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Bacterial Phospholipases C

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INTRODUCTION

Since the last review of the bacterial phospholipases C (106), several new enzymes have been discovered and the application of molecular biological techniques has transformed our knowledge of this important group of proteins. It has become apparent that many of the phospholipases C are structurally related, and this has revealed new directions for the analysis of structure-function relationships. The natural function of phospholipases C may be to secure supplies of phosphate, and the regulation of some phospholipase C genes by exogenous phosphate levels supports this hypothesis. The reasons why some phospholipases C are also toxic and cytolytic is becoming clearer, and the possible roles of some enzymes in the pathogenesis of disease has been investigated. In some cases cytolysis may be an important mechanism by which toxic effects are elicited, but equally apparent are the more subtle effects of phospholipases C on the metabolism of cells, which could play an important role in the disease process. The analysis of the roles of these enzymes is further complicated by the complex interactions which may occur with other bacterial proteins.

The interaction of phospholipases C with membrane phospholipids has been exploited in several ways. They can be used as probes to explore the phospholipid composition of membranes or to mimic the actions of eukaryotic phospholipases C on cell metabolism. Knowledge of the precise mechanisms of interaction with membranes may prove useful for delivering membrane active drugs. Unlike many bacterial toxins, internalization of the protein is not required for toxicity, and this has attracted at least one group of workers to explore the possibility that phospholipase C, linked to a suitable antibody, can form the basis of an active cytotoxic agent with potential therapeutic utility.

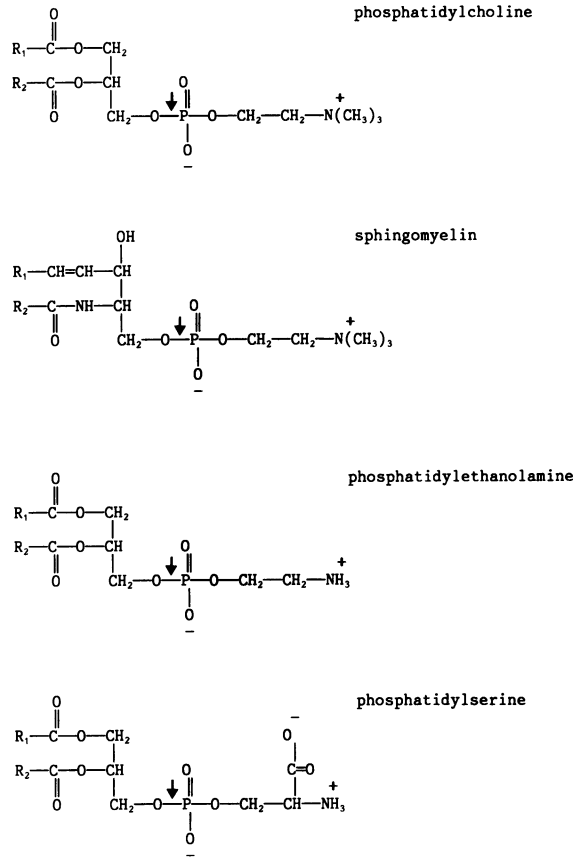

FIG. 1. Site of cleavage of the major phospholipids (arrowed) by phospholipases C.

PRODUCTION, PURIFICATION, AND ASSAY OF PHOSPHOLIPASES C

The phospholipases C are characterized by the site of cleavage of phospholipids (Fig. 1), which distinguishes them from phospholipases A and D, which are also produced by some bacteria. For the purposes of this review, sphingomyelinases C are also considered members of the phospholipase C group. Phospholipases C have been isolated from a wide variety of gram-positive and, more recently, gram-negative bacteria (Table 1). All of the enzymes are single polypeptide proteins which are found in the culture medium, and the deduced amino acid sequences of these proteins, where known, have revealed typical signal sequences (56, 70, 84, 115, 118, 129, 130, 139, 166, 175, 181, 192). On solid media the production of phosphatidylcholine-hydrolyzing enzymes has often been detected as a zone of opalescence surrounding colonies grown on an egg yolk emulsion supplemented agar, and the neutralization of this effect by specific antisera has formed the basis of a diagnostic test for *Clostridium perfringens* (the Nagler reaction). The proteins have been purified by a variety of techniques, of which perhaps the most elegant use affinity chromatography; a column of immobilized egg yolk phosphatidylcholine has been used to purify the *Bacillus cereus* phosphatidylcholine-preferring phospholipase C (PC-PLC) (88) and the *C. perfringens* enzyme (162). An alternative procedure for purifying the *Pseudomonas aeruginosa* phospholipase C relies on binding to substituted ammonium groups on DEAE-Sephacel and elution with tetradecyltrimethylammonium bromide (12).

The activity of phospholipases C can be monitored in solution by using egg yolk phosphatidylcholine (162, 166) or by spectrophotometrically measuring the hydrolysis of chromogenic derivatives of phospholipids such as *p*-nitrophenylphosphorylcholine (pNPPC) (78), a structural derivative of phosphatidylcholine, or *N*-omegatrinitrophenol-aminolauryl-sphingosylphosphorylcholine (53), a structural derivative of sphingomyelin. These chromogenic derivatives may not faithfully indicate the true degree of phospholipid hydrolysis because of the absence of extended hydrocarbon tails which are important for efficient substrate hydrolysis (45), and this is reflected in the high K_m value for pNPPC hydrolysis by *C. perfringens* alpha-toxin (78). Many workers have assumed that the hydrolysis of pNPPC was indicative only of hydrolysis of phosphatidylcholine, but this is not the case, and the reported hydrolysis of pNPPC by the *B. cereus* sphingomyelinase (121) is not surprising since the head groups of phosphatidylcholine and sphingomyelin are identical. This result further indicates the importance of hydrocarbon tails for correct substrate recognition. A hydrocarbon tail is present on the chromogenic substrate dioctanoylthiophosphatidylcholine (149), but the head group is significantly altered by the chromogen and the use of this substrate may yield equally misleading results. A similar argument may be applied to the fluorometric assay recently described by Thuren and Kinnunen (164). A more complex procedure for measuring phospholipase C activity involves separation of phospholipid digestion products by thin-layer chromatography (160). For precise measurement of phospholipid digestion, radiolabeled substrates may be used; alternatively the release of acid-soluble phosphorous can be measured (88, 160, 162). Perhaps the method which most closely mimics the interaction of the phospholipase C with substrate involves the use of artificial phospholipid bilayers (110), but the measurement of phospholipid hydrolysis, especially in mixed phospholipid bilayers, can prove complex.

GRAM-POSITIVE PHOSPHOLIPASES C

Zinc-Metalloenzymes

The *B. cereus* PC-PLC, *C. perfringens* alpha-toxin, *Clostridium bifermentans* PLC, *Listeria monocytogenes* PLC-B, and *Clostridium novyi* gamma-toxin form a group of related enzymes which contain essential zinc ions and are reversibly inactivated by EDTA or *o*-phenanthroline (54, 64, 76, 143, 160, 169). It also seems likely that the phospholipases C produced from *Clostridium absonum* and *Clostridium barati*, which are antigenically and genetically related to the *C. perfringens* alpha-toxin (112, 171), are also zinc-metallophospholipases C.

The *C. perfringens* alpha-toxin and *B. cereus* PC-PLC are the most intensively studied; investigations of the latter were prompted by the finding that the *C. perfringens* enzyme was a potent toxin (96, 97, 105) with hemolytic (139, 166), lethal (162, 168), dermonecrotic (100), vascular permeabilization (158), and platelet-aggregating (114, 157) properties. Speculation that the *B. cereus* PC-PLC enzyme may have similar properties was proved to be unfounded (123), but as a result of extensive investigations, this protein has assumed the status of a prototype phospholipase C. All of the zinc-metallophospholipases C are single polypeptides, and *B. cereus* PC-PLC and *L. monocytogenes* PLC-B are posttranslationally activated by the removal of 14 (70) or 26 (179) N-terminal amino acids, respectively. The detection of different molecular size forms of the *L. monocytogenes* PLC-B

TABLE 1. Bacterial phospholipases C[a]

Source of enzyme	Name	Gene cloned	Molecular mass (Da)[b]	Substrate specificity[c]	Ion requirements	Hemolysis
B. cereus	PC-PLC	Yes (56, 70)	28,520 (70)	PC, PE, PS (88, 123)	Zn^{2+}, Ca^{2+} (88, 123)	− (88)
	SMase	Yes (189)	34,233 (189)	SPM (67)	Mg^{2+} (67)	h[d] (67)
	PI-PLC	Yes (77)	34,466 (77)	PI, LPI (58)	None (65)	−
B. thuringiensis	PI-PLC	Yes (63)	34,515 (63)	PI, LPI (68, 161)	NR	NR
C. bifermentans	PLC	Yes (175)	42,746 (175)	NR[e]	NR	± (175)
C. novyi	γ-Toxin	No	30,000 (160)	PC, SPM, LPC, PE, PI, PG (160)	Zn^{2+}, Ca^{2+}, Mg^{2+} (160)	+ (160)
	PI-PLC	No	30,000 (159)	PI (159)		
C. perfringens	α-Toxin	Yes (84, 115, 139, 166, 175)	42,500 (176)	PC, SPM, PS, LPC (76, 162)	Zn^{2+}, Ca^{2+} (76)	+ (166)
L. monocytogenes	PLC-A	Yes (83, 101)	34,000 (83, 101)	PI (101)	Non (101)	− (83, 101)
	PLC-B	Yes (179)	39,000 (179)	PC, PE, PS, SPM (54, 179)	Zn^{2+} (54)	± (54)
S. aureus	β-Toxin	Yes (130)	34,546 (130)	SPM, LPC (136, 186, 187)	Mg^{2+} (136, 186, 187)	h (136, 186, 187)
	PI-PLC	No	20,000–30,000 (95)	PI, LPI (42)	None (66)	
P. aeruginosa	PLC-H	Yes (31)	78,352 (31)	SPM, LPC, PC (12, 118)	NR	+ (12, 118)
	PLC-N	Yes (118)	73,455 (118)	PC, PS (118)	NR	− (118)
P. cepacia	PLC	Yes (178)	72,000 (178)	PC, SPM (178)	NR	+ (178)
S. hachijoensis	PLC	No	18,000 (116)	PC (116)	Mg^{2+} (116)	NR
A. calcoacetius	PLC	No	NR	PC, SPM, PE, PS (82)	Mg^{2+} (82)	− (82)
	PLC	No	NR	PC, SPM, PE, PS (81)	Mg^{2+} (81)	+ (81)
U. urealyticum	PLC	No	NR	pNPPC (39)	NR	NR
Leptospira interrogans	SMase[f]	No	NR	SPM, PC (14)	Mg^{2+} (14)	h (14)
L. pneumophila	PLC	No	50,000–54,000 (9)	PC (9)	NR	− (9)

