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Activation of Cellular Phospholipase A2 by *Clostridium difficile* Toxin B

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Abstract *C difficile* toxin B is a potent cytotoxin known to disrupt the microfilaments of cultured cells We have recently shown also increased phospholipase A2 activity in cells treated with toxin B The activity was detected as a toxin-induced, dose-dependent release of ^{14}C-arachidonic acid from prelabeled fibroblasts Here is shown that the toxin elicited a ^{14}C-arachidonic acid release in a cell mutant resistant to the toxin B effect on the microfilaments The toxin-induced release was further characterized using fibroblasts. Within 20 min high doses of toxin B (6 μg/ml) elicited a release which increased exponentially with time Of the major membrane phospholipids the lipase activity affected mainly phosphatidyl ethanolamine Neither cycloheximide nor pertussis toxin treatment of target cells inhibited the toxin-induced release, while it could be increased with 12-*O*-tetradecanoylphorbol-13-acetate Our results also suggest a toxin-mediated increase in phospholipase C activity occurring at a later stage than the phospholipase A2 activation

We conclude that the ability of toxin B to induce phospholipase activation represents a hitherto unrecognized toxin B effect which is neither a cause nor a consequence of toxin-induced microfilament disorganization

Key words: *Clostridium difficile* cytotoxin, phospholipids, phospholipases, arachidonic acid

Clostridium difficile elaborates at least two protein toxins which together cause antibiotic-associated diarrhoea and colitis [Lyerly et al., 1988]. Toxin A is an enterotoxin and a weak cytotoxin with a cytopathic effect (CPE) morphologically similar to that of toxin B, which is a potent cytotoxin. The CPEs on cultured cells involve rearrangements of the microfilament system of the cytoskeleton, which leads to cell rounding [Mitchell et al., 1987; Fiorentini and Thelestam, 1991]. We have shown that both toxins activate phospholipase A2 (PLA2) [Shoshan et al., this issue]. The PLA2 activation was not a consequence of microfilament disruption and/or cell rounding per se, as shown with cytochalasin D, which causes cell rounding by direct interaction with microfilaments without eliciting an AA release.

PLA2 releases free fatty acid from the *sn*−2 position of phospholipids which are thus converted into lysophospholipids potentially capable of lysing cells. Some fatty acids and lysophospholipids interact with cytoskeletal components and/or protein kinase C (PKC), which in turn regulates many cellular components [Nishizuka, 1984; Huang, 1989]. PLA2s specific for arachidonic acid in the *sn*−2 position are the key enzymes of the arachidonic acid cascade, generating a variety of biologically active metabolites, among them the inflammatory mediators prostaglandins and leukotrienes. Mammalian PLA2s belong to either the well-studied low-molecular weight forms (12–18 K) which are usually secreted [Mansbach, 1990] or the more recently described cytosolic high-molecular weight forms (85–100 K) more likely to be involved in the arachidonic acid cascade [Clark et al., 1991; Leslie, 1991]. The 100K enzyme as well as a membrane-bound form of 170 K [Pind and Kuksis, 1989] also contain lysophospholipase activity. Agents known to induce PLA2 activation

Abbreviations used AA, ^{14}C-arachidonic acid, BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N ,N -tetraacetic acid, CPE, cytopathic effect, DAG, diacylglycerol, H-7, 1-(5-isoquinoline sulfonyl)-2-methyl piperazine dihydrochloride, HBSS, Hanks' balanced salt solution, PA, phosphatidic acid, PC, phosphatidyl choline, PEA, phosphatidyl ethanolamine, PGE2, prostaglandin E2, PI, phosphatidyl inositol, PKC, protein kinase C, PLA2, phospholipase A2, PLC, phospholipase C, TCD_{50}, tissue culture dose causing 50% CPE, TNF, tumor necrosis factor, TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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include bradykinin, tumor necrosis factor, and cholera toxin [Weiss and Insel, 1991; Neale et al., 1988; Peterson et al., 1991].

The purpose of this work was to characterize the toxin B-mediated activation of PLA2, including its relation to the cytoskeletal effect.

