; 10 μM) strongly delayed in a dose-dependent manner the development of both toxin CPEs in MRC-5 cells (Table I, Fig. 3). To see whether QA would inhibit another type of cell rounding cytochalasin D (50 μg/ml) was added to cells treated with 10 μM QA. As QA did not impair the cell rounding caused by cytochalasin-mediated disorganization of the microfilaments, the result suggests that the inhibition of toxin CPEs by QA was not due to unspecific membrane stabilization. When QA was added concomitantly with toxin B (200 $TCD_{50}$) the CPE was delayed, but when added 10 min after the toxin it had no effect on the CPE (data not shown). The same results were obtained in Y1 cells with both toxins. In order to see whether QA interfered with the first steps of endocytosis toxin B was first allowed to bind to, but not enter, cells by incubation at 0°C. The toxin molecules were then arrested and thus "synchronized" in the endosomes by incubating the cells at 18°C for 30 min [Florin and Thelestam, 1986]. As QA could be added after this step and still afford inhibition upon transfer to 37°C its inhibitory effect was at a point temporally beyond the endocytotic endosomes.

As PLA2 has been suggested to be involved in intracellular membrane fusion (see Moskowitz et al. [1983] for references), QA might prevent the fusion between endosomes and lysosomes, which is necessary for complete endocytosis of the toxins. The following experiment was therefore carried out (see also Table II): toxin B was arrested in the endosomes by incubation at 18°C. QA was also added as a preincubation step. The cells were then transferred to 37°C in the continued presence of QA. When the CPE appeared in parallel toxin controls, QA was replaced with $NH_4Cl$ (20 mM), which traps the toxin in the lysosome step, i.e., after the required fusion. While controls developed 100% CPE, this treatment prevented the CPE almost completely, indicating that QA had trapped the toxin in endosomes. Altogether, these experiments show that QA inhibits toxin A and B intoxication by preventing completion of endocytosis.

BPB modifies a histidine residue in the active site of PLA2, but may also have unspecific alkylation side effects. It was highly toxic to the cells, but 100 μM BPB could still prevent toxin B-induced cell rounding for at least 17 h (Table I). As with QA, BPB could be added after 18°C-treat-