[a] Numbers in parentheses are references.
[b] Molecular masses have been calculated for the mature exported protein without additional metal ions.
[c] PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SPM, sphingomyelin; PI, phosphatidylinositol; LPI, lysophosphatidylinositol; LPG, lysophosphatidylglycerol; PG, phosphatidylglycerol.
[d] h, hot-cold hemolysis on sphingomyelin-rich erythrocytes.
[e] NR, not reported.
[f] SMase, sphingomyelinase.

in culture fluid suggested that activation occurred after export from the cell (179). All of the characterized enzymes are able to hydrolyze phosphatidylcholine (54, 110, 123, 160), and other phospholipids are hydrolyzed with various efficiencies; the *C. perfringens* alpha-toxin, *C. novyi* gamma-toxin, and *L. monocytogenes* PLC-B are also able to hydrolyze sphingomyelin (54, 76, 160, 162, 179). Phosphatidylinositol and phosphatidylglycerol are additionally hydrolyzed by the *C. novyi* gamma-toxin (160). The reason why some enzymes are activated outside of the cell whereas others are produced as active enzymes suggests that some of these enzymes are potentially toxic to the cell. In this respect it is interesting that phosphatidylglycerol, an important component of the bacterial cell membrane, is hydrolyzed by the *B. cereus* PC-PLC but not by *C. perfringens* alpha-toxin (Table 1). Ether-linked phospholipids are hydrolyzed with various efficiencies; the *B. cereus* PC-PLC was able to hydrolyze these compounds (45), whereas an ether-linked phosphatidylcholine analog was a potent inhibitor of the *C. perfringens* enzyme (138). There is some evidence that subtle differences in the active-site architecture are responsible for these substrate preferences: the replacement of zinc ions in the active site of the *B. cereus* PC-PLC with cobalt ions enabled the enzyme to hydrolyze sphingomyelin (121).

The genes encoding some of the zinc-metallophospholipases C have been isolated and characterized (56, 70, 84, 115, 139, 166, 167, 175, 179), and the gene encoding the *C. perfringens* alpha-toxin has been shown to be chromosomally located (24). The deduced amino acid sequences of the proteins show significant homology up to approximately residue 250 (84, 166, 179). After this point the *C. perfringens* alpha-toxin and *C. bifermentans* PLC possess an additional C-terminal domain (Fig. 2). From the observed homologies, it seemed likely that the first 250 residues (N-terminal domain) encode the phospholipase C activity. This suggestion has recently been proven for the *C. perfringens* alpha-toxin, because a truncated form of the protein, corresponding to the *B. cereus* PC-PLC, retained the phospholipase C activity but showed markedly reduced sphingomyelinase, hemolytic, and lethal activities (168). The C-terminal domains of the *C. perfringens* alpha-toxin and *C. bifermentans* PLC appear to confer sphingomyelin-hydrolyzing and hemolytic properties on these enzymes, but only for the *C. perfringens* alpha-toxin does it also confer toxic properties on the protein. The nontoxic nature (and low hemolytic activity) of the *C. bifermentans* enzyme can be explained because the turnover rate of the enzyme is much lower than that of the *C. perfringens* alpha-toxin (Table 2), and, indeed, this was the reason for the low toxicity of this phospholipase proposed by Miles and Miles 45 years ago (104). In support of this suggestion, it has been shown that the stoichiometric relationship between phospholipase C activity (egg yolk phospholipid-hydrolyzing activity [166, 175]) and hemolytic activity (hemolytic units [166, 175]) is similar for both enzymes (1:4.5 and 1:5.2). The function of the C-terminal domains is not clear, but it is known that chemical modifi-

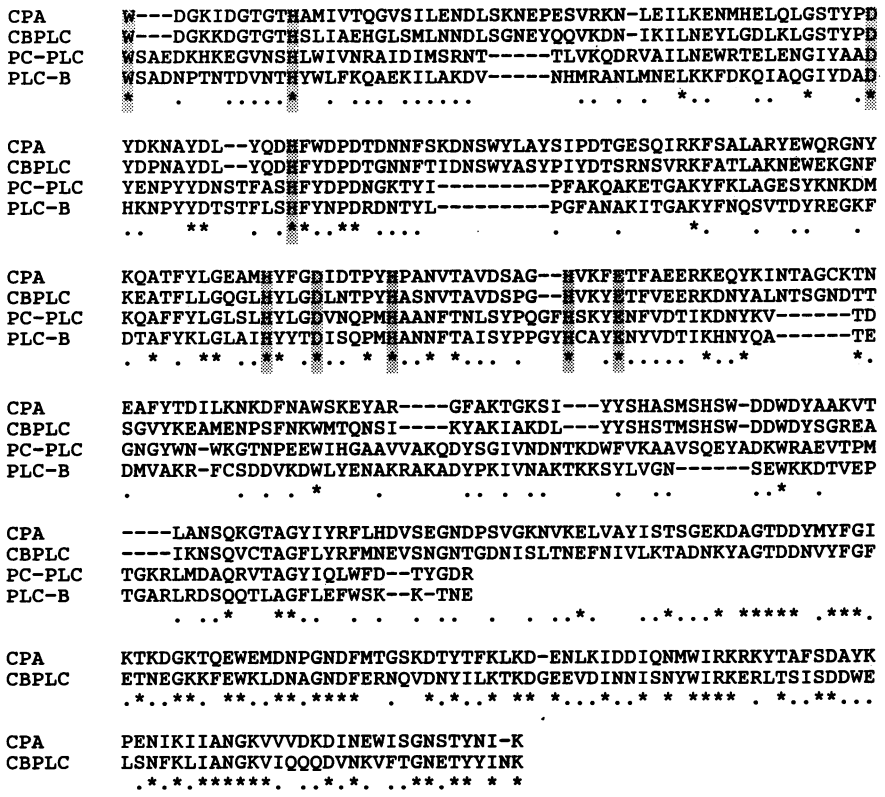

FIG. 2. Alignment of the deduced amino acid sequences of the *C. perfringens* alpha-toxin (166), *C. bifermentans* phospholipase C (175), *B. cereus* PC-PLC (56), and *L. monocytogenes* PLC-B (179). Residues known to be involved in the coordination of zinc ions in the *B. cereus* PC-PLC (64) are shaded.

cation of tyrosine residues abolishes hemolytic, lethal, and platelet-aggregating properties of the *C. perfringens* alpha-toxin (140). Perhaps significantly, the reported homology between the C-terminal domain of the *C. perfringens* alpha-toxin and the N terminus of arachidonate-5-lipoxygenase, a eukaryotic lipid-metabolizing enzyme, includes five aligned tyrosine residues (168). It seems possible that these hydrophobic tyrosine residues are similarly involved in the recognition of hydrocarbon substrates.

Crystallographic (64) and chemical modification (5, 6, 86, 87) studies of the *B. cereus* PC-PLC have provided an insight into the molecular architecture of this enzyme and, by extrapolation, the possible tertiary structures of homologous regions in the other zinc-metallophospholipases C. The enzyme is composed of seven helices, forming a twisted barrel structure. Three zinc ions, one of which is loosely bound, coordinate with amino acids from different helices and thus conformationally restrain the molecule. Zinc-coordinating histidine, glutamic acid, tryptophan, and asparagine residues are located in similar positions in other zinc-metallophospholipases C (Fig. 2), an observation which provides further confirmation of the relationship of these proteins. The importance of histidine residues for zinc coordination in the *C. perfringens* alpha-toxin has been confirmed by chemical modification of these residues. The inactivation of phospholipase C activity by diethylpyrocarbonate could be demonstrated only after EDTA pretreatment, suggesting that the zinc ions also bind to these

TABLE 2. Properties of the zinc-metallophospholipases C[a]

Enzyme	C-terminal domain	Phospholipase C activity (Eyu/mg)[b]	Sphingomyelinase activity[c]	Hemolytic activity (Hu/mg/30 min)[d]	Lethality (μg/mouse)
C. perfringens alpha-toxin	Yes	252 (175)	Yes (139, 168)	520,000 (175)	0.03–0.1 (175)
C. bifermentans PLC	Yes	5 (175)	NR[e]	12,000 (175)	1–5 (175)
L. monocytogenes PLC-B	No	Yes (54)	Yes (54)	0 (54)	>25 (54)
B. cereus PC-PLC	No	Yes (123)	No[f] (123)	0[g]	>30 (122)

[a] Numbers in parentheses are references.
[b] Eyu, egg yolk phospholipid-hydrolyzing units (175).
[c] Detected by thin-layer chromatography.
[d] Hu, hemolytic units (175).
[e] NR, not reported.
[f] Sphingomyelin hydrolysis reported on replacement of zinc ions with cobalt ions (121).
[g] Weak hemolytic activity has been reported (89).

residues in this protein (169). The protein-stabilizing effect of the zinc ions may account for the remarkable thermal stability reported for the *B. cereus* PC-PLC and *C. perfringens* alpha-toxin; either enzyme can survive heating to 100°C for short periods (123, 162).

The active site of the *B. cereus* PC-PLC has been tentatively identified by cocrystallizing the protein with phosphate ions (64). This study demonstrated phosphate binding to all three zinc ions, displacing water molecules in the process. A similar involvement of zinc ions in the binding of phosphate to *Escherichia coli* alkaline phosphatase has been reported (152), and it has been suggested that this similarity may be more than coincidental in two enzymes which together form a phosphate retrieval system (59). The architecture of the active site also revealed features which could account for lipid binding; an acidic pocket at one end could bind the head group while hydrophobic amino acids line the remainder of the active site.

The clostridial zinc-metallophospholipases C are antigenically related (104, 112, 186). Antigenic cross-reactivity with the *B. cereus* PC-PLC or *L. monocytogenes* PLC-B has not been reported, although, surprisingly, the *B. cereus* PC-PLC has been reported to show antigenic similarity with eukaryotic phospholipase C (30). Only the *C. perfringens* alpha-toxin antigenic structure has been studied in detail (92, 146), probably because of the known importance of this toxin in disease. It has been reported that a phospholipase C-neutralizing monoclonal antibody recognizes a peptide located in the N-terminal domain (ARGFAK) but that this antibody was less effective in neutralizing hemolytic and lethal activities (92). Similar results were reported by Sato et al. (142), who described other antibodies capable of neutralizing phospholipase C, hemolytic, and lethal activities. Recent studies in this laboratory have shown that whereas antibodies against the N-terminal domain neutralize only phospholipase C activity, antibodies against the C-terminal domain are highly effective in neutralizing haemolytic and lethal activities as well (180, 185). It is possible that the monoclonal antibodies described by Sato et al. (142) also bind to this domain.