MATERIALS AND METHODS

Cells

Human embryonic lung fibroblasts (MRC-5) were grown in Eagle's minimum essential medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% newborn bovine serum, 5 mM *L*-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Wildtype and mutant [Florin, 1991] cells of diploid Chinese hamster lung fibroblasts (Don cells; ATCC no CCL 16) were grown in the same medium with the same supplements although with 10% fetal bovine serum. The supplemented media will be referred to as growth media. All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Toxin B

Toxin B was prepared from *C. difficile* strain VPI 10463 according to Shoshan et al. [1990]. A Superose 6 FPLC gel filtration step was added to remove a 50 K contaminant. However, as activity losses after gel filtration approached 95% and since Superose-purified toxin elicited a release of radioactive label similar to that elicited by MonoQ-purified toxin, the latter was routinely used. For the experiments, the titer of the toxin solutions was, unless otherwise stated, 5,000 TCD_{50}, determined as described [Caspar et al., 1987], and corresponded to approximately 0.2 μg toxin/ml.

Preparations from a *C. difficile* toxin-negative strain were obtained as earlier described [Florin and Thelestam, 1991]. Goat antiserum to toxin B was a generous gift from Dr. D.M. Lyerly, VPI, Blacksburg, VA.

Chemicals

Radiochemicals were from Amersham Sweden AB, Solna, Sweden. Aquasol liquid scintillation cocktail was from DuPont NEN Products Division, Dreieich, Germany. 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and diacylglycerol kinase inhibitor I were from Calbiochem Corporation, La Jolla, CA. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), 1-(5-isoquinoline sulfonyl)-2-methyl piperazine dihydrochloride (H-7), staurosporine, calcium ionophore A23187, Quin-2AM, pertussis toxin, phospholipids, and lipid standards were from Sigma Chemical Co, St. Louis, MO. Organic solvents were from Merck, Darmstadt, Germany.

Release of AA

Cells grown to confluency in 96-well microtiter plates were incubated with ^{14}C-arachidonic acid (AA) (0.1 μCi/ml) in growth medium for 16–18 h at 37°C for optimal incorporation into the *sn*−2 position of membrane phospholipids [Furstenberger et al., 1987]. The labeling efficiency was about 40% of added AA. The medium was removed and the cells washed 3 times with Hank's balanced salt solution (HBSS) before addition of media with or without additives (see Table I), and continued incubation at 37°C. In experiments requiring preincubation of cells with a drug, preincubation ended with a change of all media, including those of controls, to fresh media with or without additives, including toxin. After the time intervals indicated in Table I, 180 μl of the medium of each sample was removed, and the radioactivity was measured by liquid scintillation counting in 1.5 ml of Aquasol. At least quadruplicate samples were assayed (triplicate in Fig. 1) at each time point, and each experiment was performed at least twice.

Analysis of Membrane Phospholipids

Cells grown to confluency in 6-well plates were labeled with AA and washed as described above before addition of toxin B and continued incubation at 37°C. After 2 and 3.5 h cells were washed 3× with HBSS and treated with EDTA (5 mM in HBSS) for 1 min, whereupon they were scraped off and removed to glass tubes. The phospholipids were analysed according to Bradova et al. [1990]. Briefly, they were extracted twice with $CHCl_3$: methanol (2:1) and the solvent was evaporated in vacuo. The obtained lipids and lipid standards were dissolved in 20–30 μl of $CHCl_3$: methanol (2:1) and spotted onto silica gel plates (DC-60, Merck, Darmstadt, Germany) preactivated by heating at 100°C for at least 1 h. The samples were chromatographed twice in $CHCl_3$: methanol:2-propanol:0.25% KCl:ethylacetate (30:9:25:6:27). The lane of each sample was divided into 3 mm strips which were scraped off into vials to which 1.5 ml Aquasol was added for liquid scintillation counting. Untreated control cells were similarly analysed. The experiment was performed twice for each time point.

