CHAPTER -1

1.1. INTRODUCTION

Phospholipids are the major building block of membranes, which are essential components of all cells. These phospholipids play multiple roles such as establishing a hydrophobic barrier, providing a matrix for many catalytic processes, and influencing the functional properties of membrane-associated processes in cells [1]. In addition to its structural role, phospholipids serve yet another function in mammalian cells, - as a reservoir of intracellular and intercellular messengers. These messengers are invariably the oxidized form of fatty acids (viz: eicosanoids and their related compounds), unusual phospholipids (viz: platelet-activating factor and lysophosphatidic acid) and lipid derivatives (diacylglycerol and phosphatidic acid) [2]. The initial discovery that hormones affect phosphoinositide metabolism was made by Hokin & Hokin in 1955 [3]. They found that stimulation of pancreas and brain cortex slices by acetylcholine resulted in an increased incorporation of $^{32}$P orthophosphate into phosphoinositide and phosphatidic acid (phosphatidylinositol cycle).

In the early 1980s, hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP2) by phospholipase C (PLC) was established as a major signalling pathway for Ca$^{2+}$-mobilizing agonists. The discovery of the second messenger roles of inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) has accelerated the progress in signal transduction research of PLC. Both IP3 and DAG, the product of PLC, function as signalling molecules by releasing intracellular calcium ions and activating protein kinases, respectively [4, 5].
1.2. TYPES OF PHOSPHOLIPASES

Phospholipases are hydrolytic enzymes, which are widely distributed in nature and serve many biological functions, as potent toxins and generators of intracellular messengers [2]. The phospholipase families of enzymes are classified on the basis of the types of bond they cleave and their site of action within a typical phospholipid molecule. The only property that all the phospholipases have in common is, they catalyse the cleavage of phospholipids [5, 6].

Several classes of phospholipases involved in signalling is shown in fig 1.1.

![Diagram of phospholipase action](image)

Fig 1.1. Classes of phospholipases and their site of action.

Phospholipase A act either on C1 or C2 position, and hence are known as A₁ and A₂ phospholipase. Phospholipase A₂ hydrolyse the carboxylester linkages at C2 position of phospholipids to produce unsaturated fatty acid and lysophospholipid.

Phospholipase C hydrolyse the phosphodiester linkage of phospholipid to produce DAG, and a phosphorylated polar head group (inositol / choline). Depending on the
specificity towards the phosphorylated heads inositol / choline, PLC is called as phosphatidylinositol specific phospholipase C (PI-PLC) or phosphatidylcholine specific phospholipase C (PC-PLC).

Phospholipase D hydrolyse the phosphodiester linkage to produce phosphatidic acid and a polar head group.

The hydrolytic products of phospholipases which are of particular interest are the (1) unsaturated fatty acid (often arachidonic acid), the product of phospholipase A2, (2) inositol tri-phosphate (IP3) generated by phosphatidylinositol - specific phospholipase C (PI-PLC), (3) diacylglycerol (DAG) released via PI-PLC/PC-PLC action, and (4) Phosphatidic acid generated via phospholipase D action [7].

1.2.1. Phosphatidylinositol specific phospholipase C (PI-PLC)

Although phosphatidylinositol (PI) constitutes only less than 10% of a cell’s membrane phospholipid content [5, 8] it is extremely important in cellular signalling pathways as the inappropriate signalling can cause diseases such as cancer and cardiovascular disease[9].

PI-PLC makes up a diverse family of enzymes. They have been isolated from a variety of organisms from protozoa and bacteria to plants and mammals. While the substrate of this enzyme varies only slightly, the function of the enzyme differs considerably depending on the organism. PI-PLCs (the focus of this thesis) from pathogenic bacteria are proposed to act as potential virulence factors [9,10], although they have been isolated from nonpathogenic bacteria as well. Using prokaryotic PI-PLCs, glycosylphosphatidylinositol (GPI) anchored proteins can also be released exploiting the glycosyl-PI cleaving ability of the enzyme. Mammalian PI-PLCs catalyzes the hydrolysis of PI (4,5)P2 to yield inositol-1,4,5-triphosphate PI(1,4,5)P3. These products are both
potent second messengers in signal transduction in regulating the release of intracellular Ca\textsuperscript{2+} and also in activating the protein kinase C [4,5].

During the past twenty years, evidence have been accumulated for the presence of phospholipids in the nuclei of eukaryotic cells. These phospholipids are distinct from those that are present in the nuclear envelope. The best characterized of the intranuclear lipids are the inositol lipids that form the components of a phosphoinositide-phospholipase C cycle. There is an extensive literature concerning nuclear PI-PLC and the isoform PI-PLC\textbeta 1 has been most highlighted to be present in the nucleus to generate DAG signals. Evidence also points out that the PI-PLC \gamma 1 isoform might also be localized partly in the nucleus and may be involved in nuclear signaling [11, 12, 13].

1.2.1.1. Mammalian PI-PLC (E.C. No 3.1.4.11)

Historically, the PI-PLC isozymes have been studied since the 1950s. Early observations by the Hokin et al [3], and later by Michell [14] and others, led to the recognition of PI-PLC as a key enzyme in agonist-stimulated phosphoinositide metabolism and calcium signalling. The direct link between PLC and the release of intracellular calcium stores was published as a seminar paper in 1983 by Streb et al. [15]. In the late 1980s and early 1990s, three mammalian PLC subtypes, \beta with a molecular weight of 140 to 155 kDa, \gamma with 145 to 148 kDa, and \delta with 85 to 88 kDa were isolated and their corresponding cDNA sequences were determined [16]. Four \beta- , two \gamma-, four \delta - isoforms, and numerous spliced variants have been described in mammals. The PI-PLCs found in yeasts, slime molds, filamentous fungi and plants closely resemble mammalian
PI-PLC δ [17]. Recently a novel class of human PI-PLC, named PLC-ε (epsilon), a structurally distinct fourth family of PI-PLC was identified [18].