When all of these results are considered together, it seems likely that phospholipase C activity alone is not sufficient for the toxicity of these proteins. The C-terminal domains of the *C. perfringens* alpha-toxin and *C. bifermentans* enzyme confer hemolytic properties on these enzymes; the *L. monocytogenes* PLC-B has been reported to be weakly haemolytic (54), and its activity may be comparable to the weak hemolytic activity reported for the *B. cereus* enzyme (88, 89) (Table 2). In the *C. perfringens* alpha-toxin, removal of this domain reduces but does not abolish sphingomyelinase activity, whereas hemolytic and lethal activities are not detectable (168, 170). It may be that these domains facilitate protein interaction with cell membranes and that poor effectiveness of phospholipase C-neutralizing antibodies in neutralizing lethal activity could be explained if the protein underwent a conformational change on interaction with membranes.

Sphingomyelinases

The sphingomyelinases from *Staphylococcus aureus* and *B. cereus* share many properties. Both enzymes are single polypeptides, require magnesium for activity (55, 67, 136, 189), and cause hot-cold lysis of sphingomyelin-rich erythrocytes (67, 136). In view of this, it is not surprising that a comparison of the deduced amino acid sequences (56, 130) revealed 56% similarity over 200 residues (130). The *S. aureus* sphingomyelinase (beta-toxin) has been the subject of intensive studies in past decades, and several reviews on this toxin have been written (136, 188, 189).

Circular dichroism spectrum studies and protein structure prediction studies both indicated that the *B. cereus* protein was largely in a beta-sheet conformation (172), and observations that the enzyme was inactivated by reducing agents (189) suggested that the protein may be stabilized by a disulfide bridge formed between the two cysteine residues in the protein. Magnesium ions have been reported to be loosely bound to the *B. cereus* protein, but their presence had little effect on the circular dichroism spectrum, suggesting that the ion(s) did not play a significant structural role (172). It has been suggested that the ions, which can be substituted for by calcium ions, facilitate substrate binding (172).

It is perhaps surprising that the toxic properties of the *S. aureus* sphingomyelinase have attracted so much attention, even though it is between 1 and 0.1% as toxic as the *C. perfringens* alpha-toxin (100, 136), whereas the *B. cereus* enzyme has not been considered in this context. The closely related bacterium *Bacillus anthracis* has also been reported to possess hemolytic and phospholipase C activities (35, 147); although the anthrax toxin is certainly of major significance in the pathogenesis of disease, it may now be of interest to examine the phospholipases C produced by this bacterium.

Phosphatidylinositol-Hydrolyzing Phospholipases C

The phosphatidylinositol phospholipases C (PI-PLCs) from *B. cereus*, *Bacillus thuringiensis*, and *L. monocytogenes* (PLC-A) show extensive deduced amino acid sequence similarity, particularly at the N termini (63, 83, 101). Along with the *C. novyi* PI-PLC, these enzymes have been classed as type I enzymes since they are able to hydrolyze phosphatidylinositol phosphates but are not membrane associated (23, 101). Sequence homology has also been reported between the *B. cereus* PI-PLC and the similarly sized *Trypanosoma brucei* enzyme (77) and between these proteins and eukaryotic PI-PLCs from rats and *Drosophila* species (101). An important feature of the PI-PLCs is their ability to cleave the phosphatidylinositol-glycan-ethanolamine anchor to which many eukaryotic membrane proteins are attached (23, 69, 75, 101). Unlike other phospholipases C, the PI-PLCs have not been reported to require divalent cations for activity (55, 77, 101). Other than this, little is known about structure-function relationships in this group of enzymes.

GRAM-NEGATIVE PHOSPHOLIPASES C

Pseudomonad Phospholipases C

The phospholipases C produced by *P. aeruginosa* have been the subject of considerable investigation over the past decade, and the gene encoding the hemolytic enzyme was the first bacterial phospholipase C gene to be cloned (177). Later, Ostroff and Vasil reported that the insertional inactivation of the *P. aeruginosa* hemolytic phospholipase C (PLC-H) did not completely abolish phospholipase C activity and thereby identified a second, nonhemolytic phospholipase C (PLC-N) produced by this bacterium (119). Using conventional protein purification techniques, Chin and Watts (25) also reported that two phospholipases C were

produced by a fleecerot isolate of *P. aeruginosa* but that only one of these was hemolytic; this enzyme has also been termed the heat-labile hemolysin by some workers, since activity is destroyed by heating to 100°C (3, 12). PLC-H and PLC-N were separable by ion-exchange chromatography (25). The genes encoding PLC-H and PLC-N have been isolated, and their nucleotide sequences have been determined (31, 93, 118, 129). *plcS*, which encodes the PLC-H enzyme, and *plcN*, which encodes PLC-N, are distally located on the chromosome (118), and the encoded proteins are of similar molecular weights, but whereas PLC-N is a basic protein (pI 8.8), PLC-H is acidic (pI 5.5) (118). Only PLC-H is hemolytic for sheep, human, and rabbit erythrocytes (12, 118). The protein is posttranslationally modified, by one of the *plcR* gene products via an unknown mechanism, to yield a product with altered charge and greater hemolytic activity (144). Neither PLC-H nor PLC-N is able to digest phosphatidylethanolamine (118), a major phospholipid component of the prokaryotic cell membrane (1), and it has been suggested that a substituted ammonium group on the phospholipid is required for binding by PLC-H (12).

It is interesting to speculate why two phospholipases are produced by this organism. The similarity of the deduced amino acid sequences (40% identity) suggests that these proteins could have risen from an early gene duplication event (99%). Since homology is greatest within the N-terminal regions of these proteins, it seems likely that differences in the C-terminal regions are responsible for the different substrate specificities. The requirement for different enzymes, produced by the same organism, to digest different phospholipids is not unusual and presumably reflects the different roles of these enzymes in the ecology or pathogenicity of the bacterium. Since both enzymes are able to digest phosphatidylcholine but only PLC-H can digest sphingomyelin, this difference in substrate specificity must be due to differences in recognition of the hydrocarbon tails rather than of the head group (which is identical). This contrasts with the ability of only PLC-N to hydrolyze phosphatidylserine, which must be due to a difference in the recognition of the head group of the phospholipid.

A DNA fragment which reacted with the *P. aeruginosa* *plcN* or *plcS* gene probes has been isolated from the related pathogen *Pseudomonas cepacia* and appears to be located within a highly variable region of the genome. On expression in *E. coli*, a 72-kDa protein was produced (178). However, this gene product alone did not possess phospholipase C activity. To generate a phospholipase C (and hemolytic) activity, the coexpression of a second gene, located on the same DNA fragment and encoding a 22-kDa protein, was required. The simple mixing of the two gene products did not result in phospholipase C activity (178). This result may reflect a mechanism similar to the reported activation of the *P. aeruginosa* PLC-H by the *plcR* gene product. Whether the 22-kDa gene product plays a role directly in phospholipase C activity or indirectly by posttranslationally modifying the 72-kDa protein could be resolved by purifying and characterizing the active phospholipase C produced by this organism.

A variety of other *Pseudomonas* species have been reported to produce phospholipases C (106); however, most of these species are low-grade pathogens or nonpathogens. One notable exception is the highly virulent *Pseudomonas pseudomallei*, several strains of which produced large amounts of phospholipase C when grown on egg yolk-containing medium (165). The properties of this enzyme await investigation.

Legionella Phospholipase C

There have been several reports of the production of phospholipase C by *Legionella pneumophila* (8–10) and by other *Legionella* species (43). The enzyme is produced extracellularly (43), and the *Legionella pneumophila* enzyme has been purified, partially characterized, and shown to hydrolyze phosphatidylcholine (9). It is not clear whether this enzyme is related to any of the phospholipases described above, but it is apparently not a zinc-metallophospholipase C or a phosphatidylinositol-specific enzyme. Zinc ions inhibit activity (9), and EDTA stimulates activity severalfold (9). The purified protein is not hemolytic for dog erythrocytes (9), which are rich in phosphatidylcholine (113), and the hemolytic activity produced by this bacterium has been shown to be due to other moieties, notably the metalloprotease and legiolysin (43). The role of this enzyme in the pathogenesis of Legionnaires' disease has not been investigated, but in view of the aerosol route of infection of this pathogen, it seems possible that it damages the phospholipid-rich lung surfactant in a manner similar to that suggested for the *P. aeruginosa* phospholipases C.

OTHER PHOSPHOLIPASES C

The production of phospholipases C by a variety of other bacteria has been reported, but in most cases these enzymes, or their encoding genes, have not been characterized in detail. The enzyme produced by *Ureaplasma urealyticum* was unusual in that it appeared to be membrane bound (39), and it is not clear from this report whether the protein would be surface exposed, which would presumably be a prerequisite for a role in pathogenesis.

REGULATION OF GENE EXPRESSION

Phosphate-Regulated Genes

Both of the phospholipases from *P. aeruginosa* and the *B. cereus* PC-PLC have been reported to be phosphate regulated (59, 118, 129, 145). The *P. aeruginosa* enzymes are induced under low-phosphate conditions, and regulation appears to be at the transcriptional level (118, 129, 145). The low level of expression of *plcS*, cloned into *E. coli*, has made it difficult to determine whether the gene is similarly regulated in this host (93, 129). Other proteins in a *P. aeruginosa* putative phosphate-scavenging pathway, such as alkaline phosphatase and P_i transport proteins, are P_i regulated (58); PLC-H and PLC-N may form part of a P_i regulon (145), which would be important for phosphate retrieval from the environment. The molecular basis of regulation in *P. aeruginosa* has been partially elucidated by using a variety of isogenic mutants. Of particular significance was the finding that *plcA* regulates the expression of both phospholipases C and that the gene encodes a homolog of the PhoB regulatory protein in *E. coli* (4). Significantly, a sequence resembling the *E. coli pho* box was located upstream of *plcN* (4). In addition to the regulation of the phospholipases C by P_i, several compounds derived from the enzyme product, notably choline, betaine, and dimethylglycine, can induce phospholipase C production, but the mechanisms of induction of PLC-H and PLC-N are different. Induction of PLC-H was independent of P_i concentration and PhoB, whereas PLC-N induction was seen only under low-P_i conditions and required PhoB (145). Since these product derivatives can also act as osmoprotectants, it has been suggested that induction

of phospholipases C can form part of a protective response of the bacterium when grown under conditions of high osmotic strength (145). The significance of PLC induction by product derivatives in relation to pathogenesis is discussed in more detail below. A third mechanism of regulation may involve the *plcR* gene, which is located downstream of *plcS*, and the gene products (PlcR1 and PlcR2) may also play a role in phosphate regulation of the phospholipases C and other phosphate-regulated proteins. Although the deletion of these genes in *P. aeruginosa* results in an increase in phospholipase C activity, production of the enzymes is still phosphate repressible (144), suggesting that they are not directly involved in phosphate regulation. Perhaps it is more likely that the *plcR* gene products play a role in the export or activation of phosphate-regulated proteins (144).