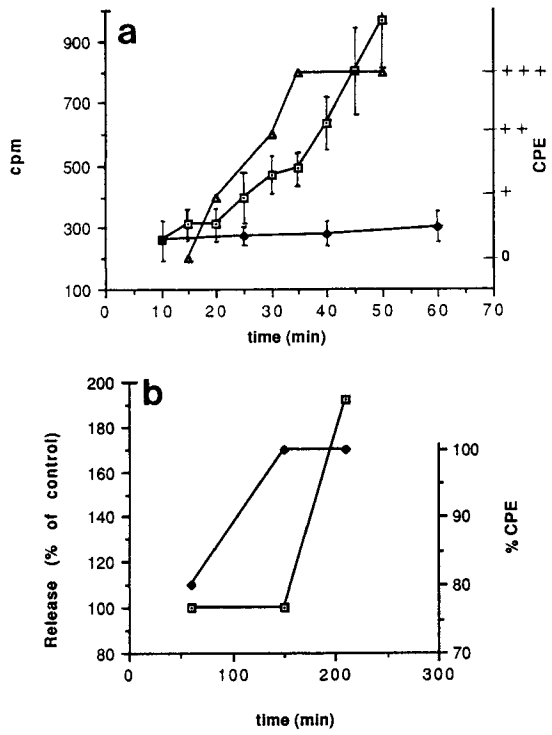

Fig. 1. AA release induced by toxin B. Toxin was added to prelabeled cells and the AA release monitored as described in Materials and Methods. The parallel development of the CPE is shown on the right-hand Y-axis. The figures each show one experiment of two. **a:** Toxin dose 10^5 TCD_{50}. Triplicate samples were taken every 5 min. The equation of the graph is $y = 179.5556 \times 10^{(0.014x)}$ ($r = 0.99$). ⊡, growth medium with toxin B; ◆, growth medium only; ▲, CPE (+ denotes a CPE between 10 and 25%, and +++ a CPE of 75% or more). **b:** Toxin dose 2×10^3 TCD_{50}. Quadruplicate samples were taken at each time point. ⊡, release from intoxicated cells (% of release in control cells) ◆, % CPE.

Analysis of Diacylglycerol Levels

Cells grown to confluency in 6-well plates were labeled with AA and washed as described above before addition of toxin B and continued incubation at 37°C. After 90 min diacylglycerol kinase inhibitor I and staurosporine (5 μM and 20 nM, respectively) were added simultaneously to samples in order to i) inhibit conversion of diacylglycerol (DAG) to phosphatidic acid (PA), and ii) minimize possible PKC activity induced by raised levels of DAG. At 2.5 or 3.5 h after toxin addition cells were washed 3× with HBSS and the levels of DAG were analysed as follows (Dr. E. Nånberg, Department of Pathology, University Hospital, Uppsala, Sweden; personal communication): The cells were treated with cold methanol at 4° for 30 min, whereupon they were scraped off and removed to a glass tube. One volume of $CHCl_3$ and ½ vol of 1 M NaCl were added and the extracts were vortexed vigorously. The $CHCl_3$ phase was collected, washed with 1 M NaCl, and evaporated in vacuo. The obtained lipids and lipid standards were dissolved in 20–30 μl of $CHCl_3$:methanol (2:1) and spotted onto silica gel plates pretreated with 1% K^+-oxalate and heating to 100°C for at least 1 h. The samples were developed in hexane:diethylether:methanol:acetic acid (45:10:1.5:1). After exposure to ammonia vapor (20 min) lipid spots were detected with iodine vapor. The lane of each sample was divided into 3 mm strips which were scraped off into vials to which 1.5 ml Aquasol was added for liquid scintillation counting. Untreated control cells were similarly analysed. The experiment was performed twice.

RESULTS

Toxin B-Induced Release of ^{14}C-Arachidonic Acid From Labeled Cells

The CPE caused by the toxin dose used in all experiments (5×10^3 TCD_{50}, unless otherwise stated) appeared after 45 min, while the same dose elicited a barely detectable AA release (~120% of controls) only after 90 min. A high dose (10^5 TCD_{50}) of toxin B added to AA-labeled MRC-5 cells caused an exponential release of label into the surrounding medium which was detectable after 25 min, i.e., shortly after the onset of the CPE (Fig. 1a). With lower doses the release appeared after a longer lag period (Fig. 1b). Thus, for each specific dose, the dose-dependent lag period of the CPE [Florin and Thelestam, 1983] was invariably shorter than the likewise dose-dependent lag period of the release.

The results presented in the tables and figures represent samples taken at various time points up to 3.5 h after toxin addition. Assays after longer time may be misleading, as released AA is recycled into cells [Furstenberger et al., 1987]. Under these conditions radioactive label release was not due to unspecific leakage of membrane phospholipids (Shoshan et al., this issue), or to leakage of cytosolic free AA, since no radioactive leakage occurred from MRC-5 cells prelabeled according to Thelestam [1988] with ^{3}H-uridine (1 μCi/ml) for 1 h before addition of toxin (data not shown).