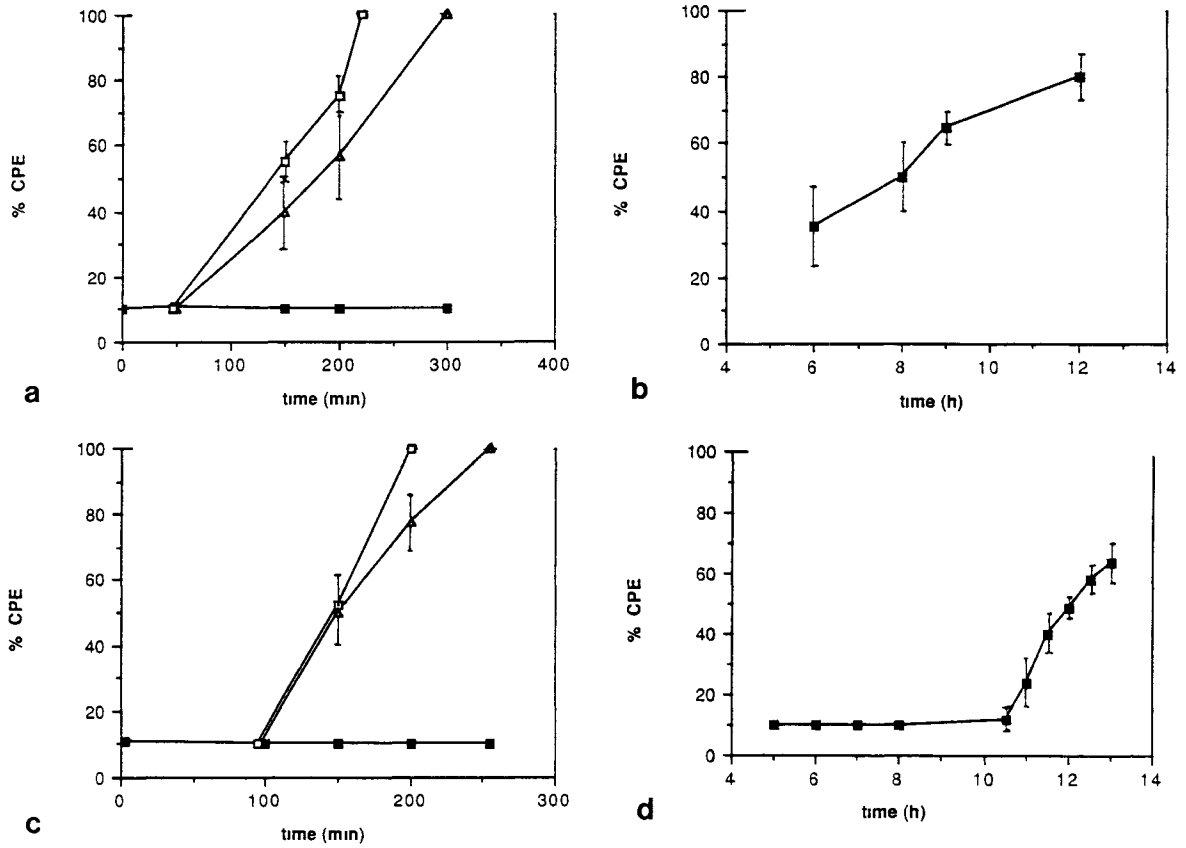


Fig 3 Effect of quinacrine on development of CPEs MRC 5 cells were preincubated for 1 h with quinacrine (QA) at different concentrations before addition of toxin A or B (1 5 μg/ml and 100 $TCD_{50}$) respectively The CPE was scored by light microscopy as described [Caspar et al 1987] **a** With toxin A □ toxin only △ toxin and 1 μM QA ■ toxin and 10 μM QA **b** Extent of delay of toxin A CPE effected by 10 μM QA (continuation of a) **c** With toxin B □ toxin only △ toxin and 1 μM QA ■ toxin and 10 μM QA **d** Extent of delay of toxin B CPE effected by 10 μM QA (continuation of c)

ment of toxin-treated cells and still afford inhibition upon transfer to 37°C Most likely, BPB has the same effect as QA on intracellular membrane fusion

Neither indomethacin nor esculetin affected the CPE of either toxin (Table I) This confirms the report that indomethacin was unable to protect CHO cells against toxin A [Lima et al , 1988] Thus, the CPEs of the toxins do not require AA metabolites

### Activation of PLA2 by Toxins A and B

To examine the possibility that the inhibitory effect of QA was at least in part due to another role for PLA2, MRC-5 cells were prelabelled with $^{14}$C-arachidonic acid (AA) and the toxin-induced release of radioactive label into the medium was measured This assay is based on the preferential incorporation of AA into the sn-2 position of phospholipids, which is also the site of PLA2-mediated hydrolysis [Furstenberger et al , 1987] Toxin B caused a dose-dependent release of AA (Fig 4) With toxin A, the ratio between levels of released radioactivity and the cytotoxic titer was greater than that of toxin B, e g , 20 $TCD_{50}$ of toxin A induced a release roughly equal to that induced by 2,000 $TCD_{50}$ of toxin B However, as this corresponds to very high protein amounts of toxin A further studies on PLA2 activation by toxin A were not performed

To make sure that the released radioactivity was due mainly to free AA, thin layer chromatography analysis of the supernatant was performed according to Flower and Blackwell [1976] The results (Fig 5) show that it was indeed so with both toxins An additional, minor radioactive peak migrated to the same position as authentic prostaglandin E2