The *B. cereus* PC-PLC and sphingomyelinase-encoding genes form a cistron. It is known that the products of these genes can act synergistically to yield a hemolytic complex termed cereolysin A-B (56), suggesting that coexpression of these proteins is advantageous to the organism. Whether this is because cereolysin A-B plays a particular role in the ecology of this organism or whether the coregulation simply reflects that phosphatidylcholine and sphingomyelin are likely to be found together in the environment awaits investigation.

Non-Phosphate-Regulated Genes

Although production of many of the phospholipases C is not regulated by exogenous phosphate levels, other factors may control gene expression. The *L. monocytogenes plc-A* promoter contained a 14-bp palindrome within the −35 promoter region. This motif is characteristic of genes which are positively regulated by the *pfrA* gene product (83, 179), and this gene may therefore be coordinately regulated by the *pfrA* gene product along with other virulence determinants such as listeriolysin O, whose gene (*hlyA*) is located back-to-back with *plcA* (83). The *L. monocytogenes plcB* gene was located within an operon which also contained the metalloprotease gene (*mpl*), the actin-polymerization gene (*actA*), and the unassigned open reading frames ORF-X, ORF-Y, and ORF-Z (179). Control of *plcB* may be regulated since it is known that the gene can be expressed from the *mpl* or *actA* promoters, both of which are regulated by the *pfrA* gene product (179). Transposon mutagenesis into *mpl* reduced PLC-B production significantly, but the total loss of *plcB* expression resulted from disruption of the *actA* promoter (179). This result can be explained because the operon is regulated by the *mpl* promoter but transcription of *plcB* can also take place from the *actA* promoter, which is located downstream of *mpl* (179). It is possible that this arrangement allows the bacterium to regulate the expression of *plcB* (and *actA*) partly independently of other *pfrA*-regulated genes, and this may reflect the different roles of these gene products in the pathogenesis of listeriosis.

Studies by Murata et al. (111) and Rood and Cole (137) failed to show that environmental phosphate levels also affect alpha-toxin production, but it seems unlikely that expression of this gene is unregulated, since *C. perfringens* is a normal member of the gut flora and it seems unlikely that large quantities of toxin would be produced by the organism in this commensal state. In addition, wide variations in the levels of phospholipase C production by different strains have been reported (106, 107). Phospholipase C production, in vitro, does not correlate with virulence, since some clinical isolates produce almost undetectable levels of the enzyme (106, 171). Some progress has been made in elucidating mechanisms of gene regulation. A possible relationship between environmental iron levels and alpha-toxin production by *C. perfringens* (111) has not been followed up, and it is only recently that mechanisms of gene regulation have been investigated at a molecular level. Determination of mRNA levels in a heat-resistant strain of *C. perfringens* (139) showed that the alpha-toxin gene was expressed constitutively and that production was about threefold higher in the stationary phase than in the logarithmic phase. This pattern of gene transcription may not be typical, since previous reports have indicated that maximal toxin production occurs in the logarithmic phase, with a reduction on entry into the stationary phase (148). In other type A and type B strains, the gene was expressed maximally during the logarithmic phase of growth, and it was demonstrated that alpha-toxin production is regulated at the transcriptional level (191). The mechanisms of regulation have not been characterized, but the previously identified A+T-rich region upstream of the −35 promoter region appears to negatively regulate expression of the gene (174). Deletion of this region increased gene expression 10-fold in *E. coli* and was attributed to the DNA-bending potential of this region. Whether this effect is also seen in *C. perfringens* awaits investigation, but other factors must also regulate gene expression, since it has been shown that this region is identical in high- and low-producing strains of *C. perfringens* (191).

INTERACTION OF PHOSPHOLIPASES C WITH PHOSPHOLIPIDS AND MEMBRANES

Hydrolysis of Membrane Phospholipids

The interactions of the *B. cereus* PC-PLC and the *C. perfringens* alpha-toxin with phospholipids and their analogs has been studied in some detail. Not surprisingly, interaction with the polar head group and also, in the case of the *B. cereus* PC-PLC, the associated carbonyl group appears to be important for phospholipid recognition (45). The fatty acyl chains also play a significant role in substrate binding and must be of sufficient length (greater than six carbons) for hydrolysis of phospholipid to take place (45). Presumably, hydrophobic side chains close to the active site (64) mediate this binding. The ester bonds which link the fatty acyl chains also play a major role in the binding of the *B. cereus* PC-PLC to phosphatidylcholine (45) but a less important role in the binding of the *C. perfringens* alpha-toxin (138). Although these ester bonds are considered to be within the interfacial region (45), they are presumably less accessible than the head group (Fig. 3). These observations could partially explain why the *C. perfringens* alpha-toxin can hydrolyze membrane phospholipids whereas hydrolysis by the *B. cereus* PC-PLC is limited. The sphingomyelin molecule lacks one of the carbonyl side chains, and one would therefore predict that, unlike the *C. perfringens* alpha-toxin, the *B. cereus* PC-PLC would not be able to hydrolyze this molecule. Experimental data confirm this hypothesis to some extent, but our observations that a truncated form of the *C. perfringens* alpha-toxin is not able to efficiently digest sphingomyelin suggest that there are other parts of the sphingomyelin molecule which bind to the active-site region (168). The enhancing effect of detergents on activity of the *C. perfringens* alpha-toxin and *B. cereus* PC-PLC (46, 76) appears to be due to phospholipid solubilization (78) or solubilization of the diacylglycerol reaction product (46) rather than to an effect on the protein.

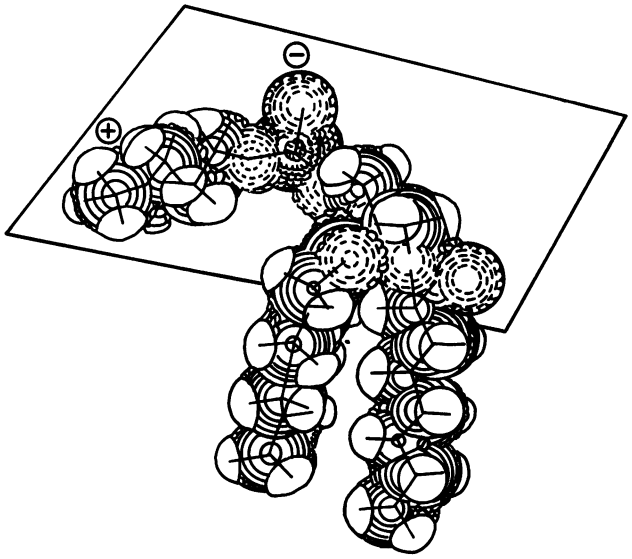

FIG. 3. Interfacial area of phosphatidylcholine (boxed) in the membrane, accessible to phospholipases (adapted from reference 45 with permission). Charged regions on the phospholipid head group are also shown.

Calcium ions are required for the binding of the *C. perfringens* alpha-toxin or the *B. cereus* PC-PLC to phosphatidylcholine films (110), especially at high lateral surface pressures (11), and for the binding of *B. cereus* sphingomyelinase to erythrocytes (67). Other phospholipases C, such as the *Legionella pneumophila* enzyme, have also been reported to be stimulated by calcium ions (9). The most commonly cited reason for this effect is that the calcium ion binds to the phosphate head group, altering the charge on this region of the phospholipid (11, 173). The ability of quinine to enhance phospholipid hydrolysis has been attributed to a similar mechanism (74). There is some evidence, from a kinetic analysis of the hydrolysis of a lysophosphatidyl analog, that the calcium ion binds to the *C. perfringens* alpha-toxin before substrate binding (193). Calcium ions are also required by numerous other eukaryotic lipid-binding proteins and enzymes, including intracellular phospholipases C and A_2 and snake venom phospholipases A_2 (133). It seems possible that the calcium ion performs a similar role in lipid binding with all of these proteins, although the sequence motifs of the suggested calcium-binding domains in the eukaryotic phospholipases C (29, 133) do not appear to be highly conserved in the prokaryotic phospholipases C.

Because phospholipids are the major structural components of the cell membrane, it seems simple to predict the effects of phospholipases C on cells by examining the spectrum of phospholipids degraded by the enzyme. Although this rationale may provide a starting point for such predictions, it is obvious that other factors are equally important in determining the interaction of phospholipases C with lipids and membranes.

It is known that the distribution of phospholipids in the cell membrane bilayer is not symmetrical and this asymmetry can explain why some phospholipases C are cytolytic. The outer leaflet is made up mainly of phosphatidylcholine and sphingomyelin (Table 3). Although the potential susceptibility of inner-leaflet phospholipids to hydrolysis by *S. aureus* beta-toxin and the *B. cereus* sphingomyelinase and PC-PLC has been demonstrated by using erythrocyte ghosts (67, 94), phospholipids in the inner leaflet are not normally accessible to the phospholipase and movement of phospholipids across the membrane is restricted (27).

TABLE 3. Distribution of phospholipids between the outer and inner leaflets of the human erythrocyte membrane bilayer[a]

Phospholipid	% of total in:	
	Outer leaflet	Inner leaflet
Sphingomyelin	22	5
Phosphatidylcholine	22	7
Phosphatidylethanolamine	5	22
Phosphatidylserine	0	13

[a] Data from reference 180.