An excess of partly purified preparations from a toxin-negative strain of *C. difficile*, did not elicit any release (not shown), an observation which confirms that the AA release was not elicited by contaminants (see Toxin B, in Materials and Methods section). There was further-

TABLE I. Effects of Tested Compounds on Toxin B-Induced AA Release*

Treatment	Concentration	Preincubation	AA Release (% of release induced by toxin only)[a]	Parallel effect on CPE
Compounds affecting phospholipases				
Quinacrine	5 μM	60 min	65–73	None
Neomycin	15 mM	45 min	64–84	None
Compounds affecting PKC				
TPA	50 ng/ml	0 min	192 ± 42	stim.onset[b]
H-7	50 μM	60 min	88–94	None
Staurosporine	10 nM	60 min	92–106	None
Treatments affecting Ca^{2+}				
A23187[c]	50 μM		189 ± 5	None
Depletion of extracellular Ca^{2+}			100 ± 15	N.D.[d]
BAPTA	5 μM	45 min	90–91	None
Quin-2AM	100 μM	0 min	90–94	None
Other compounds				
Cycloheximide	100 μM	2 h	100–103	None
Pertussis toxin	1 μg/ml	4 h	78–119	None

*The toxin dose is 5,000 TCD_{50} Samples for liquid scintillation assays of the supernatant were taken 3 5 h after addition of toxin B to cells

[a]The values are the averages of quintuplicate samples At least two experiments per treatment were performed When three or more experiments were done, the average ± SD is given

[b]See Shoshan et al , this issue

[c]A23187 was added at 45 min after toxin addition

[d]N D = no data

more no AA release after exposure to toxin treated with neutralizing antitoxin (dil. 1:75) or heated to 56°C for 30 min.

As a positive control of increased cellular PLA2 activity cells were preincubated 50 min with 50 ng/ml TPA before addition of 50 μM A23187 [Fürstenberger et al., 1987]. Samples taken 70 min after toxin addition showed a release of the same magnitude as that elicited by 5×10^3 TCD_{50} toxin B after 3.5 h. When AA-labeled MRC-5 cells were preincubated with the PLA2 inhibitors quinacrine (5 μM for 1 h) (Table I) or 4-bromophenacylbromide (10 μM for 25 min) the toxin-induced AA release after 3.5 h was reduced. However, since PLA2 has been suggested to be involved also in intracellular membrane fusion and endocytosis [see Moskowitz et al., 1983, for references], the interpretation of these results is uncertain. We believe the main effect of quinacrine on intoxication with toxin B is to inhibit the later stages of endocytosis (Shoshan et al., this issue). The use of PLA2 inhibitors in studies on endocytosed agents is therefore of limited value.

The development of the CPE in Don wildtype cells is similar to that in MRC-5 cells [Florin, 1991]. A mutant of Don cell origin which is 10^4 times less sensitive than the wildtype to the CPE of toxin B has been isolated [Florin, 1991]. In labeled wildtype Don cells high doses of toxin B (1.5×10^4 TCD_{50} or more) caused cell rounding and AA release (Fig. 2a), while the same doses applied to the mutant did not cause cell rounding but did elicit an AA release of the same order as that from the wildtype (Fig. 2b). Thus, the CPE and PLA2 activation are separate toxin activities.

The ability of toxin B to activate target cell PLA2 is neither a cause, nor a consequence of the toxin effect on the cytoskeleton. This conclusion is supported also by results presented below (see below: Toxin-Induced Cell Lysis).

Characterization of Toxin-Induced AA Release

The role of protein synthesis. AA-labeled MRC-5 cells were preincubated with cycloheximide (100 μM) for 2 h before toxin addition. This treatment did not affect the subsequent toxin-induced release (Table I) indicating that, unlike PLA2 activation induced by tumor necrosis factor (TNF) [Neale et al., 1988] or by cholera toxin [Peterson et al., 1991], the toxin B-induced activation of PLA2 did not require protein synthesis. The short lag period for AA release (approx 25 min including endocytosis) observed with high toxin doses is consistent with this result.