1.2.1.1.1. The structure of mammalian PI-PLC

There is a δ isoyme -like core sequence present in all of the ten mammalian PI-PLC isozymes that have been so far reported (fig 1.2).

![Diagram of mammalian PI-PLC isozymes](image)

**Fig 1.2. Structure of mammalian PI-PLC isozymes:** The sequence of mammalian PI-PLC contain a string of modular domains organised around a catalytic domain. They include a pleckstrin homology (PH) domain, EF-hand motifs and a single C2 domain. Additional regulatory regions are present in the β and γ subtypes of PI-PLC but absent in PI-PLC δ.

The β isozymes have a C-terminal extensions of about 400 residues, and the γ isozymes have an insertion of about 500 residues between the two halves of the catalytic domain.

The crystal structure of the mammalian PLC-δ1 isoenzyme had been solved. The N-terminal domain was identified separately from the catalytic core of the enzyme [19]. The N-terminal region of the enzyme contains the pleckstrin homology (PH) domain of about 120 residues folded into seven antiparallel β-strands arranged into a barrel-like structure. This PH domain of the enzyme is responsible for the localization of PLC-δ1 to the plasma membrane. After the PH domain, is the EF-hand domain, which forms a
flexible link between the PH domain and the catalytic core. It consists of four helix-loop-helix motifs arranged in two lobes. Experimental evidence show that Ca^{2+} may not bind to EF-hand, unlike most EF-hands where the metal ion binds. The catalytic core of the enzyme consists of an irregular (βα)_8 - barrel and is typically divided into two halves referred to as the X and Y regions which are separated by a linker sequence called the X/Y-linker. PLC-γ isozymes have evolved extensively and contains two [SRC-Homology (SH)] SH2 domains and an SH3 domain within the X/Y linker domain of the enzyme. This X/Y linker domain of the PLC-γ isozymes is hypothesized to play a role in both activation and in repression of the enzyme by tyrosine kinases. The C-terminal domain of PLC-δ1 consists of an antiparallel, eight-stranded β-sandwich which is commonly referred to as a ‘C2’ domain. This domain has three metal binding sites and may be involved in membrane binding [17,20-22].

Human PI-PLC-ε contains the conserved catalytic (X and Y) and regulatory domains (C2) similar to other eukaryotic PLCs. In addition it also contains two Ras-associating (RA) domains and a Ras guanine nucleotide exchange factor (RasGEF) motif [18].

1.2.1.2. Bacterial PI-PLC (E. C. No. 3.1.4.10)

Bacterial PI-PLCs are the smallest among the different isolates with an approximate size of 35kDa and about 300 amino acids in length [23]. They have been purified from cultures of Bacillus cereus [24-26], Bacillus thuringiensis [26], Staphylococcus aureus [27] and Clostridium novyi [28]. The sequences of five bacterial PI-PLCs have been so far elucidated and the identities are highest among PI-PLCs within the same species [23]. The crystal structure of B. cereus PI-PLC has been solved [29] to...
have a single domain. Thus the structural functional characterization of bacterial PI-PLC has emerged as a useful model for studying eukaryotic PI-PLCs as well. Sequence data of \textit{B. cereus} PI-PLC shows the highest homology with PI-PLC from \textit{B. thuringiensis}, differing by only eight amino acids. It also shows a 37-38\% sequence homology with trypanosome GPI-PLC and an eukaryotic PLC. There is also a relatively high degree of similarity between \textit{B. cereus} and other eukaryotic conserved regions (26\% identity) supporting the idea of convergent evolution [23]. The crystal structure of PI-PLC from \textit{Listeria monocytogenes} (PDB 1AOD) has also been solved [30].

Since the focus of this thesis is PI-PLC from \textit{Bacillus thuringiensis}, much emphasis is given to the crystal structure of the closely related PI-PLC from \textit{B. cereus} and not on other PLCs.

1.2.1.2.1. The crystal structure of the PI-PLC from \textit{Bacillus cereus}

The crystal structure of the PI-PLC from \textit{Bacillus cereus} as complexed with myo-inositol had been solved [29] and refined at 2.6 Å resolution (fig 1.3).

![Crystal structure of B. cereus PI-PLC](image)

Fig 1.3. The crystal structure of \textit{B. cereus} PI-PLC in complex with myo-inositol at the active site. The ribbon diagram representation shows the (βα)8-barrel fold of the protein (α-helices, A-H; β-strands, I-VIII). Myo-inositol (Ins) occupies the active site of the enzyme, located at C-terminal end of the β-barrel. The catalytic base, His32, and catalytic acid, His82, are shown in ball-and-stick representation.
It consists of a single globular domain approximately 40 x 40 x 50 Å. It folds an imperfect (βα)₈ - barrel that closely resembles the triose phosphate isomerase (TIM)-barrel. The parallel eight-stranded β-barrel consists of residues 29-34 in (strand I), 64-72 (II), 108-114 (III), 155-163 (IV), 173-176 (V), 182-188 (Vb), 193-201 (VI), 226-236 (VII), and 269-274 (VIII). The α-helices consist of residues 4-8 (helix A), 42-48 (B), 55-61 (C), 91-107 (D), 127-139 (E), 204-222 (F), 243-264 (G) and 284-294 (H). Hydrogen bonds between strands V and VI are not present, therefore a gap exists between the strands. There is also an antiparallel β-strand (Vb) consisting of residues 182-188, different from the typical TIM barrel. PI-PLC is the first phospholipase to be identified as having a TIM-barrel like topology [29].