Thus, it seems feasible to propose that the most hemolytic phospholipases C preferentially degrade outer-leaflet phospholipids. The *C. perfringens* alpha-toxin and *P. aeruginosa* PLC-H hydrolyze phosphatidylcholine and sphingomyelin and are hemolytic, whereas the structurally related nonhaemolytic *B. cereus* PC-PLC and *P. aeruginosa* PLC-N are not able to effectively hydrolyze sphingomyelin. Additional evidence for this hypothesis is provided by reports that the *B. cereus* sphingomyelinase and PC-PLC can act synergistically to cause hemolysis (56).

Two exceptions to this rule concern the *S. aureus* beta-toxin and the *B. cereus* sphingomyelinase, which are not able to digest phosphatidylcholine. However, the only erythrocytes sensitive to lysis by these enzymes have a high proportion of sphingomyelin in the erythrocyte membrane (Table 4) (13, 37, 67) and, more specifically, within the outer leaflet. Even after sphingomyelin hydrolysis at 37°C, cell lysis ensues only when the erythrocytes are cooled (this is known as hot-cold hemolysis). Hot-cold hemolysis has also been reported when the alpha-toxin acted on sheep erythrocytes (162), but hot-only hemolysis was observed when mouse erythrocytes (166) or rabbit erythrocytes (110) were tested. Since sheep erythrocytes are rich in sphingomyelin but almost devoid of phosphatidylcholine (37), it is possible that hot-cold lysis is observed when a phospholipase C hydrolyzes only the membrane sphingomyelin whereas hemolysis at 37°C is indicative of hydrolysis of both sphingomyelin and phosphatidylcholine.

The exact mechanism of hot-cold lysis has been attributed to the generation of fragile erythrocytes after the cleavage of membrane sphingomyelin (13). On cooling, the phase change in the membrane lipids may cause stresses which lead to cell

TABLE 4. Sensitivity of erythrocytes to lysis by *S. aureus* beta-toxin[a]

Species	Lytic activity (Hu/ml)[b]	Sphingomyelin (% of total lipids)
Sheep	7.6×10^5–2.1×10^6	51
Ox	5.7×10^5	46
Goat	4.25×10^5	46
Human	4.2×10^3	27
Rabbit	2.9×10^2	19
Horse	<10	14
Dog	<10	11
Guinea pig	<10	11

[a] Data from reference 13.

[b] Hu/ml, hemolytic activity expressed as the reciprocal of the dilution resulting in 50% lysis of erythrocytes when exposed to *S. aureus* beta-toxin.

lysis. Other treatments, such as EDTA, can also lead to lysis of erythrocytes pretreated with *S. aureus* beta-toxin at 37°C (136), and it seems likely that in this case the chelation of metal ions weakened the membrane. Even in the absence of EDTA, the reduced membrane-stabilizing ability of magnesium ions at low temperatures (106) can lead to hot-cold lysis.

The appearance of erythrocytes treated with phospholipases C has often revealed electron-dense intralamellar droplets, which have been presumed to arise from aggregation of the ceramide or diglyceride products of phospholipid hydrolysis (13, 19, 32, 33, 94, 180). The droplets disappear after digestion with pancreatic lipase (33) or bound lipophilic dyes (124), lending further credence to this suggestion. It has been suggested that for erythrocytes treated with *S. aureus* beta-toxin, the appearance of intracellular vesicles is caused by the invagination of the inner membrane as a result of the shrinking of the sphingomyelin-depleted outer leaflet (94, 180). However, other workers have suggested that the ceramide or diglyceride product remains in the outer leaflet and the membrane shape changes to accommodate the stresses induced (27). Perhaps the stresses induced on hydrolysis of phosphatidylcholine can be accommodated more easily than those induced on hydrolysis of sphingomyelin, whereas hydrolysis of both phospholipids leads to an energetically unfavourable situation.

Although the phospholipid composition of the outer leaflet undoubtedly influences the outcome of enzyme-membrane interaction, other membrane components such as sulfatides have been reported to play an additional moderating role in *C. perfringens* alpha-toxin binding (16), especially at high surface pressures (16). This has been attributed to the negatively charged head group on the phospholipids (16), which could presumably bind the enzyme via sulfatide ions. Other workers have shown that proteins can moderate the interaction of phospholipases C with membranes. α-Lactalbumin bound to lipid monolayers may inhibit phospholipid hydrolysis by the *C. perfringens* alpha-toxin (62), and the increased susceptibility of lipids to hydrolysis by the *B. cereus* PC-PLC in pronase-treated cells has been attributed to increased accessibility of the substrate (67). One suggested role of the *L. monocytogenes* PI-PLC is to remove GPI-anchored membrane proteins and enhance the cytolytic effect of other membrane-damaging proteins such as listeriolysin O (101).

Membrane Lateral Pressure and Phospholipase C Action

Ultimately, the phospholipase C must gain access to the membrane for phospholipid hydrolysis to take place, and the lateral pressure within the membrane reportedly plays a significant role in moderating this interaction. The hydrolysis of phosphatidylcholine by alpha-toxin, in a phosphatidylcholine monolayer or a mixed phosphatidylcholine-cholesterol film, could take place at surface pressures up to 40 dynes/cm (11) and 35 mN/m (=35 dynes/cm [110]), respectively, which is similar to the surface pressure within the erythrocyte membrane (31 to 35 mN/m [38]). In contrast, nonhemolytic phospholipases cannot hydrolyze phospholipids at high surface pressures (Table 5). It seems that some phospholipases C can gain access to the membrane more easily than others. From studies of the zinc-metallophospholipases C, it is tempting to speculate that the C terminus of the *C. perfringens* alpha-toxin and *C. bifermentans* phospholipase C is involved in such a role, and this could also explain how this domain facilitates interaction with sphingomyelin. This would certainly explain the weak hemolytic activity of *L. monocytogenes* PLC-B despite its ability to hydrolyze both phosphatidylcholine and sphingomyelin (54).

TABLE 5. Hydrolysis of phospholipids in monolayers by hemolytic and nonhemolytic phospholipases[a]

Source of phospholipase	Type	Hemolysis of human erythrocytes	Maximal surface pressure at which phospholipid hydrolysis can take place (dyne/cm)
Pig pancreas	A_2	−	16.5
Cabbage	D	−	20.5
Crotalus adamanteus	A_2	−	23
B. cereus	C	−	31
Naja naja	A_2	+	34.8
Bee venom	A_2	+	35.3
S. aureus	C	+	>40
C. perfringens	C	+	>40

[a] Data from reference 106.

The precise mechanism by which some phospholipases C bind to membranes remains unclear. Presumably these proteins possess hydrophobic regions, and it is possible that a conformational change on interaction with the membrane surface exposes these regions. Such changes have been reported for snake venom phospholipase A_2 and *Rhizomucor miehei* lipase (17, 22), which enable the enzymes to retract phospholipids above the membrane surface before cleavage. It is not clear whether this enzyme (or other phospholipases C) has a similar phospholipid-retracting mechanism or whether the protein becomes embedded within the membrane. A conformational change of the *C. perfringens* alpha-toxin on phospholipid binding has been previously suggested from studies with enzyme inhibitors (138), but presumably such a change would be transient when the enzyme interacted with dispersed substrate. On binding to a membrane, the conformational change may be attained for longer periods, and this would explain why a monoclonal antibody which neutralizes *C. perfringens* alpha-toxin phospholipase C activity was less effective in neutralizing hemolytic activity (92).

All of the studies with purified phospholipases C have suggested that only outer-leaflet phospholipids are hydrolyzed, and a dearth of information on the interactions of phospholipases C with membranes at the molecular level makes it difficult to speculate whether these enzymes can cross the membrane. However, these studies have all been based on the external application of phospholipases C and have been colored by studies with extracellular pathogens such as *C. perfringens*. When intracellular pathogens such as *L. monocytogenes* and *Legionella pneumophila* are considered, it is apparent that the site of production of the enzymes could be within the cell, after escape of the organism from the phagolysosome. In these situations it may be more appropriate to consider the effects of phospholipases C on the inner-leaflet phospholipids. A similar argument can be applied to the membrane-localized phospholipase C produced by *U. urealyticum*, but in this case it seems possible that the enzyme also plays a role in the initial stages of entry into the host cell.

An additional mechanism which could influence the cytolytic potential of phospholipases C involves the activation of the eukaryotic cell phospholipases C by the diacylglycerol product of the bacterial enzyme. Since many of these eukaryotic phospholipases C are membrane bound, it is

conceivable that these activated enzymes contribute to cell autolysis. This possibility has yet to be investigated.

Cell Membrane Repair

One further consideration which may influence the cytolytic potential of phospholipases is the speed with which the cell can repair membrane damage; ATP-depleted erythrocytes were more susceptible to cell lysis by alpha-toxin and *B. cereus* PC-PLC, perhaps because of their inability to repair membrane damage. Recovery from membrane damage may be quite protracted, taking at least 24 h for *S. aureus* sphingomyelinase-treated lung fibroblast membranes (126). Ultimately, cell lysis or extensive membrane damage by phospholipases C may reflect the fine balance between cell membrane damage and repair (127). An intriguing report by Kanfer and Spielvogel (71) suggested that the *C. perfringens* alpha-toxin was able to catalyze the limited formation of sphingomyelin from phosphatidylcholine and ceramide. Although this possibility does not appear to have been followed up, such a mechanism would presumably result in the alteration of the membrane structure.

SYNERGISTIC AND ANTAGONISTIC EFFECTS INVOLVING PHOSPHOLIPASES C

Only some phospholipases C have been reported to be hemolytic and lethal and to have necrotizing activities. However, it is now apparent that nonhemolytic phospholipases C can act in conjunction with other proteins to cause cell lysis. Individually the *B. cereus* PC-PLC and sphingomyelinases are only weakly hemolytic (56), but, acting together, the enzymes are able to cause hemolysis; this complex has been termed cereolysin A-B (56). A similar result has been obtained with the *B. cereus* PC-PLC and *S. aureus* sphingomyelinase (13, 135). A mechanism to explain this result has been proposed, involving the initial degradation of sphingomyelin, with a resultant lowering in membrane lateral pressure, which then allows the phosphatidylcholine-hydrolyzing enzyme access to susceptible membrane phospholipids (106, 135).