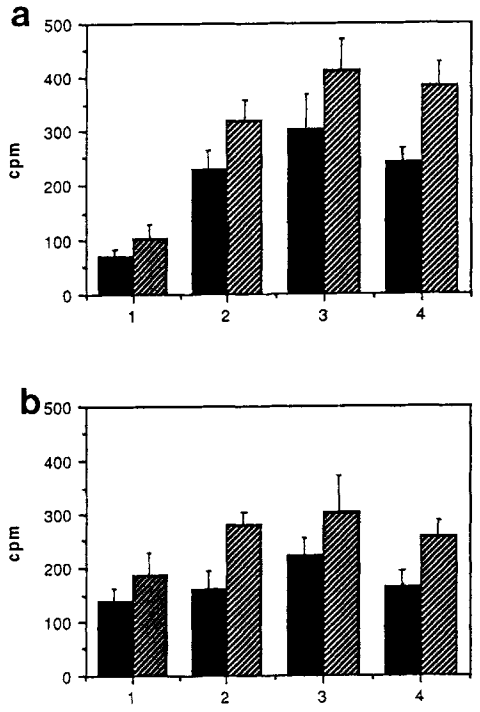

Fig. 2. Toxin-induced AA release from Don wildtype and toxin-resistant mutant cells. Toxin B at different doses was added to prelabeled cells and the AA release measured as described in Materials and Methods. Quintuplicate samples were taken after 40 and 60 min. All wildtype samples showed 100% CPE at these time points. The figure shows one experiment of two. **a:** AA release from Don wildtype cells. **b:** AA release from Don mutant cells. **1:** Control cells. **2:** 1.5 × 10^4 TCD_{50}. **3:** 3 × 10^4 TCD_{50}. **4:** 1 × 10^5 TCD_{50}. ■, release at 40 min after toxin addition; ▨, release at 60 min after toxin addition.

The role of pertussis toxin-sensitive G proteins. Membrane PLA2 may be regulated by G-protein(s) which in turn are coupled to a membrane receptor [Axelrod et al., 1988]. Labeled fibroblasts were preincubated with pertussis toxin (1 μg/ml) for 4 h [Clark et al., 1988] before toxin addition. This treatment did not alter the toxin-induced AA release (Table I), indicating that pertussis toxin-sensitive G-proteins are not involved in toxin B activation of PLA2.

The role of PKC. Tumor-promoting phorbol esters, such as TPA, activate protein kinase C (PKC) [Huang, 1989] and stimulate PLA2 activity via an unidentified mechanism [Fürstenberger et al., 1987; Weiss and Insel, 1991]. When TPA (50 ng/ml) was added to AA-labeled cells concomitantly with toxin B in doses up to 4 × 10^4 TCD_{50}, there was a significant increase in released label after 2 h of incubation (Fig. 3a). The time course of the release was followed when TPA was added to cells together with toxin (3 × 10^4 TCD_{50}) (Fig. 3b). The resulting release was exponentially increased by TPA, suggesting a synergistic effect rather than an additive. However, PKC did not appear directly involved in the toxin-induced AA release, as preincubation of labeled fibroblasts with the PKC inhibitors H-7 (50 μM) or staurosporine (10 nM) for one hour did not prevent the release (Table I).

The role of calcium. In general, PLA2s are regulated by intracellular Ca^{2+} levels, but Ca^{2+}-independent PLA2s have been found in a variety of cells [Pind and Kuksis, 1989; Hazen and Gross, 1991]. Preincubation of cells for 45 min with either of the intracellularly acting Ca^{2+} chelators 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (15 μM) or Quin-2AM (100 μM) did not prevent toxin-induced AA release. Increasing intracellular Ca^{2+} with the calcium ionophore A23187 only slightly increased toxin-induced AA release, as compared to TPA-stimulated toxin-induced release (Fig. 3a). Possibly the A23187 effect is an unspecific stimulation of all Ca^{2+}-dependent cellular phospholipases, which may also explain why A23187 promoted toxin-induced lysis of cells, while TPA had no such effect (not shown).

To study the role of extracellular Ca^{2+} the following experiment was done: toxin (10^4 TCD_{50}) was allowed to bind to cells by incubation in Ca^{2+}-containing growth medium (in order to ensure normal binding) at 0°C for 15 min before removal of toxin. The cells were then washed with 5 mM EDTA in Ca^{2+}-free HBSS and transferred to Ca^{2+}-free HBSS supplemented with *L*-glutamine and 1% glucose for continued incubation at 37°C. The toxin-induced AA release from cells thus treated was approximately equal to that from similarly toxin-treated cells in Ca^{2+}-containing medium (HBSS or Eagle's minimal medium with 1.3 mM $CaCl_2$, *L*-glutamine and 1% glucose).