Recently, the structure of a second bacterial PI-PLC from Listeria monocytogenes has been solved. It shares only about 24% amino acid sequence identity with the enzyme from B. cereus. This shows that they are among the most dissimilar bacterial PI-PLCs known today [30].

1.3. Purification of PI-PLC

1.3.1. Mammalian PI-PLC purification

PI-PLC from several sources have been purified and the genes were cloned and sequenced. Mammalian PI-PLCs have been purified from various sources viz., rat liver [31], sheep seminal vesicles [32], bovine iris sphincter [33], bovine brain [34].

Preliminary purification of PI-PLC from animal cells revealed that instead of a single activity, multiple activities were seen. One of the first purified PI-PLC reported, was a 70 kDa protein from rat liver cytosol [31]. This was followed by a study in sheep
seminal vesicles in which a 65 kDa PI-PLC protein was purified to homogeneity and a second PI-PLC activity of 85 kDa was partially purified [32]. A, 143 kDa protein was purified from bovine platelets, suggesting that many different PI-PLC may exist [35]. In 1986 [36], purification of a membrane bound and cytosolic activity of PI-PLC from calf thymocytes (both 70 kDa) were reported. Three bovine brain PI-PLC’s of MW 150, 145 and 85 kDa were purified and their corresponding cDNA’s were cloned [37]. Multiple purification steps were employed for the purification of these isozymes.

Since this thesis mainly focuses on the bacterial PI-PLC, importance is given to the literature concerning the purification of PI-PLC from bacterial sources.

1.3.2. Bacterial PI-PLC purification

Bacterial PI-PLCs have been purified to homogeneity as extracellular enzymes from Bacillus cereus [24, 25, 26], Bacillus thuringiensis [26, 38], Staphylococcus aureus [27, 39], Clostridium novyi [28], and Streptomyces antibioticus [40].

Bacillus cereus and Bacillus thuringiensis PI-PLC have been purified using a combination of ammonium sulfate precipitation, CM-Sepharose and DEAE-Cellulose ion exchange chromatography followed by phenyl-Sepharose column with a yield of 27 and 23 % respectively [41]. However, an improved purification of PI-PLC from wild type Bacillus thuringiensis as well as recombinant clone have been achieved by Kupke et al (1989) using a two step ion exchange column, DEAE-Sepharose followed by Mono-Q on FPLC with a yield of 50 % [38].

PI-PLC from Staphylococcus aureus was purified in 1977 by Low and Finean [39] using Amberlite CG-50 and Sephadex G-75 with a recovery of only 6 %. However, Staphylococcus PI-PLC have also been purified using Amberlite CG-50 ion exchange
column as well as on Mono-S HR-5/5 cation exchange column with an yield of 25% [42]. Purification of PI-PLC from Clostridium [28], Listeria [43] as well as Streptomyces antibioticus [40] were also done using a combination of ammonium sulfate precipitation followed by ion-exchange columns. The isoelectric point of Bacillus cereus PI-PLC was found to be 5.3 [41].

1.4. Substrates for bacterial PI-PLC's assay system

Bacterial PI-PLCs have been assayed by using different substrates as these enzymes are capable of hydrolysing PI as well as its glycosylated derivatives. [3H]inositol-labelled PI which is relatively easy to prepare and also commercially available is widely used as the substrate of choice. In addition GPI anchored proteins such as alkaline phosphatase and acetylcholinesterase (AChE) are also used as the substrate for assaying the bacterial PI-PLCs [42, 44]. After the action of the enzyme on substrate, the technique to separate the substrate and product formed is dependent on two factors: the nature of the substrate and the part of the molecule, which will be detected or quantified. There are essentially three different ways of separation of the substrate and product. They are

1. Chloroform:methanol:water phase system. This is ideal for measuring phospholipids that are labeled in the polar head group, e.g., [3H]inositol 1,2-cyclic phosphate which partitions into the upper aqueous phase.

2. The second system uses ‘Butanol:water’. This technique is ideal for measuring the degradation of [3H]myristate-labeled Variable surface glycoprotein (VSG) by GPI-PLC, as the product [3H]1,2-dimyristoylglycerol partitions into upper butanol rich phase.
3. The third phase separation uses 'Triton X-114'. This separation procedure is clearly the method of choice when the substrate is a protein and when the detection method measures the protein. Many GPI-anchored proteins partition into triton-poor aqueous phase after degradation of GPI anchor by PI-PLC. In this method the proteins are generally not denatured and therefore the distribution of the enzyme after phase separation (e.g. alkaline phosphatase, acetylcholinesterase) can be determined by measuring its enzyme activity in aqueous phase.

The pictorial representation of the three phase separation techniques is shown in the figure 1.4.