Incubation of the prelabeled cells with cytochalasin D (50 μg/ml) did not induce any release of label (data not shown), indicating that rounding-up per se does not induce PLA2 activation

**TABLE II. Quinacrine Prevents Completion of Endocytosis***

| 0 min | 15 min | 60 min | 165 min | 180 min |
|---|---|---|---|---|
| | Wash | Wash | | Wash |
| 0°C | 18°C | 37°C | | |
| toxin B | QA + $NH_4Cl$ | QA | QA+ $NH_4Cl$ | $NH_4Cl$ |
| Binding step | Toxin trapped in endosomes | In the presence of QA, is the toxin trapped in endosomes by inhibition of vesicle fusion? | | When QA is removed, can $NH_4Cl$ then trap the toxin in the lysosomes? |

| Samples | Toxin | QA | QA | $NH_4Cl$ | % CPE at 3.5 h | % CPE at 7 h |
|---|---|---|---|---|---|---|
| 1) | + | + | + | + | 0 | 0 |
| 2) | + | − | − | − | 100 | 100 |
| 3) | + | + | − | − | 100 | 100 |
| 4) | + | − | + | − | 0 | 100 |
| 5) | + | + | + | − | 0 | 100 |
| 6) | + | − | − | + | 100 | 100 |

*The table shows an overview of the experimental procedure, as described in Materials and Methods. The toxin B dose was 1,000 $TCD_{50}$. Quinacrine was used at 10 μM, and $NH_4Cl$ at 20 mM. Negative controls had no morphological effect on cells.

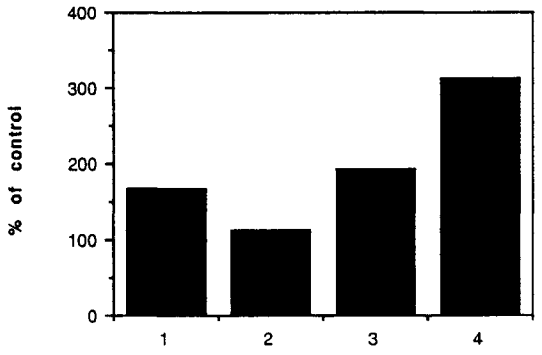


**Fig. 4.** Toxin-induced release of $^{14}C$-arachidonic acid (AA). MRC-5 cells were labeled with AA and treated with toxin as described in Materials and Methods. The radioactivity in supernatants after 3.5 h of toxin treatment was quantified by scintillation counting. The background release in control cells was set to 100% and corresponded to 157 ± 31 cpm. **1:** toxin A (9.2 μg/ml), **2:** toxin B (200 $TCD_{50}$), **3:** toxin B (2,000 $TCD_{50}$), **4:** toxin B (10,000 $TCD_{50}$).

Further studies of factors governing toxin B-induced activation of PLA2 are presented in a companion paper in this issue.

## DISCUSSION

The aim of this work was to find some signal transduction pathway engaged in the development of the cytopathic effects (CPEs) of *C. difficile* toxins A and B, and to thereby obtain a clue to the molecular modes of action of these toxins.

Neither pertussis toxin-sensitive G proteins, arachidonic acid cascade metabolites, nor raised levels of cGMP or cAMP were found to affect the development of the CPEs of the *C. difficile* toxins, as summarized in Table I. Neither did the phosphoinositol cycle appear involved, as the CPEs were not affected by neomycin or LiCl which both perturb the phosphoinositol cycle (Fig. 1) by different mechanisms. The negative results obtained with pertussis toxin is in accordance with this conclusion, since this agent may interfere with G-protein-coupled phosphoinositide hydrolysis.

The positive results obtained with stimulators of PKC and with inhibitors of PLA2 suggest that these two major and ubiquitous enzymes are somehow involved in the intoxication process.