In summary, depletion of extracellular or cytosolic Ca^{2+} had no significant effect on toxin-induced AA release. Raising intracellular Ca^{2+} levels increased the release, but this increase may be unspecific.

The role of phospholipase C. AA can be released from cells via an alternative route involving phospholipase C (PLC), which converts phosphatidyl choline (PC) and phosphatidyl inositol (PI) into DAG, from which DAG lipase may liberate AA. DAG may also be rapidly converted

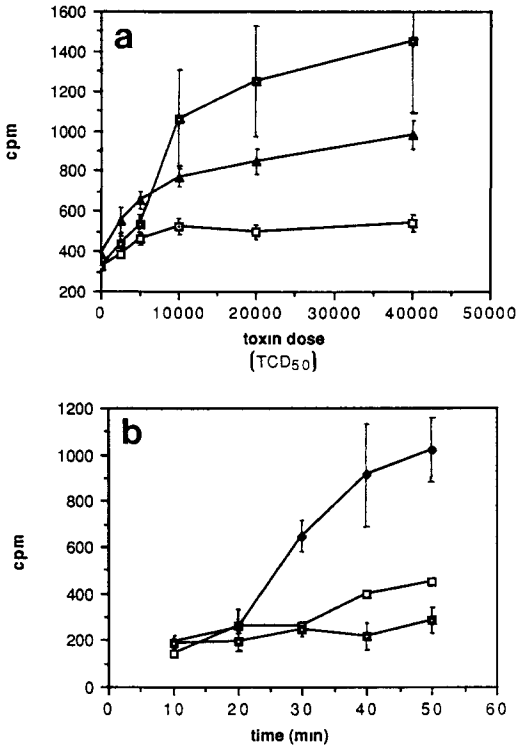

Fig. 3. Effects of TPA and A23187 on toxin-induced AA release **a:** Toxin B at different doses and TPA (50 ng/ml) or A23187 (50 μM, added 45 min after toxin) was added to prelabeled cells and after 2 h the AA release in quadruplicate samples was measured as described in Materials and Methods The figure shows one experiment of two □, toxin only, ■, toxin + TPA, ▲, toxin + A23187 **b:** Time course of toxin-induced (3×10^4 TCD) AA release stimulated with TPA (50 ng/ml) added concomitantly with toxin The figure shows one experiment of two □, toxin only, ◆, toxin + TPA, ■, TPA only

by phosphorylation into phosphatidic acid (PA). In addition to DAG production, PLC activity on a certain species of PI (phosphatidylinositol 4,5-bisphosphate) results in increased levels of inositol 1,4,5-trisphosphate (IP_3). To assess the role of PLC in toxin B-mediated AA release, several experiments were performed.

Indications of PLC activity in target cells were seen in the following experiments: AA-labeled cells were preincubated with neomycin (15 mM; 45 min) in order to block PLC activity. This treatment decreased the toxin-induced AA release by, on average, 25% (Table I). Thus, PLC might to some extent be involved in toxin B-induced AA release. In another experiment analysis of cellular phospholipids after 3.5 h of toxin treatment showed that the normally very low PA levels had doubled, as compared to control cells (Table II). However, PA appeared only when PC and the joint PI + PS (phosphatidyl serine) levels were decreasing, i.e., when most of the preferred substrate of PLA2 (phosphatidyl ethanolamine; PEA) had already been hydrolyzed. Thirdly, cellular DAG levels were analyzed in conjunction with measurements of AA release. To minimize conversion of DAG into PA, a DAG kinase inhibitor was added, and to minimize AA release caused by DAG-activated PKC, staurosporine was added. This treatment altered neither basal nor toxin-induced AA release. At 2.5 h after toxin addition no change in DAG levels was detected in toxin-treated cells as compared to control cells (Table III), while after 3.5 h there was an increase in DAG (Table III). Finally, an experiment kindly performed by Dr. P. Gerwins (Department of Pharmacology, Karolinska Institute, Stockholm, Sweden) showed that within the first 1.5 h after toxin addition (10^4 TCD_{50}) there was no increase in cellular levels of IP_3.