Fig 1.4. Separation techniques used in the assay of PI-PLCs (For details see text).
1.4.1. PI-PLC kinetic studies

Kinetic studies of PI-PLC have been hampered by the lack of continuous assay. An early kinetic study of B. cereus PI-PLC using a discontinuous radioisotope assay was reported by Sundler et al in 1978 [45]. Shashidhar et al in 1991 developed a continuous fluorimetric assay using a synthetic 2-napthyl-myoinositol 1 phosphate [46]. Since the specificity of the substrates were not up to the expectations (only 0.003 % that of the natural substrate) they developed an assay using chromogenic substrate 4-nitrophenyl-myoinositol 1 P which showed a higher specific activity [47]. Later, Hendrickson et al [48, 49] used a thiophosphate analog of PI, the C16-thio PI as well as a fluorescent analog pyrene-PI as the substrates for PI-PLC. Rukavishnikov et al reported the first fluorescein-containing substrate for the continuous fluorescence assay of PI-PLC [50]. This compound is the only reported fluorogenic substrate that is cleaved by both the bacterial and mammalian PI-PLC.

Lot of work is going on in the field of substrate development, which is now one of the hotspots in the field of PI-PLC study.

1.5. CATALYTIC MECHANISM OF PI-PLC

The catalytic domains of all PI-PLCs may share not only a common fold but also a similar catalytic mechanism utilizing general base/acid catalysis [51, 52].
1.5.1. Bacterial PI-PLC mechanism of action

Fig 1.5. Conversion of phosphatidylinositol (PI) to inositol cyclic phosphate (IcP) and inositol phosphate (IP) by two step reaction.

The catalytic action (fig 1.5) of bacterial PI-PLC consists of two steps: fast cleavage of PI into lipid-soluble diacylglycerol and water-soluble myo-inositol 1,2-cyclic phosphate (IcP), and slow hydrolysis of IcP to myo-inositol 1-phosphate (IP). Conversion of PI to IcP is 100-1000-fold faster than hydrolysis of IcP to IP [51]. On the basis of the stereochemical course of the reaction and the X-ray structure of PI-PLC complexed with myo-inositol, a mechanism involving general base-general acid catalysis, has been proposed for both steps as shown in fig 1.6.

Fig 1.6. Mechanism of general base-general acid catalysis proposed on the crystal structure [29].
According to the proposed mechanism, His-32 abstracts the 2-OH proton and His-82 donates a proton to the leaving group O3 oxygen of diacylglycerol in the first step (PI to IcP).

The roles of His-32 and His-82 are reversed in the second step (IcP to IP): His-82 serves as a general base to activate the water molecule, whereas His-32 serves as a general acid to protonate the inositol O2 oxygen and thereby facilitate the ring opening.

Despite many structural similarities between bacterial and the mammalian PI-PLCs, the mechanism of both enzymes differs in two aspects: the eukaryotic enzymes require a calcium cofactor whereas the bacterial PI-PLCs as well as T. brucei GPI-PLC are metal independent. The principal reaction products of the eukaryotic PI-PLCs are both cyclic and acyclic inositol phosphates while the bacterial enzyme yields mainly the cyclic product.

Mammalian PI-PLCs use Ca\(^{2+}\) as a cofactor during catalysis, whereas bacterial enzymes use a spatially conserved guanidinium group from Arg 69 to mediate this reaction [51]. The recent report on engineering a catalytic metal binding site into a calcium-independent bacterial enzyme showed an high steroioselectivity of substrate similar to Ca\(^{2+}\) dependent mammalian PI-PLCs [53].

1.5.2. Scooting model of substrate catalysis

PI-PLC is a water-soluble lipolytic enzyme that binds reversibly to phospholipid surfaces and exhibits a membrane binding specificity. Examination of the active site reveals four regions that may be responsible for the ability of PI-PLC to bind the lipid membranes. The binding of PI-PLC to the membrane surface, and cleaving the substrate
at a higher catalytic rate is known as interfacial activation [23]. However, the ability of PI-PLC to complete several rounds of substrate catalysis before dissociating suggests that the enzyme is capable of acting in a 'scooting mode of interfacial catalysis' (fig 1.7) [54]. Jain et al. suggested that the average time PI-PLC spends associated to the membrane at any given instance is approximately 0.25 seconds, and averages approximately 40-50 catalytic cycles every time it associates with the membrane [55].

![Fig 1.7. Scooting model of PI-PLC action: The PI-PLC attach to the membrane/vesicle surface through phosphatidylinositol (PI), its substrate to give diacyl glycerol (DAG) and 1,2 cyclic inositol phosphate (IcP). Several such cycles of substrate turnover continues before dissociation of the enzyme from membrane surface.](image)

1.5.3. Substrate specificity

Bacterial PI-PLC's specificity towards the substrate depends on

(i) myo-inositol phosphate head group

(ii) and those that vary the fatty acids and glycerol moiety.
1.5.3.1. Myo-inositol phosphate head group

Inositols at first glance have the appearance of sugars, but they are cyclohexanes with one OH group at each carbon atom. The most abundant natural stereoisomer of inositol is myo-inositol. The D-enantiomer of myo-Inositol present in PI and phosphorylated PI are the substrates for bacterial and eukaryotic PI-PLC respectively. Rates of cleavage by bacterial PI-PLCs on the D- and L-enantiomers of myo-inositol show that the enzymes are specific for D-myo-inositol [56, 57]. In order for PI to be a substrate for the bacterial PI-PLCs, either the 4- or 5-OH group of PI must be phosphorylated [58]. The active site cleft in which the inositol fits is too deep and too narrow to allow substrates with phosphoryl groups at these positions to fit within the cleft. Therefore bacterial PI-PLC is extremely specific for phospholipids containing a myo-inositol moiety that can be glycosylated (glycosyl-phosphatidylinositol) but not phosphorylated [23]. The crystal structure of PI-PLC from B. cereus has been determined both in free form (at 2.5 Å resolution; PDB 1PTD) and in complex with myo-inositol (at 2.6 Å resolution; PDB 1PTG) [29, 59].