The CAMP reaction described by Christie et al. (28) provides another example of hemolysis occurring as a result of interaction between proteins from different bacterial species: the initial treatment of erythrocytes with a sphingomyelinase resulted in hemolysis only on the addition of a second, nonenzyme protein produced by group B streptococci (termed CAMP factor). Since that discovery, CAMP-like factors have been reported to be produced by a variety of bacteria (48). Investigation of the molecular biology of the CAMP factor has suggested that four regions of the 226-amino-acid polypeptide, all located in the N-terminal part of the molecule, can form amphiphilic helices (48). The lipid-binding potential of this region has been demonstrated by using a 9-kDa CNBr fragment of the CAMP factor (153). It has been proposed that CAMP factor interacts with lipids in the membrane, which has already been partially destabilized by the sphingomyelinase, and this leads to complete membrane destabilization (48). By analogy with the synergistic process described in the previous paragraph, it may be the case that sphingomyelinase treatment of erythrocytes leads to a lowered membrane lateral pressure, which then allows membrane penetration by the CAMP factor and leads to cell lysis.

Not surprisingly, antagonistic effects between phospholipases C and other proteins have been less well described. Nevertheless, there are some examples (44, 150). The pretreatment of cells with phospholipase D from *Corynebacterium haemolyticum*, *Corynebacterium ovis*, or *Corynebacterium ulcerans* reduced their sensitivity to lysis by *S. aureus* sphingomyelinase (150), probably because the products of phospholipase D hydrolysis (phosphatidic acid and/or ceramide phosphate) are not suitable substrates for the *S. aureus* enzyme.

Whether any of these synergistic or antagonistic effects are significant in vivo remains open to question, but some recent evidence suggests that this may be the case for the alpha- and beta-toxins of *S. aureus* (21). It therefore seems pertinent to pursue this line of investigation, which could reveal novel pathogenic mechanisms exploited in mixed infections.

EFFECTS OF PHOSPHOLIPASES C ON CELLS OTHER THAN ERYTHROCYTES

Cytotoxicity

Hemolysis of erythrocytes has often been used to measure the cytolytic activity of phospholipases C, but it is apparent that some phospholipases C are cytotoxic for other cell types. The *S. aureus* beta-toxin, *P. aeruginosa* PLC-H, and *C. perfringens* alpha-toxin are the most intensively studied in this respect. The beta-toxin shows a marked degree of specificity, being cytotoxic for human thrombocytes but not leukocytes (182). Platelets were lysed rapidly by this toxin (181). It is not clear whether these differences simply reflect differences in sphingomyelin content or accessibility in the cell membranes or whether toxicity is elicited by an effect other than membrane breakdown. Degradation of membrane sphingomyelin in fibroblasts was accompanied by redistribution of cellular cholesterol away from the cell surface, indicating the importance of sphingomyelin as a modulator of cholesterol distribution in cells (126). This result may be especially significant when considering the roles of sphingomyelinases and thiol-activated toxins on cell surfaces. In a study with the *P. aeruginosa* PLC-H, significant cytotoxic effects were observed in mouse peritoneal cells and human leukocytes (103). The effects of the *C. perfringens* alpha-toxin may be more subtle than cell lysis. Nonlethal membrane damage to human diploid fibroblasts was monitored by measuring the release of a low-molecular-weight label (aminoisobutyric acid) (108, 163), and it is possible that the membrane damage in these cells is rapidly repaired, preventing cell lysis.

Activation of Arachidonic Acid Cascade

Several lines of evidence suggest that phospholipases C are able to stimulate the arachidonic acid cascade in cells. It has been demonstrated that sublytic concentrations of the *C. perfringens* alpha-toxin can lead to limited membrane damage and the accumulation of diacylglycerol in the cell membrane. The diacylglycerol may enter one of two pathways in the cell (Fig. 4). In rat aorta cells or colon mucosal cells, the arachidonic acid cascade may be activated directly, after conversion of diacylglycerol to monoacylglycerol, by intracellular diacylglycerol lipase (41, 51). In cells from the small intestine, activation appears to take place indirectly after stimulation of intracellular phospholipase A_2 activity (60, 61). By whichever mechanism, activation of the arachidonic acid cascade by the *C. perfringens* alpha-toxin can lead to the production of thromboxane A_2, a potent mediator of

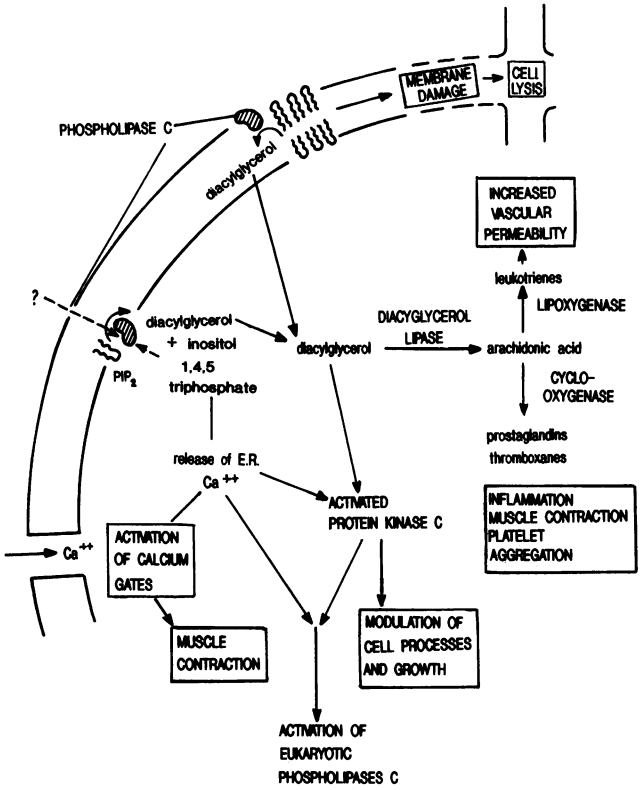

FIG. 4. Effects of bacterial phospholipases C on eukaryotic cells. The bacterial phospholipase C is hatched. Enzymes are shown in capital letters, and possible effects on eukaryotic cells are boxed. E.R., endoplasmic reticulum.

inflammatory responses (51), or prostaglandins which can induce chloride ion secretion in rat colonic cells via activation of chloride ion channels (41).

Similar results have been reported with other phospholipases C. The nonhemolytic *B. cereus* PC-PLC has also been reported to both activate the arachidonic acid cascade and stimulate prostaglandin formation in several mammalian cell types (85). The magnitude of this response is not clear, but in view of the proven limited accessibility of cell membrane phospholipids to cleavage by this enzyme, it may be much lower than that observed with the *C. perfringens* alpha-toxin. The *P. aeruginosa* PLC-H can activate the lipoxygenase and cyclooxygenase pathways in mouse peritoneal cells, leading to the production of an array of thromboxanes, leukotrienes, and prostaglandins (102). Similar results were obtained in vitro with mouse peritoneal cells or human granulocytes (102).

The induction of arachidonic acid metabolites by bacterial phospholipases C could account for many of the effects observed with these toxins in vitro and in vivo. Leukotrienes C_4 and D_4 (LTC_4 and LTD_4) increase vascular permeability and promote the exudation of fluid into the extravascular space (141, 182), and these effects have been observed after the intradermal administration of purified *C. perfringens* alpha-toxin in the guinea pig (156) and *P. aeruginosa* PLC-H in the mouse (102). The contraction of the isolated rat aorta and isolated rat ileum could be attributed to the induced production of LTC_4 or thromboxane A_2 (TXA_2) (51). These mechanisms may also be of significance in the aggregation of platelets, which has been reported as an effect of the alpha-toxin (114, 157) and *P. aeruginosa* PLC-H (36), since the thromboxanes are known to induce platelet aggregation (176). The failure of *S. aureus* beta-toxin to induce platelet aggregation (182) would be expected, because the ceramide product of sphingomyelin hydrolysis would not serve as a substrate for the arachidonic acid pathway.

It remains to be discovered whether other phospholipases C are able to stimulate the arachidonic acid pathway in cells, but this question seems pertinent in view of the potential roles of some of these enzymes in the pathogenesis of disease.

Activation of Protein Kinase C

Several workers have shown that exogenously applied *B. cereus* PC-PLC can elicit mitogenic responses in fibroblasts (80) or that *C. perfringens* alpha-toxin can give selective advantages to transformed keratinocyte cells in culture (125). It seems possible that the generation of diacylglycerol by exogenously applied bacterial phospholipases C mimics the effects of normal eukaryotic cell enzymes, where the generated diacylglycerol serves as a secondary messenger (15). The molecular basis of these effects has not been fully elucidated, but it is known that protein kinase C (PKC) can be activated by diacyglycerol and/or increased intracellular calcium levels (15). Thus it seems possible that bacterial phospholipases C activate PKC via the generation of diacylglycerol. Since it has been suggested that PKC can activate the eukaryotic cell phospholipases C and D, this pathway would serve as a positive-feedback loop. In this respect the effect of the bacterial phospholipase C could be considered to mimic the effects of hormones and neurotransmitters, such as epidermal growth factor, platelet-derived growth factor, vasopressin, and the interleukins (47, 126), which activate PKC (47). PKC is known to modulate a wide variety of cell processes, but Larrodera et al. (80) have shown that the mitogenic response elicited by the *B. cereus* PC-PLC is also apparent in PKC down-regulated cells, suggesting that the diacylglycerol may activate other pathways. Whatever the precise action of the bacterial phospholipases C, it seems that the effects observed may be of significance in the proliferation of cells and could therefore play a role in two-stage carcinogenesis; the effect of *C. perfringens* alpha-toxin was shown to be similar to that observed when keratinocytes were treated with a phorbol ester tumor promoter (which directly activates PKC), because transformed cells were selectively advantaged in cell culture (125). Similar conclusions were reached by Diaz-Lavida et al. (40), who showed that treatment of fibroblasts with the *B. cereus* PC-PLC (but not the *B. thuringiensis* PI-PLC) led to increased levels of activated PKC in a manner similar to that seen in cells transformed by the *ras* or *src* oncogenes.