TABLE II. Toxin-Induced Changes in Membrane Phospholipids*

Incubation with toxin (h)	Resulting label in cell phospholipids after toxin treatment (% of controls)			
	PC	PS + PI	PEA	PA
2	100	100	22	100
3.5	92	30	10	215

*AA-labeled fibroblasts were treated with toxin (5×10^3 TCD_{50}) for the time intervals indicated The phospholipids were extracted and analyzed by thinlayer chromatography as described in Materials and Methods The results represent averages of two experiments

TABLE III. Toxin-Induced Change in Diacylglycerol Levels*

Incubation with toxin[a]	DAG[b] levels (% of total radioactive label)	
	Control cells	Toxin-treated cells
I		
2 5 h	0 8	0.9
3.5 h	0 9	2.0
II		
2 5 h	ND[c]	2.0
3 5 h	2 0	3.0

*AA-labeled fibroblasts were treated with toxin (5×10^3 TCD_{50}) for the time intervals indicated DAG kinase inhibitor and staurosporine were added simultaneously after 90 min The DAG species were extracted and analyzed by thinlayer chromatography as described in Materials and Methods
[a]I and II denote two separate experiments
[b]The label comigrated with 1,2-dioctanoyl-sn-glycerol, but not with 1,2-dioleyl-sn-glycerol or 1,2-dioleyl-rac-glycerol
[c]ND = no data

Consequences of Toxin-Induced PLA2 Activation

Toxin-induced alterations in membrane phospholipids. The membrane phospholipid composition of AA-labeled fibroblasts treated with toxin B for 2 and 3.5 h was analysed by thinlayer chromatography, and compared with that of control cells without toxin (Table II). After 3.5 h of toxin treatment the amount of PEA was reduced to 10% of that in control cells. After 3.5 h of toxin treatment the joint amount of PS and PI had decreased to 30% of that in control cells. The resulting lysophospholipids are not radioactive and could thus not be quantitated, but spots co-migrating with lyso-PEA could be seen. There was an increase in PA after 3.5 h (Table II), suggesting an activation of PLC (see above). An additional radioactive peak (average R_f-value 0.66, i.e., closer to the nonpolar end of the chromatogram than all the other peaks) also appeared. It remains unidentified as yet, but did not comigrate with authentic AA (R_f-value 1.0).

Toxin-induced cell lysis. When the amount of toxin-induced release of label reached a certain level in MRC-5 cells (approximately a third of the initial cellbound label), the cells lysed. We propose that toxin B-induced cell lysis is due to increased PLA2 activity. Table IV shows the doses required to cause at least 50% cell lysis within 2 h in four cell types. Although three of them were equally sensitive to the cytoskeletal effect of toxin B, their sensitivities to toxin-induced lysis differed widely, e.g., on Y1 cells the highest dose tested (5×10^5 TCD_{50}) was not lytic although all cells were round. In comparison, a 50 times lower dose lysed MRC-5 cells. In support of the proposed relationship between toxin-induced PLA2 activation and cell lysis, the toxin-induced AA release from Y1 cells was lower than from the other cell types, e.g., a toxin dose of 2×10^4 TCD_{50} was required to double the release of control samples within 3.5 h. This dose would on Don cells double the release within approximately 45 min.

TABLE IV. Sensitivities of Different Cell Types to the Toxin B Lytic Effect

Cell type	Dose which caused lysis within 2 h (TCD_{50})[a]	Dose which caused 50% CPE within 20 h (TCD_{50})
MRC-5	1×10^4	1
Y-1	No lysis[b]	1
Don wt	3×10^4	1
Don mutant	3×10^4	1×10^4

[a]As determined on MRC-5 cells.
[b]Highest dose tested was 5×10^5 TCD_{50}.

The results on the lytic effect of toxin B also support the conclusion that toxin B-induced PLA2 activation is separate from the toxin's cytoskeletal effect, as exemplified by the Don mutant cells which lysed readily despite their high resistance to the CPE.

DISCUSSION

We have shown that the *C. difficile* toxins A and B both induce activation of target cell PLA2 as seen by release of incorporated AA (Shoshan et al., this issue). As the cytoskeletal effect of toxin B, i.e., the CPE, occurred at far lower doses than did the AA release, and as the CPE appeared much earlier than the release with all but the highest toxin doses, the two effects appeared separate. In the present work we confirm this, using a Don cell mutant 10^4 times less sensitive than the wildtype to the CPE of toxin B. In this mutant, toxin B elicited an AA release without a concomitant CPE. Furthermore, cell lysis (the likely consequence of high PLA2 activity) was shown to be independent of development of the CPE in four different cell types (Table III).