In contrast to the strict requirements for the head group conformation, bacterial PI-PLCs are insensitive to the stereochemistry around the glycerol sn-2 carbon, the chiral center of the lipid portion of the substrate molecule [60, 61]. A broad spectrum of naturally occurring variations of the lipid moiety is tolerated by bacterial PI-PLC’s. Such variations are abundant in glycosylphosphatidylinositol (GPI) where ether- or ester-linked saturated or unsaturated lipids, ceramides and monoacylglycerol are present in the DAG portion [62]. This suggests that the lipid portion of the substrate, binds to the enzyme via non-specific hydrophobic interactions. The release of membrane proteins by
bacterial PI-PLCs led to the discovery of the GPI-anchored proteins as substrates in the early 1960s [63].

1.5.3.2. Glycosylphosphatidylinositol (GPI) substrates

Many proteins of eukaryotic cells are anchored to membranes by covalent linkage to glycosyl-phosphatidylinositol (GPI). These proteins lack a transmembrane domain, have no cytoplasmic tail, and are, therefore, located exclusively on the extracellular side of the plasma membrane.

Hundreds of structurally and functionally diverse proteins from a variety of eukaryotic sources (mammalian, plant, yeast, and protozoan) have been identified as GPI anchored. These proteins include lymphocyte surface antigens such as Thy-1, protozoan surface coats (trypanosomal VSG), adhesion molecules (e.g., lymphocyte function associated molecule, LFA-3), Decay accelerating factors (DAF), exofacial ectoenzymes such as 5'-nucleotidase (5'-NTase), alkaline phosphatase (APase), acetylcholinesterase (AChE), the prion protein and certain receptors [64-69].

Although several other proteins are also linked through GPI anchor, no exact function and characteristics of GPI anchor to such proteins are known. However, at least in one human disease, paroxysmal nocturnal hemoglobinuria, it has been reported that defective addition of GPI anchor to the plasma membrane proteins caused the disease [70]. The presence of a GPI anchor does confer some functional characteristics to proteins:

1. It is a strong apical targeting signal in polarized epithelial cells
2. GPI-anchored proteins do not cluster into clathrin-coated pits but instead are concentrated into specialized lipid domains in the membrane, including so-called smooth pinocytic vesicles, or caveolae.
(3) GPI-anchored proteins can act as activation antigens in the immune system when the GPI anchor is cleaved by PI-phospholipase C or PI-phospholipase D, second messengers for signal transduction are generated.

(5) the GPI anchor can modulate antigen presentation by major histocompatibility complex molecules. [71-73]

1.5.3.2.1. Structure of the GPI anchor

The initial observation that alkaline phosphatase (thought to be an integral protein) could be released from the membrane bilayer by bacterial PI-PLC was the first clue that there was another form of membrane attachment for proteins, likely via covalent linkage to an inositol phospholipid. Since then, bacterial PI-PLCs (from Bacillus cereus, Bacillus thuringiensis, Clostridium novyi, and Staphylococcus aureus) have been used extensively to identify proteins with GPI anchors. Bacterial PI-PLC have a high affinity for the GPI anchor and catalyses cleavage in vitro to release a soluble protein and diacylglycerol, which remains in the membrane.

The GPI anchor consists of a PI portion where the 6-OH group of myo-inositol is glycosidically linked to a linear oligosaccharide bearing an ethanolamine phosphate group at the distal end. To anchor a protein via GPI, the C-terminal carboxyl group of the protein forms an amide bond with ethanolamine of GPI (fig 1.8).
In some cases, palmitate is esterified to the 2-OH group of myo-inositol. This modification prevents the formation of I(1,2)cP and hence these GPI anchors are not substrates for bacterial PI-PLC [74, 75].

To understand how PI-PLC interacts with the GPI anchor, the crystal structure of PI-PLC from *B. cereus* was determined at 2.2 Å resolution in complex with glucosaminyl (α1→6)-D-myoinositol (PDB 1GYM), which is part of the glycan core of all GPI anchors [76]. The myo-inositol moiety of glucosaminyl (α1→6)-D-myoinositol occupies the same position as free myo-inositol, whereas the glucosamine moiety lies exposed to solvent at the entrance of the active site, indicating that the catalytic mechanism of cleavage of PI is similar to that of GPI. The glycan moiety has very little contact with the enzyme, which explains why bacterial PI-PLCs can cleave GPI anchors with varying glycan structures [69].
GPI-PLC activity is not found with any of the eukaryotic PI-PLCs. It is unique to the bacterial PI-PLCs and a GPI-PLC from *T. brucei*.