NADPH oxidase catalyzes the formation of O^{2-} from molecular oxygen and therefore plays an important role in the respiratory burst of phagocytic cells which is associated with bacterial killing. It is not known whether PKC can activate NADPH oxidase, but it is known that one component of NADPH oxidase is a substrate for PKC (7). In this context it is significant that the *C. perfringens* alpha-toxin did not affect the viability of polymorphonuclear phagocytes, but it was suggested that membrane perturbation leads to activation of NADPH oxidase in the cells (154). Activation of this enzyme could have explained the production of O^{2-} by neutrophils exposed to *C. perfringens* alpha-toxin and the *B. cereus* PC-PLC (155). Such a mechanism

TABLE 6. Membrane-bound enzymes released by bacterial phosphatidylinositol-specific phospholipases C[a]

Enzyme	Source of PI-PLC
Alkaline phosphatase	*B. cereus*, *S. aureus*, *C. novyi*, *B. thuringiensis*
5'-Nucleotidase	*S. aureus*, *C. novyi*, *B. thuringiensis*
Alkaline phosphodiesterase I	*B. thuringiensis*
Acetylcholinesterase	*S. aureus*, *B. thuringiensis*

[a] Data from reference 66.

may be of significance in the pathogenesis of disease caused by phospholipase C-producing bacteria, since this event would lead to a premature activation of phagocytic cells. The extensive vacuolization of neutrophils treated with the *P. aeruginosa* PLC-H was also attributed to lysosomal discharge following a phospholipase C-induced respiratory burst (103). If these (and other) phospholipases C acted on cells before they contacted bacteria, the resultant respiratory burst might subsequently limit the extent of this response after ingestion of the bacteria.

These effects of phospholipases C on PKC activation, with the attendant modulation of a variety of cell functions (41), require further investigation, especially in the context of the possible roles of some bacterial enzymes in pathogenicity and even in the development of tumors.

Effects on Inositol Triphosphate and Intracellular Calcium

The hydrolysis of phosphatidylinositol-4,5-bisphosphate by phospholipase C yields diacylglycerol and inositol-1,4,5-triphosphate. The secondary-messenger role of diacylglycerol has been referred to above. Although bacterial phosphatidylinositol-hydrolyzing phospholipases C have not been shown to increase the levels of inositol triphosphate in eukaryotic cells, there is no reason why this effect could not be elicited, especially if the phospholipase C could gain access to the inner leaflet of the plasma membrane. Indeed, it has been suggested that the *L. pneumophila* phospholipase C could act in this manner (43). One of the clearly defined effects of inositol triphosphate and of its derivative inositol tetraphosphate (15) is to stimulate the release of calcium from the endoplasmic reticulum (15). The alteration of intracellular calcium levels may in turn stimulate calcium influx across the plasma membrane through calcium gates (Fig. 4). The contraction of the isolated rat aorta and isolated rat ileum has been attributed to this mechanism (51), and the activation of membrane calcium gates (and chloride ions channels) may have been responsible for the changes in frog muscle resting and action potentials which have previously been reported (18). Such effects may be of significance in regulating the blood supply to tissues infected with bacterial pathogens. For most pathogens this would be advantageous, since access of immune system cells to the site of infection would be restricted. For anaerobes, the anoxic conditions generated might facilitate growth of the organism. The role of inositol triphosphate, especially in relation to the role of diacylglycerol and arachidonic acid metabolites, requires further investigation.

Release of Cell Membrane Proteins

Phosphatidylinositol is only a minor component of many cell membranes, but it performs an essential role in anchoring a variety of proteins. The phosphatidylinositol-glycan-ethanolamine anchor can be cleaved by membrane-active phosphatidylinositol-specific phospholipases C (75) to release a variety of cell membrane-bound enzymes (Table 6). The reported increase in blood alkaline phosphatase levels following intravenous administration of the *B. cereus* PI-PLC (66) suggested that these enzymes elicit similar effects in vivo and in vitro. The significance of these released enzymes has not been identified, but it seems possible that the eukaryotic alkaline phosphatase could be used by the bacterium as part of a phosphate-scavenging pathway. This would be especially significant since, as noted above (66), the level of P_i in the blood is below that required for the growth of many bacteria. The phosphatidylinositol phospholipases C have also been reported to moderate the growth of cells in tissue culture (66). It is possible that, like the *C. perfringens* alpha-toxin, these enzymes are able to activate secondary-messenger pathways to cause these effects.

Other Effects

Other effects of phospholipases C on cell metabolism have not been described specifically, but it is worthwhile considering the central roles of diacylglycerol and inositol triphosphate on the short-term regulation of cell metabolism and the secretion and contraction of cells. These second messengers may also play a role in longer-term events such as growth and perhaps information storage in the brain (15). It seems timely to consider the roles of bacterial phospholipases C in these contexts rather than simply as agents of cytolysis.

ROLES OF PHOSPHOLIPASES C IN DISEASE

C. perfringens Alpha-Toxin

C. perfringens is the bacterium most frequently associated with gas gangrene in humans (186). The disease usually results from the growth of the bacterium in tissues which become anoxic either as the result of traumatic damage or from obliterative arterial disease in the limbs (187, 188). During past armed conflicts, the disease was a major cause of death of wounded soldiers (99, 183, 186). It has long been suspected that the *C. perfringens* alpha-toxin plays a key role in gas gangrene. However, the precise role of this protein in disease and the molecular basis of toxicity have not previously been understood. The observation that hemolytic and lethal activities of the alpha-toxin are intimately linked has prompted speculation that the role of the toxin in the pathogenesis of gas gangrene is simply to cause cytolysis. Hemolysis may occur in tissues close to the focus of infection, but the effects may be more subtle in distal tissues. The activation of the arachidonic acid cascade and cell calcium gates may lead to blood vessel contraction (50, 51). Both of these effects would reduce the blood supply to tissues and promote the anoxic conditions required for the further growth of *C. perfringens*.

An abnormally high level of *C. perfringens* in the gut or excessive production of the alpha-toxin has been noted in patients with rheumatoid arthritis (117), and it has also been suggested that *C. perfringens* alpha-toxin-mediated inflammatory responses play a role in ileitis and Crohn's disease (60). As described above, these effects could be elicited via activation of the arachidonic acid pathway with the production of thromboxanes and leukotrienes. It has also been reported that alpha-toxin treatment of tissue cultures can confer selective advantages on transformed cells (125). Since *C. perfringens* is a member of the normal intestinal flora, and

since strains isolated from this source have been shown to have the potential to produce phospholipase C, it may be appropriate to examine the role of this enzyme in carcinomas of the gastrointestinal tract in more detail.

L. monocytogenes Phospholipases C

L. monocytogenes causes a variety of opportunistic infections in humans and animals. The pathogenesis of listeriosis involves a number of critical steps, including penetration of the gut mucosa, dissemination by the vascular system to other tissues, establishment of abscesses of infection, and, in some cases, passage across the blood-brain barrier to cause meningoencephalitis (98, 131, 132). A central feature of the pathogenic process is the ability of the organism to enter cells, replicate within them, and invade adjacent cells (131, 132). This process allows the bacterium not only to establish foci of infection in tissues but also to evade host phagocyte-killing mechanisms, even after uptake by these cells (98). It is known that listeriolysin O plays an important role in virulence of the bacterium by permitting escape from phagolysosomes (52, 128), and recent evidence suggests that the phospholipases C are also important determinants of pathogenicity. The precise interpretation of many studies with PLC$^-$ mutants is made difficult because of the pleiotropic effects of mutations in these genes (see above). Notwithstanding these difficulties, several workers have constructed PLC-A$^-$ and PLC-B$^-$ mutants to attempt to elucidate the roles of these phospholipases; a *plcA* mutant of *L. monocytogenes* has been shown to be between 10-fold (101) and 1,000-fold (23) less virulent in the mouse. The role of PLC-A may be most significant after ingestion by professional phagocytes; *plcA* mutants are apparently able to invade liver hepatocytes, replicate, and cause a progressive infection in mice which have been treated with monoclonal antibody 5C6 (34). This antibody, which is specific for the type 3 complement receptor on these cells, prevents the accumulation of neutrophils at the site of infection (34). Additional evidence for the role of PLC-A in the evasion of phagocyte host defenses was reported by Camilli et al. (23), who showed that the *plcA* mutant was able to invade, but did not replicate in, mouse peritoneal macrophages. This was suggested to be the result of a reduced ability of the bacterium to escape from the host cell phagosome (23). It is possible that the enzyme removes GPI-anchored host cell membrane proteins and that this potentiates the membrane to the damaging effects of listeriolysin O (101) or PLC-B.

To study the role of PLC-B in disease, Vazquez-Boland et al. (179) constructed a *plcB* mutant of *L. monocytogenes* by the insertional inactivation of *plc-B*. By using in vitro tissue culture systems, it was demonstrated that PLC-B did not play an important role in the initial infection of J774 macrophages, and the *plcB* mutant was able to lyse the phagosome single membrane and escape into the cytoplasm as effectively as the wild-type bacterium did. However, it appeared that later in the infection cycle, cell-to-cell spread of the *plcB* mutant was reduced and the bacteria accumulated in the resultant double membrane vacuoles. In a plaque assay the size of plaques surrounding infected L2 or 3T3 fibroblasts was significantly reduced with the *plcB* mutant. The role of PLC-B may be to partially disrupt the cell-cell fusion vacuole membrane, but complete disruption may be additionally dependent on the action of listeriolysin O or PLC-A.

Thus, both PLC-A and PLC-B appear to play significant roles in the pathogenesis of listeriosis, perhaps by their actions on host cell phagolysosome membranes. It is also intriguing to speculate that phospholipases C delivered from within infected cells may perturb host cell metabolism in the ways suggested above.

P. aeruginosa Phospholipases C

A variety of diseases of humans and animals are caused by *P. aeruginosa*. Fleecerot is an economically important disease of sheep; it is characterized by skin lesions and superficial inflammation of the skin. Chin and Watts have reported that intradermal inoculation of a hemolytic phospholipase C is able to reproduce many of the symptoms of fleecerot in sheep (25). This phospholipase C may be similar to that reported to induce skin lesions in rabbits (90). In a study with highly purified PLC-H administered by an intravenous or an intraperitoneal route, the enzyme was shown to reproduce many of the effects seen with crude culture filtrates. Necrosis of the liver and kidney was observed, and it was concluded that PLC-H was the main lethal toxin produced by the bacterium (103). Although this route of administration may not be directly relevant to diseases caused by the bacterium, it seems likely that phospholipase C is produced by the bacterium in vivo. Patients with chronic *P. aeruginosa* infections show high antibody titers against phospholipase C (57), and sheep which have suffered repeated episodes of fleecerot are reported to contain high levels of circulating nonneutralizing antibody against phospholipase C (25). In the latter case it has therefore been suggested that a crude toxoid may not be able to induce protection against the disease.