Kinases have been described as "switches" that turn cellular functions on and off [Hunter, 1987]. Cytoskeleton functions regulated by phosphorylation include focal point integrity [Turner et al., 1989] and microfilament integrity [Lyass et al., 1988]. The morphological similarities between, on the one hand, toxin B-treated cells, and on the other hand, cells transformed with src kinase or treated with the phosphatase inhibitor Na-orthovanadate [Wang and Goldberg, 1976; Kellie, 1988] prompted us to include phosphorylation-related agents in this study. Furthermore, the rapid cellular effects of changes in phosphorylation are in agreement with the short

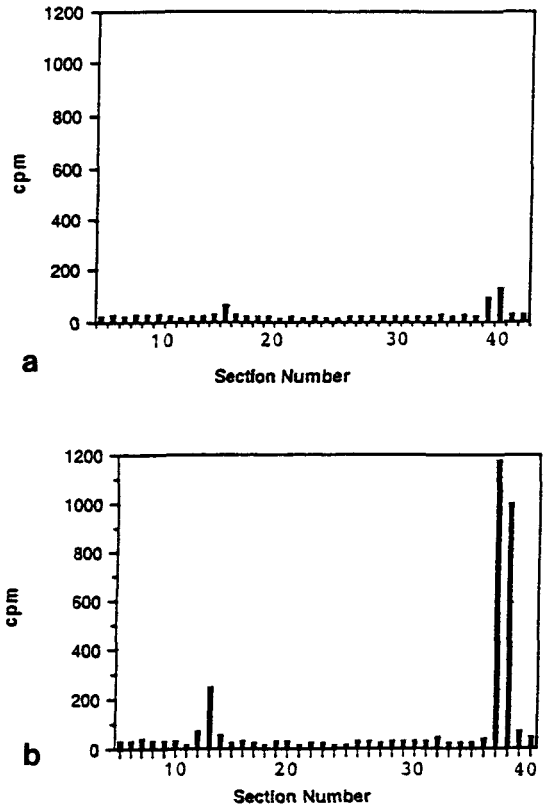


Fig. 5. Specific release of AA and production of PGE2 The media from AA-labeled and intoxicated cells were analyzed for the presence of free AA as described in Materials and Methods The minor peak in the toxin B diagram migrated to the same position as standard PGE2 The radioactivity in similarly treated controls but without toxin was 29 cpm/section (mean) and did not exceed 44 cpm in any sample **a:** Supernatant after toxin A treatment (4 6 μg/ml, 3 h) **b:** Supernatant after toxin B treatment (5,000 $TCD_{50}$, 3 h)

latency period (17–20 min including internalization) of at least toxin B in high doses.

Using well-established inhibitors of PKC-induced phosphorylation we show here that cellular intoxication with *C. difficile* toxins A or B is independent of PKC activity. Neither is cAMP-dependent protein kinase A (PKA) likely to be involved, as increased cellular levels of cAMP were without effect on the intoxication rate. In addition, being an inhibitor of ser/thr-phosphorylation, H-7 inhibits also PKA, but had no effect on intoxication.

In contrast, stimulation of PKC had a strong stimulating effect on the development of the CPEs. However, the toxins did not appear to be PKC-like in that pretreating them with TPA did not potentiate their effects. Neither do the deduced amino acid sequences of the toxins show any significant homology with PKC [Dove et al., 1990; Barroso et al., 1990]. The toxin A molecule contains one sequence motif typical of the phosphorylation site in PKC substrates [Kemp and Pearson, 1990] which may explain the TPA effect on the CPE. On the other hand, toxin B, whose CPE was in fact more sensitive to TPA stimulation, does not contain any such sequence motif. We therefore suggest the toxin effects on their own may overlap at least one effect of PKC; i.e., the toxins share part(s) of a PKC-dependent pathway. However, judging by the conserved amino acid sequences characteristic of many kinases [Hunter, 1987] the toxins themselves are probably not kinases.