1.5.3.3. *Trypanosoma brucei* GPI-PLC

African trypanosomes, the parasite causing ‘sleeping sickness’ in humans and ‘nagana’ in livestock are well known to survive the host immune system by expressing large number of variant surface glycoprotein (VSG) genes. On the plasma membrane of *T. brucei* is a dense protein coat composed of approximately 10 million of VSG which are attached by a glycosylphosphatidylinositol (GPI) anchor. These trypanosomes contain a GPI specific PLC that can cleave the VSG. Although the physiological role of GPI-PLC in this organism is unclear, deletion of its gene in one strain of *T. brucei* reduces the virulence of the parasite [77, 78].

GPI-PLC is unique and distinct from most mammalian phosphatidylinositol-specific phospholipases C (PI-PLC): it is an integral membrane protein, does not require Ca$^{2+}$ for activity, and does not have protein sequence similarity with the mammalian enzymes. The *T. brucei* enzyme is highly specific for GPI while, phosphatidylinositol (PI) is an extremely poor acting substrate. The active site of the *T. brucei* enzyme has not been identified. When the amino acid sequence of *T. brucei* GPI-PLC was compared with the PI-PLC from *Bacillus thuringiensis, Listeria monocytogenes* and *Staphylococcus aureus*, a peptide region of high similarity (75%) and identity (50%) was found [23]. Glutamine at position 81 in *T. brucei* GPI-PLC is conserved in all the prokaryotic PI-PLC [79].
1.5.3.4. Active-site amino acids of PI-PLC

Structural analysis of PI-PLC from \textit{B. cereus} bound to \textit{myo}-inositol shows the binding of the inositol’s hydroxyl groups in the 2-5 positions strongly interact with specific amino acids of the PI-PLC active site. Atleast one hydrogen bond is formed between the hydroxyl groups of the \textit{myo}-inositol and the side chains of amino acids His32, Arg69, Arg163, Lys115 and Asp198 [23, 29]. These side chains are held in the correct orientations by hydrogen bonding to the side chains of Asp67, Glu117, Asp180, Trp178 and Asp274, and are required for the stereoselectivity of the D-\textit{myo}-inositol head group. These strong interactions with the \textit{myo}-inositol hydroxyl groups indicate the reason behind bacterial PI-PLCs specificity towards non-phosphorylated substrates and not on the phosphorylated substrates. From the available crystal structure and NMR evidence, His32-Asp274 functions as the catalytic diad of \textit{Bacillus cereus} PI-PLC.

1.5.3.4.1. The interfacial contact region of bacterial PI-PLC

For substrate binding and catalysis, PI-PLC must make physical contact with the lipid surface of a vesicle or micelle containing the substrate. This contact region which is called as interfacial binding surface, are contributed by three different parts of the enzyme which was identified based on the crystal structure of \textit{B.cereus} PI-PLC. They are

(a) the short K-helix B with residues Pro 42, Ile 43, Val 46 and Trp 47,

(b) Pro 84 and Leu 85 contained in the loop connecting strand II and helix D, and

(c) Ala 241, Trp 242 and Pro 245 of the loop between strand VII and helix G (fig. 1.9).
In addition to these hydrophobic interactions, an electrostatic contribution involving three lysine residues was also implicated. They are Lys 44, Lys 122 and Lys 201. Other than this, Trp 47 and Trp 242 which are clustered around the hydrophobic ridge are also implicated in the interfacial binding of the substrate and are likely responsible for the intrinsic fluorescence increase observed when PI-PLC binds to an interface [23, 80].

**1.6. Roles of PI-PLC**

In addition to its role in signal transduction at cellular level and as a virulent factor in pathogenic organism, PI-PLC also has some additional functions. They are,
1. A role of PI-PLC in vivo has been demonstrated for *Drosophila melanogaster*, in which mutations in PI-PLC β isoform disrupts phototransduction reactions which results in their blindness [16].

2. Disruption of mouse PI-PLC γ gene results in embryonic lethality indicating an essential requirement for developmental activities [81].

3. Growth factors and certain oncogenes activate phospholipid-mediated signalling resulting in an increase of intracellular free Ca\(^{2+}\). This leads to activation of protein kinase C setting in a series of profound cellular changes, facilitating cellular proliferation and neuronal activity. Abnormal functions of PI-PLC have been linked to cancer. Increased PI-PLC activity has been reported in a number of human tumours, especially in the more aggressive malignant tumours [82-88].

Some examples are

- PI-PLC activity in rat hepatomas is increased in the more aggressively growing tumors. It has been reported that 76% of human breast cancers have detectable PI-PLCγ immunoreactive protein compared to only 6% of benign breast tissue [82,83].

- Cytosolic PI-PLC activity was found to be increased up to 4-fold in human lung cancer and renal cell cancer compared to normal tissue [83].

- An association between PI-PLCγ immunoreactivity and EGF receptor expression has been reported in human glial tumors [85].

The growth inhibition of GH4C1 cancer cells by tamoxifen has been linked to inhibition of PI-PLC. Therefore selective small molecule inhibitors of over activated PI-PLC signalling pathways may provide potential therapies for cancer [82-89].
1.7. Modulators of PI-PLC

1.7.1. Commercial inhibitors of PI-PLC

Three compounds which function as inhibitors of PI-PLC are now commercially available. One is the surfactant-like molecule 1-O-octadecyl-2-O-methyl-ras-glycero-3-phosphorylcholine (IC$_{50}$ - 15 μM) and the other is the steroid analogue \{1-[6-((17b-3-methoxy-esta-1,3,5(10)-trien-17yl)aminohexyl]-1H-pyrrole-2,5-dione\} U73122 with an IC$_{50}$ of 2.1 μM concentration. U73122, a specific PI-PLCy inhibitor, was found to greatly diminish invasiveness of DU-145 prostate tumor cells [90].