In humans, *P. aeruginosa* is an opportunistic pathogen, causing disease mainly in debilitated individuals. Lung infections are particularly prevalent in cystic fibrosis patients and several lines of evidence indicate that the phospholipases C are produced in vivo; not only has it been shown that clinical isolates from the lungs produce phospholipase C (12), but also it has been shown that phospholipase C is produced when the bacterium is grown in saline bronchial washings (90, 91) and that cystic fibrosis patients had circulating antibody against phospholipase C (57). Skin infections with *P. aeruginosa* are common in burn patients, and it has been shown that partially purified preparations of phospholipase C can reproduce the pathologic changes associated with these infections (90, 102). The role of phospholipase C in urinary tract infections is not proven, but it may be significant that clinical isolates from this site produce higher levels of phospholipase C than those isolated from the lungs or from blood (12). Since production of the phospholipases C (and other proteins such as alkaline phosphatase) is induced under low phosphate conditions, it has been suggested that these enzymes function as part of a phosphate-scavenging pathway. Such a phosphate retrieval system has been suggested to be especially significant in vivo, because it has been shown that the level of free phosphate in sera from humans infected with gram-negative pathogens is suboptimal for bacterial growth (184). However, this phosphate retrieval system may be of limited significance in *P. aeruginosa* infections of the lungs in cystic fibrosis patients, since the lung surfactant is phosphate rich. In the lungs the phospholipase C could act by degrading the phospholipid-rich lung surfactant, enhancing the colonization of tissues (151) and therefore contributing directly to the pathology of disease (90, 91). Also, the finding that phospholipases C are induced under environmental conditions of high osmolarity (145) may be especially significant in the lungs, where these conditions would be found. In this environment the phospholipase C

products may be converted into glycine betaine, which can be accumulated intracellularly as an osmoprotectant (79). In addition to these direct effects of the enzymes on host tissues and bacterial metabolism, the ability of PLC-H to induce leukotriene and thromboxane release from host cells could partially explain the inflammatory responses seen in many *P. aeruginosa* infections (102).

These proposed roles of the phospholipases C in pathogenicity could be investigated by the construction of isogenic phospholipase C mutants. *plcS* mutants of strain PAO1, grown under phosphate-limiting conditions, showed a 200-fold increase in 50% lethal dose (LD_{50}) compared with the wild-type strain in the mouse burn model (120). *plcR* mutants were also attenuated, and these strains produced greater amounts of phospholipase C and hemolysin (120), although the relative production of PLC-H, PLC-N, and the glycolipid hemolysin was not reported. This apparent enigma indicates a role for the *plcR* gene products in virulence, or the regulation of virulence, but the observation that the *plcS plcR* mutant is even more attenuated (10,000-fold increase in LD_{50} compared with the wild type [120]) would suggest that the PLC-H does play a significant role in pathogenicity.

S. aureus Beta-Toxin

Considerable controversy has been evident over the past 20 years concerning the toxic properties of beta-toxin, and interpretation of data has been made difficult because of the likely contamination of many beta-toxin preparations with other *S. aureus* toxins. An attempt to clarify the role of this toxin in disease was made by determining the pathogenicity of an *hlb* (beta-toxin-negative) mutant of *S. aureus* in the mouse mammary gland (21). The mutant was recovered in significantly lower numbers from the mammary gland, suggesting that this toxin plays a role in virulence. The exact mechanism by which the beta-toxin elicits these effects is not clear, and, surprisingly, the toxin appeared to stimulate neutrophil influx into the site of infection, although increased neutrophil killing and epithelial-cell damage was attributed to the toxin (21). Epidemiological evidence also suggested that the beta-toxin played a significant role in pathogenicity, and selection for beta-toxin-producing strains seemed to occur in vivo (65).

RESEARCH AND THERAPEUTIC APPLICATIONS OF PHOSPHOLIPASES C

Vaccines

Although phospholipases C have been implicated as virulence determinants of several bacterial species, they have rarely been investigated as vaccine components. In many cases this may be because they have been perceived to play only a minor role in the disease process. One notable exception concerns the *C. perfringens* alpha-toxin. Because this toxin is thought to play a major role in the pathogenesis of gas gangrene, various workers have investigated the efficacy of a toxoid vaccine in protection against the disease. Kameyama et. al. (72) reported that a formaldehyde toxoid induced protection against *C. perfringens*-mediated gas gangrene, but it is not clear whether the toxoid preparation also contained other *C. perfringens* antigens. In a study with a mixed *C. perfringens-C. septicum-C. novyi* toxoid, sheep were found to be almost totally protected (83 to 94 percent) against gas gangrene, even up to 1 year postvaccination (20). The major problem with such a vaccine is that the time taken for an antibody response to develop is usually greatly in excess of the time taken for symptoms of the disease to appear (134). Such a vaccine would, however, be of great use for at-risk groups, particularly if used prior to surgery on the lower limbs.

Membrane Probes and Models for Eukaryotic Phospholipases C

The bacterial phospholipases C are generally readily available in research quantities and have therefore attracted considerable attention as reagents not only for exploring the structure of cell membranes, particularly the structure of the erythrocyte membrane (2, 27, 49, 180), but also for some other novel applications. Some of these earlier studies may have yielded equivocal results because of the questionable purity of the enzymes used. The production of purified phospholipases C by recombinant DNA techniques may significantly enhance the utility of these reagents for future studies.

The report that monoclonal antibodies raised against the *B. cereus* PC-PLC also reacted with human monocytic-cell phospholipase C (30) indicates that the *B. cereus* enzyme may serve as a readily available model for investigating eukaryotic phospholipases C. In particular, the reported crystal structure of the *B. cereus* protein should facilitate the design of novel enzyme inhibitors which could have significant clinical utility. Various other workers have used the *C. perfringens* alpha-toxin to measure the potency of inhibitors of phospholipase C activity (190). Additionally, the bacterial phospholipases C could be used to investigate mechanisms by which the arachidonic acid pathway and PKC are activated in mammalian cells (73). In one report exemplifying this approach, Parkinson (125) used the *C. perfringens* alpha-toxin to stimulate PKC activity in cells and mimic the effect of phorbol ester tumor promoters on cells.

Immunotoxins

Almost all of the immunotoxins evaluated to date have been generated by fusing antibodies with diphtheria toxin, ricin, or *Pseudomonas* exotoxin A. These immunotoxins rely on the delivery of the active fragment of the toxin into target cells, often an inefficient process, and the subsequent inhibition of protein synthesis. The clinical utility of these immunotoxins has so far been limited. The fusion of antibody with a membrane active toxin could yield an immunotoxin with enhanced properties, but the chemical coupling of polyclonal antibody with the *C. perfringens* alpha-toxin has yielded disappointing results (109). Chovnick et al. (26) have reported the generation of a hybrid anti-Tac-*C. perfringens*-alpha-toxin antibody by fusing *cpa* with the 5′ end of the segment encoding V_HC_H1 from the anti-Tac heavy chain. The hybrid toxin retained approximately 50% of the hemolytic activity and retained the ability to recognize the T-cell IL-2 receptor. Inhibition of protein synthesis was used to measure the activity of the immunotoxin, and the 50% inhibitory concentration was found to be 1.8 ng/ml when measured against cells expressing the interleukin-2 receptor. This potency compared favorably with an anti-Tac-*Pseudomonas* exotoxin A antibody. The utility of such immunotoxins remains to be determined, and it is not yet clear whether nonspecific hemolytic activity of the alpha-toxin immunotoxin in vivo will prove problematic.

CONCLUSIONS

The considerable resurgence of interest in the bacterial phospholipases C over recent years can be attributed to several factors. Many of the newly characterized enzymes such as the *L. monocytogenes* PLC-A and PLC-B are thought to play important roles in the disease process, and this has stimulated new interest in phospholipases C such as the *C. perfringens* alpha-toxin which were already thought to be determinants of pathogenicity. These studies have revealed that phospholipases C may have varied roles in the disease process, ranging from promoting the intracellular spread of *L. monocytogenes* to eliciting toxic and lethal effects in *C. perfringens*-mediated gas gangrene. In general, there is a large body of circumstantial data indicating the roles of these enzymes in the pathogenesis of disease but precious little unambiguous data. In addition, many previous studies may have been colored by simply assessing whether a phospholipase C was toxic and judging its importance in pathogenicity on this basis. The use of defined mutants may further clarify the roles of these enzymes in disease, but the results of such studies must be interpreted with caution because of the complex pleiotropic effects that may be associated with such gene deletions. Thus the examination of mechanisms of gene regulation must proceed in tandem with such gene deletion studies, to enable valid conclusions to be drawn from these experiments.

The finding that eukaryotic phospholipases C can play an important role in the regulation of cell metabolism, via the generation of secondary messengers, suggested that the bacterial phospholipases C may cause similar effects. It is apparent that some bacterial phospholipases C elicit toxic effects by mimicking the actions of the eukaryotic enzymes on host cells. The combined effects on arachidonic acid metabolite levels and diacyglycerol and inositol triphosphate levels may be to restrict the extent of the immune response either by limiting the blood supply to these tissues or by "confusing" the phagocytic cells. An additional area which merits attention is the possible contribution of bacterial phospholipases C to the transformation of eukaryotic cells. This effect may be due to the ability of these enzymes to activate PKC and may be especially relevant in chronic infections (e.g., caused by *P. aeruginosa* in the lungs) or commensal states (e.g., *C. perfringens* in the gut).

In spite of the advances over the past decade, the molecular mechanisms by which some bacterial phospholipases C interact with membranes is still not clear. Much of our knowledge concerning the interaction of phospholipases C with substrates has been gained by the use of chemically modified substrates, but we cannot unequivocally state why some phospholipases C are potent lethal toxins whereas others are apparently nonlethal. The comparison of deduced amino acid sequences of several phospholipases C has provided a basis for examining these differences because structurally related enzymes with different toxicities have now been identified. To examine these differences at a molecular level will require the crystallization of these proteins alone and in the presence of substrates. Only then will we be able to examine substrate specificities in a rational manner and perhaps design enzymes with novel properties. Even at this stage, the extrapolation to the interaction of these enzymes with membranes will prove challenging, and greater emphasis should be placed on the use of synthetic bilayers.

Many of the studies to date have considered the effects of externally applied phospholipases C, but since many phospholipase C-producing bacteria are now known to be intracellular pathogens, this may not be the most appropriate route for investigating the effects of these enzymes. The prokaryotic enzymes are generally available in research quantities, and the reported antigenic cross-reactivity of the *B. cereus* PC-PLC with eukaryotic phospholipase C suggests that the bacterial enzyme may serve as a useful model for examining eukaryotic enzyme structure-function relationships. In the longer term, the rational design of enzyme inhibitors or vaccines with potential therapeutic use will become feasible, and this must surely be the goal of many microbiologists. Such studies will provide an exciting challenge over the next decade.

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