Cellular PLA2s may be of several kinds, and can be found in the plasma membrane or in membranes of intracellular vesicles. Intact PLA2 activity was found to be required for the toxin-induced CPE, most likely for the fusion of endocytotic compartments. Consistent with this, the major arachidonic acid metabolites were not found to be involved in the CPE.

It has been shown recently that despite their morphologically similar cytopathic effects toxins A and B do have different cytoskeletal effects [Fiorentini et al., 1989]. Nevertheless, some similarity in their molecular modes of action is supported by the high amino acid homology between the two toxins [von Eichel-Streiber et al., 1992]. The present work suggests that the molecular mechanisms of action of both toxins at some point intersect a PKC-dependent pathway. Both toxins were also found to activate PLA2. This novel activity, as caused by toxin B, is further characterized in a companion paper [Shoshan et al., this issue].

## ACKNOWLEDGMENTS

We thank Dr. Paola Mastrantonio for providing purified toxin A, and Dr. Per Askelöf for the gift of pertussis toxin. Greger Blomquist and Kerstin Andreasson are acknowledged for excellent assistance in the preparation of purified toxin B. This work was supported by the Swedish Medical Research Council (05969), the Italian National Council of Research (203.04.15) and the funds of the Karolinska Institute.

## REFERENCES

Barroso LA, Wang S-Z, Phelps CJ, Johnson JL, Wilkins TD (1990) Nucleotide sequence of *Clostridium difficile* toxin B gene Nucl Acid Res 18(13) 4004

Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling Nature 341 197–205

Caspar M, Florin I, Thelestam M (1987) Calcium and calmodulin in cellular intoxication with *C difficile* toxin B J Cell Physiol 132 168–172

Chardin P, Boquet P, Madaule P, Popoff MR, Rubin EJ, Gill DM (1989) The mammalian G protein *rho*C is ADP ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells EMBO J 8(4) 1087–1092

Dove CH, Wang S-Z, Price SB, Phelps CJ, Lyerly DM, Wilkins TD, Johnson JL (1990) Molecular characterization of the *Clostridium difficile* toxin A gene Infect Immun 58(2) 480–488

von Eichel-Streiber C, Lauffenberg-Feldmann R, Sartingen S, Schulze J, Sauerborn M (1992) Comparative sequence analysis of the *Clostridium difficile* toxins A and B Mol Gen Genet 233 260–268

Fiorentini C, Thelestam M (1991) Review article *Clostridium difficile* toxin A and its effects on cells Toxicon 29(6) 543–567

Florin I, Thelestam M (1983) Internalization of *Clostridium difficile* cytotoxin into cultured human lung fibroblasts Biochim Biophys Acta 763 383–392

Florin I, Thelestam M (1986) Lysosomal involvement in cellular intoxication with *Clostridium difficile* toxin B Microbial Pathogen 1 373–392

Flower RJ, Blackwell GJ (1976) The importance of PLA2 in prostaglandin synthesis Biochemical Pharmacol 25 285–291

Furstenberger G, Rogers M, Fabermann J, Ganss M, Richter H, Marks F (1987) Effects of the phorbol ester TPA and of the ionophore A23187 on phospholipase A2 and C activities in the mouse epidermal cell line HEL 30 Cancer Res Clin Oncol 113 310–318

Hannigan GE, Williams BRG (1991) Signal transduction by interferon-alpha through arachidonic acid metabolism Science 251 204–207

Haystead TAJ, Sim ATR, Carling D, Honnor RC, Tsukitani Y, Cohen P, Hardie DG (1989) Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism Nature 337(5) 78–81

Henriques B, Florin I, Thelestam M (1987) Cellular internalization of *Clostridium difficile* toxin A Microbial Pathogen 2 455–463

Hewlett EL, Sauer KT, Myers GA, Cowell JL, Guerrant RL (1983) Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin Infect Immun 40 1198–1203

Houslay MD (1987) Ion channels controlled by guanine nucleotide regulatory proteins Trends Biochem Sci 12 167–168