Recently, inhibitors (6-aza-steroids) based on U73122 have been synthesized and found to be very potent against HT-29 colon cancer cells and MCF-7 breast cancer cells [90].

1-O-octadecyl-2-O-methyl-ras-glycero-3-phosphocholine (ET-18-OCH$_3$), a synthetic analogue of lysophosphatidylcholine showed antitumor activity and was found to be an specific inhibitor of PI-PLC. ET-18-O-CH$_3$ selectively inhibits PI-PLC-B1 more than PI-PLC $\gamma$1 (IC$_{50}$ = 9.6 μM) and does not inhibit PC-PLC [91].
1.7.2. Substrate analogues as inhibitors of PI-PLC

Substrate analogues of phosphatidylinositol were synthesized and evaluated as potential inhibitors of the bacterial PI-PLC from *Bacillus cereus*. A recent report showed that D-*myo*-inositol 4-(hexadecyloxy)-3(S)-methoxybutanephosphonate (C4-PI), an isosteric phosphonate analog of phosphatidylinositol inhibits the activity of purified recombinant PI-phospholipase C-β at all concentrations tested; it enhanced the activity of PI-PLC-γ and PI-PLC-δ at low concentrations (10 μM), while severely inhibiting their activities at higher concentrations [92].

1.7.2.1. *myo*-inositol(Ins)-1-O-dodecylphosphonate as inhibitor of GPI-PLC

Deletion of the gene for GPI-PLC from *T. brucei* results in parasites with reduced ability to grow in a mammalian host [93]. This lead to the possibility that inhibition of GPI-PLC could be beneficial in the management of sleeping sickness, the disease caused by *T. brucei*. GPI-PLC of *T. brucei* was inhibited by *myo*-inositol(Ins)-1-O-dodecylphosphonate (VP-602L) a derivative of glucosaminylinositol (GlcN(a1-6)Ins), the core group GPI anchor. Several novel fluoro-substituted analogs of 2-deoxy-*myo*-Ins-1-O-dodecylphosphonate were studied among which 2-deoxy-2-fluoro-scyll-o-Ins-1-O-dodecylphosphonate (VP-616L) was found to be most powerful.
1.7.3. Interaction of aminoglycoside antibiotics with PI-PLC

Reports have shown that aminoglycosides cause lysosomal phospholipidosis in cultured cells by inhibiting lysosomal phospholipases. These aminoglycosides by binding to the negatively-charged phospholipids of the membrane make the phospholipases unable to establish contacts with the membrane, thereby causing their impairment and an accumulation of undigested phospholipids [94, 95]. This is one of the causes for the development of phospholipidosis, which is the hallmark of the renal toxicity of aminoglycosides. A recent report shows that a GPI-PLC from the protozoan parasite *Trypanosoma brucei*, was activated to 6-fold by aminoglycoside antibiotics of neomycin class [96].

1.7.4. Interaction of glucocorticoids on PI-PLC

Studies in humans and animals suggested the involvement of glucocorticoids in many functional aspects of the CNS, such as behavior, mood, emotion, and learning [96]. Recent report shows that, when dexamethasone (DEX), a synthetic glucocorticoid, was given as a single dose or as a repeated administration (0.5 or 1.0 mg/kg for 10 days), caused a significant increase in PI-PLC activity as well as in protein expression of the PLC β1 isozyme in rat brain. The increase in PLC β1 protein was associated with an increase in its mRNA level which suggest that DEX up-regulates PI-PLC in rat brain. This presumably is due to the increase in expression of the PI-PLC-β1 isozyme. The observed changes in PI-PLC was related to the hypothalamic–pituitary–adrenal axis-mediated changes in mood and behavior in rats [97].
1.8. Interaction of pertussis toxin with PI-PLC

Most extracellular signals interact with receptors at the cell surface, which in turn transduce signals to the interior of the cell. One such receptor is a G protein (guanine nucleotide binding protein) linked cell surface receptor. Rat mast cell was the first system reported to show the interaction of G protein with PI-PLC [98]. Numerous stimuli are capable of inducing histamine by activating PI-PLC, one such was a compound 48/80, which induce histamine release by activating PI-PLC. It has been shown that the histamine release induced by compound 48/80 can be blocked by pertussis toxin (produced by *Bordatella pertussis*), which catalyses the transfer of ADP-ribosyl moiety of NAD to a site on α-subunit of G protein. This inturn interacts with PI-PLC thereby blocking the histamine release. Similarly, pertussis toxin blocks the activation of PI-PLC in HL60 cells also [99]. Pertussis toxin has been used to find out the involvement of G protein in a large number of PI-PLC receptor coupled system [21]. The interaction of the protein toxin (pertussis) with PI-PLC prompted us to study the interaction of diphtheria toxin (DT) with PI-PLC, since both pertussis and DT’s mode of actions are similar i.e. ADP-ribosylation to inactivate the protein.

1.8.1. Diphtheria toxin

Many toxic proteins of bacterial origin are known to act by enzymatically modifying their substrates within the cytosol of mammalian cells, but the mechanism by which these toxins crosses a membrane to gain access to its substrates is not yet understood. These intracellularly acting toxins are generally bipartite proteins, containing the enzymic and receptor-binding functions on separate polypeptides, designated A and
B, respectively. In some toxins, the B moiety also mediates the translocation of the A moiety across membranes.