Our results also suggest involvement of PLC, as the AA release after 3.5 h was slightly reduced by pretreatment of the cells with neomycin, and as the cellular phospholipid pattern of intoxicated cells showed a concomitant increase in DAG or PA. However, although PLC can initiate an alternative route to release of AA, this is most likely not the major origin of toxin B-induced AA release. The preferred substrates of PLC isozymes are PC and PI species, while the PLA2 activated by toxin B treatment hydrolyzed mainly PEA. Thus, increases in DAG or its phosphorylated form PA appeared only when PC and joint PI + PS levels were decreasing, i.e., when most of the PEA had already been hydrolyzed.

Although secreted PLA2s are generally activated by Ca^{2+} [Mansbach, 1990], cellular PLA2s differ in their sensitivity to intracellular Ca^{2+} levels [Pind and Kuksis, 1989; Hazen and Gross, 1991]. At least one cellular PLA2 species is activated by raised intracellular pH [Harrison et al., 1991]. Exogenous agents may activate cellular PLA2s via a receptor-coupled G-protein, e.g., bradykinin-induced PLA2 activation [Slivka and

Insel, 1988], or via a G-protein independently of a receptor [Axelrod et al., 1988]. Cholera toxin activates PLA2 by a mechanism involving de novo synthesis of an unidentified protein [Peterson et al., 1991]. Tumor necrosis factor (TNF) induces synthesis of a PLA2-activating protein (PLAP) with sequence homology to melittin of bee venom, which activates PLA2 by an unknown mechanism [Clark et al., 1988]. Finally, PKC-activating phorbol esters are known to stimulate PLA2 activity [Fürstenberger et al., 1987; Halenda et al., 1989; Weiss and Insel, 1991]. The complex interactions between these two enzymes are as yet far from clear.

Unlike cholera toxin and TNF, toxin B did not mediate its PLA2-activating effect via synthesis of another protein. Neither did pertussis toxin-sensitive G proteins or activation of PKC appear involved. Phorbol ester treatment synergistically increased the toxin-induced AA release, in agreement with results in other systems [Axelrod et al., 1988; Halenda et al., 1989; Slivka and Insel, 1988]. The finding that PKC inhibitors did not affect toxin B-induced AA release is not contradictory, since PKC activity is not an absolute requirement for PLA2 activation [Halenda et al., 1989]. Increasing or decreasing intracellular Ca^{2+} did not affect toxin-induced AA release, suggesting that the involved PLA2 is either not Ca^{2+}-dependent, or that the toxin releases Ca^{2+} from endogenous stores by an as yet unknown mechanism.

Although toxin B did not appear to induce AA release by a G_i receptor-coupled mechanism, it might still affect such a process from within the cell. One possibility might then be that toxin B acts intracellularly on several GTP-binding proteins (G-proteins), one of which is coupled to PLA2 [Axelrod et al., 1988], while toxin B interaction with other G-proteins, e.g., rho proteins, could account for the effect on microfilaments [Chardin et al., 1989; Ridley and Hall, 1992]. Since G-proteins are implicated as targets for PKC action [Sagi-Eisenberg, 1989], they could be a link between TPA-stimulated CPE and TPA-stimulated AA release.

Apart from the outright cell disintegration caused by high doses of toxin B, phospholipase activation may contribute to toxin-induced alterations in the membrane, e.g., blebbing [Malorni et al., 1990], which, together with phospholipid hydrolysis, may precede toxic cell death [Boobis et al., 1989]. A recent report on the ability of toxin B to induce release of TNF from monocytes [Flegel et al., 1991] is in agreement with toxin-mediated PLA2 activation: toxin B-induced production of AA metabolites, e.g., prostaglandins, has been suggested (see Shoshan et al., this issue) and these substances have been shown to induce TNF gene expression [Mohri et al., 1990]. In short, toxin B activities in cell systems more pertinent than fibroblasts to the in vivo pathophysiological effects of toxin B may lead to release of cytotoxic and/or inflammatory agents of importance for long-term in vivo effects. AA metabolites might also be involved in toxin A or B-mediated increase in vascular permeability [Lyerly et al., 1988], since PLA2 activation and AA metabolites have recently been suggested to mediate the hitherto unexplained effects of cholera toxin on vascular permeability and electrolyte transport [Peterson et al., 1991].

In summary, the present work describes a novel toxin B activity which is neither the cause of the cytoskeletal effect of the toxin, nor a consequence of it. The described activation of PLA2 may have consequences for the in vivo effects of the toxin.

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