Huang K-P (1989) The mechanism of protein kinase C activation Trends Neurobiol Sci 12(11) 425–432

Hunter T (1987) A thousand and one protein kinases Cell 50 823–829

Johnson GL (1982) Cholera toxin action and the regulation of hormone-sensitive adenylate cyclase In Cohen P, van Heyningen S (eds) "Molecular Action of Toxins and Viruses" Amsterdam Elsevier

Kellie S (1988) Cellular transformation, tyrosine kinase oncogenes, and the cellular adhesion plaque BioEssays 8(1) 25–30

Kemp BE, Pearson RB (1990) Protein kinase recognition sequence motifs Trends Biochem Sci 15 342–346

Kimura Y, Okuda H, Arichi S, Baba K, Kozawa M (1985) Inhibition of the formation of 5-hydroxy 6,8,11,14 eicosatetraenoic acid from arachidonic acid in polymorphonuclear leukocytes by various coumarins Biochim Biophys Acta 834 224–229

Lassing I, Lindberg U (1985) Evidence that the phosphatidylinositol cycle is linked to cell motility Exp Cell Res 174 1–15

Lima AAM, Lyerly DM, Wilkins TD, Innes DJ, Guerrant RL (1988) Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro Infect Immun 56(3) 582–588

Lyass LA, Bershadsky AD, Vasiliev JM, Gelfand IM (1988) Microtubule-dependent effect of phorbol ester on the contractility of cytoskeleton of cultured fibroblasts Proc Natl Acad Sci USA 85 9538–9541

Lyerly DM, Krivan HC, Wilkins TD (1988) *Clostridium difficile* Its disease and toxins Clin Microb Rev 1(1) 1–18

Mitchell MJ, Laughon BE, Lin S (1987) Biochemical studies on the effect of *Clostridium difficile* toxin B on actin in vivo and in vitro Infect Immun 55(7) 1610–1615

Moskowitz N, Shapiro L, Schook W, Puszkin S (1983) Phospholipase A2 modulation by calmodulin, prostaglandins and cyclic nucleotides Biochem Biphys Res Comm 115(1) 94–99

Nishizuka Y (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion Nature 308 693–698

Rivedal E, Mikalsen S-O, Sanner T (1990) The non-phorbol ester tumor promoter okadaic acid does not promote morphological transformation or inhibit junctional communication in hamster embryo cells Biochem Biophys Res Comm 167(3) 1302–1308

Shoshan MC, Åman P, Skog S, Florin I, Thelestam M (1990) Microfilament-disrupting *Clostridium difficile* toxin B causes multinucleation of transformed cells but does not block capping of membrane Ig Eur J Cell Biol 53(2) 357–363

Sullivan NM, Pellett S, Wilkins TD (1982) Purification and characterization of toxins A and B of *Clostridium difficile* Infect Immun 35 1032–1040

Takai Y, Kaibuchi K, Matsubara T, Nishizuka Y (1981) Inhibitory action of guanosine 3 ,5 -monophosphate on thrombin-induced phosphatidylinositol turnover and protein phosphorylation in human platelets Biochem Biophys Res Comm 101(1) 61–67

Thelestam M, Florin I, Shoshan MC, Fiorentini C (1992) Interaction of *Clostridium difficile* toxin B with cellular functions In Witholt B (ed) "Fifth European Workshop on Bacterial Protein Toxins" Gustav Fischer Verlag, Stuttgart

Turner CE, Pavalko FM, Burridge K (1989) The role of phosphorylation and limited proteolytic cleavage of talin and vinculin in the disruption of focal adhesion integrity J Biol Chem 264(20) 11938–11944

Wang E, Goldberg R (1976) Changes in microfilament organization and surface topography upon transformation of chick embryo fibroblasts with Rous sarcoma virus Proc Natl Acad Sci USA 73 4065–4069

Zollner H (1990) "Handbook of Enzyme Inhibitors" Weinheim, Germany VCH