Diphtheria is an acute, communicable respiratory disease caused by toxigenic strains of *Corynebacterium diphtheriae* which produces the causative diphtheria toxin (DT). DT, the earliest example of an AB toxin, is a single, 535-residue polypeptide containing three distinct domains: the amino-terminal or catalytic domain (residues 1–185) called C domain; the intermediate or transmembrane domain (residues 202–378) known as T domain and the carboxyl-terminal or receptor-binding domain (residues 386–535) known as R domain. After binding to its cell-surface receptor via the R domain, DT is proteolytically cleaved within the arginine-rich loop, yielding two disulfide-linked fragments: fragment A (corresponding to domain C) and fragment B (corresponding to domains T and R). Fragment A enters into the cytosol (10–12) through receptor mediated endocytosis where it catalyzes the ADP-ribosylation of elongation factor 2, causing inhibition of protein synthesis and cell death. [100-107].

The cellular effects of several toxins have not been analyzed in detail. Unexpected findings stimulate novel approaches to study the toxin-protein interaction within cells. One such interesting observation resulted from our study involving DT’s interaction with acetylcholinesterases. This finding will open up new avenues in DT’s action on cellular proteins, which will allow us to understand the extended mode of action of these bacterial toxins.

1.9. Cholinesterases

Cholinesterases belong to the family of serine hydrolases. In vertebrates, they exist in two forms: acetylcholinesterase (AChE, EC 3.1.1.7), which hydrolyzes the
neurotransmitter acetylcholine and butyrylcholinesterase (EC 3.1.1.8), for which the physiological role is not well known. Rapid hydrolysis of acetylcholine by AChE is essential for cholinergic neurotransmission \( \frac{k_{\text{cat}}}{K_m} \approx \frac{k_{\text{cat}}}{k_m} \times 10^9 \text{ M}^{-1} \text{s}^{-1} \) which ranks it as one of the highest catalytically efficient enzyme known [108].

The dimeric membrane form of AChE is attached via a GPI anchor which is the mode of attachment of AChE in the electric organ of Torpedo, flounder muscle, human and bovine erythrocyte membrane as well as in Drosophila heads. Except from human erythrocyte membrane all these enzymes can be converted to a soluble form by treatment with bacterial PI-PLC or GPI-PLC from Trypanosoma brucei. The resistance to PI-PLC of membrane form AChE from human erythrocytes is due to the existence of an additional fatty acyl chain esterified with inositol. Because of the AChE’s high catalytic efficiency, bovine erythrocyte AChE was used as the substrate for PI-PLC from Bacillus thuringiensis in the present study.

Ligand binding and X-ray crystallography studies have revealed a narrow active site gorge which is 20 Å deep with two separate ligand binding sites (acyl binding site; choline binding site) and a peripheral anionic site (PAS) near the mouth of the gorge. Recent investigations have shown that the peripheral site contributes to catalytic efficiency by transiently binding substrates on their way to the acylation site [109]

Inhibitors of AChE act on two target sites in the active site gorge and the PAS. Inhibitors directed to the active site prevent the binding of a substrate molecule, or its hydrolysis, either by occupying the site with a high affinity (edrophonium and tacrine) or by reacting irreversibly with the catalytic amino acid serine (organophosphates and carbamates). The PAS is located at the entrance of the gorge and the inhibitors that bind to this site include small molecules, such as propidium, curare, gallamine, and peptide
toxins from Mamba venoms, the fasciculins [7, 8]. Bis-quaternary inhibitors, e.g. decamethonium and BW284c51, simultaneously bind to the active and PAS, thus occupying the entire catalytic gorge. The mechanism by which PAS inhibitors block the catalytic activity of AChE is explained either by steric blockade of the catalytic gorge entrance or by an allosteric mechanism. It has been proposed that occupancy at the PAS induces a conformational change which allosterically modify the orientation of a tryptophan residue, Trp-84, which serves as the choline binding site [109-117].

When the interaction of DT on PI-PLC (section 1.8.1) was carried out, we found to our surprise that the AChE from bovine erythrocyte was inhibited rather than PI-PLC. This prompted us to study the interaction of DT on AChE rather with PI-PLC. Further studies involving DT were carried out using the AChE from various sources which are discussed in detail in chapter - 4.

1.10. Scope of the thesis

Phospholipases, in general, provides to many bacteria an imposing armada of virulence attributes. In addition, in unequivocal terms it has also been demonstrated to be involved in signal transduction, where the specific molecular interaction with cellular components under physiological conditions and several exogenous compounds under pharmacological conditions remains to be defined. To know more about the role of phospholipases in bacterial (with virulent/non-virulent) as well as in human diseases, regarding the structural-functional relationship, interaction with inhibitors/activators/drugs must be known. The characterization of PI-PLC from Bacillus thuringiensis undertaken in the present study is an attempt in understanding the above functional characteristic of the complex multidomain enzyme phospholipase C.
In summary the thesis discusses:

1. the production of GPI-PLC from *Bacillus thuringiensis* using response surface methodology.

2. the interaction of GPI-PLC with aminoglycoside antibiotics and possible physiological relevance of these antibiotics interaction with PI-PLC.

3. the interaction of chloroquinoline based antibacterial/antifungal compounds on GPI-PLC and its possible implications on mammalian PI-PLC.

4. the DT activation of catalytic activities of AChE and its possible role in the pathogenicity of DT.