Review of Literature
Fig. 11 Sites of Action of the Second Messengers IP$_3$ and DAG: Shown here are the potential ways in which IP$_3$ can bring about release of calcium from internal stores including the nucleus. Cytoplasmic IP$_3$ mobilizes calcium from the endoplasmic reticulum/vacuole or the nuclear envelope while DAG activates protein kinase C which becomes membrane bound. Protein kinase C in turn phosphorylates cytosolic and/or nuclear proteins An analogous cycle is also present in the nucleus. (adapted from Irvine, 2000)
External signals arriving at the cell surface engage receptors to initiate signalling pathways whereby information flows from one component to the next until the final effector system is activated. Calcium ions play an important role in various cellular functions such as signal transduction and regulation of mechano-chemical reactions in plant and animal cells. Transient increases in intracellular calcium concentrations have been observed in response to a variety of stimuli in plant and mammalian cells. Calcium entry into cells can be regulated by a number of mechanisms, for example operating through channels operated by voltage, by receptors or second messengers. Mammalian cells have two intracellular channels for regulating calcium release from internal stores; inositol phosphate and ryanodine receptors. Inositol phosphate is a second messenger that controls many cellular processes by generating internal calcium signals and operates through the intracellular inositol phosphate receptors. Production of the second messenger inositol phosphate is brought about by the action of phospholipase C (PLC). The second messenger generation brought about by PLC at the membrane surface and in the nucleus is illustrated in Fig II and is now well established in plants. PLC, by virtue of its ability to produce the second messengers, inositol phosphate and diacylglycerol plays an important role in calcium signalling. In this review we have attempted to compile all the relevant literature on PLC in plants. An overview of PLC in other systems is also presented.

2.1 Classification of Phospholipases

Most cells are in a state of flux in relation to their membranes. Glycerophospholipids are important components of membranes and their hydrolysis by enzymes called phospholipases in response to stimuli constitutes an important part of a signalling process leading to the generation of second messengers. These phospholipases are classified depending on their site of action (Sopory et al., 2001). Thus, phospholipases PLA₁ and PLA₂ act respectively to remove the fatty acids esterified at the sn-1 and sn-2 hydroxyl groups of the glycerol backbone to give lyso-phospholipids. Other phospholipases termed C and D act at the phosphodiester linkage in the glycerophosphate backbone at different sites to generate bio-active molecules. PLD activity leads to the formation of phosphatidic acid (PA) and free headgroup while PLC activity leads
to the production of diacylglycerol (DAG) and a phosphorylated headgroup. About two decades ago, a special kind of PLC was discovered that hydrolysed the minor lipid Phosphatidylinositol (4,5) bisphosphate (PIP$_2$) to generate the second messengers DAG and inositol (1,4,5) trisphosphate and has now been implicated to be activated in response to diverse stimuli in a manner that seems to be conserved across species (Rhee et al., 1989). This PIP$_2$-dependent PLC will be referred to as PI-PLC in the review.

2.2 PI-PLC Isozymes

PI-PLCs comprise a diverse family of enzymes. They have been isolated from bacteria, protozoa (Heinz et al., 1995; Williams, 1999), yeast (Flick and Thorner, 1993) fungi (Jung et al., 1996), invertebrates (Shortridge and McKay, 1995; Shibatohge et al., 1998) and mammals (Rhee et al., 1989).

2.2.1 Mammals

The first mammalian PI-PLC purified to homogeneity was from rat liver and its molecular mass as judged from SDS-PAGE was 68 KDa. (Rhee et al., 1989). Subsequently numerous “PI-PLC activities” were resolved chromatographically from a variety of tissues and shown to differ in size and behaviour (molecular mass, pI), activity (pH optima, calcium dependency), localization and antigenicity, pointing to the existence of multiple PLC isozymes. Despite a wide variation in properties, all PI-PLCs had one biochemical feature in common – all enzymes were specific for phosphatidylinositol (PI) and polyphosphoinositides (including PIP and PIP$_2$) and did not hydrolyze other phospholipids. Subsequently, PI-PLC related cDNA clones were isolated and sequenced and showed differing amino acid sequences (Rhee et al., 1989). These isozymes of PI-PLC with different primary sequences were designated using Greek letters ($\alpha$, $\beta$, $\gamma$ and $\delta$). The letters were assigned in the chronological order of purification. PLC$\alpha$ is an anomaly in that despite its ability to utilise PI and other polyphosphoinositides as substrates, it shows wide sequence divergence from other PI-PLC isozymes. Recently a new isozyme, PLC$\varepsilon$ has been reported and phylogenetic comparison reveals that it shows closest homology to the PLC$\beta$ isozymes (Kelley et al., 2001; Lopez et al., 2001; Song et al.,
2001). Thus far, eleven mammalian PI-PLC isozymes have been reported (PLCβ1-β4, PLCγ1-γ3, PLCδ1-δ4 and PLCε). PLCε is the largest PI-PLC known with the homolog from rat having a molecular weight of 255 KDa (Kelley et al., 2001). PLCβ and PLCγ have average molecular weights of around 150 KDa while PLCδ has a molecular weight of around 85 KDa in mammals (Essen et al., 1996). Sequence comparison reveals the presence of two domains X and Y separated by a spacer region that are conserved across all classes of PI-PLC (Rhee et al., 1989).

2.2.2 Bacteria

The smallest PI-PLCs are about 35 KDa in size and are produced by a variety of aerobic or anaerobic Gram positive bacteria including the pathogens Bacillus cereus, B. thuringiensis, Listeria monocytogenes, L. ivanovii, Staphylococcus aureus, Clostridium novyi and Rhodococcus equi and are considered to be potential virulence factors (Heinz et al., 1995; Griffith and Ryan, 1999). These enzymes are also found in non pathogenic species such as L. seeligeri, Streptomyces antibioticus and Cytophaga spp. In contrast to mammalian PI-PLCs, bacterial PI-PLCs are secreted, prefer PI as substrate, cannot hydrolyse phosphorylated phosphoinositides and do not require calcium for activity. The sequence for five bacterial PI-PLCs are known (Griffith and Ryan, 1999). All are single polypeptide chains of about 300 amino acids. The highest degree of similarity among PI-PLCs is seen among members of the same genus. Thus the sequences reported from B. cereus and B. thuringiensis differ from each other at only eight amino acid residues (Heinz et al., 1995). Similarities to the mammalian PI-PLCs are limited to a few residues within the N-terminal half of the bacterial PI-PLC sequences and the X-region.

2.2.3 Lower eukaryotes and plants

Six putative PLC genes have been cloned from invertebrates; four of these are from Drosophila and one each from Caenorhabditis elegans and Artemia (Shortridge and McKay, 1995; Singer et al., 1997; Shibatohge et al., 1998). The Drosophila norp A and plc21 genes and one partial sequence reported from Artemia are all β-subtypes. The PLC-γD, also from Drosophila, is a gamma subtype. PLCε was first reported from C. elegans and was identified in a screen as a Ras binding protein, let-60.
Fig. III: Linear alignment of the members of the family of eukaryotic phosphoinositide-specific Phospholipase C: PLCs comprise different structural modules and from various studies it is now possible to assign specific functions to them. During the normal catalytic cycle, PLCs appear to bind stably to membrane interfaces, as a pre-requisite for efficient substrate hydrolysis. Three modules (PH domain, X/Y TIM barrel and C2 domain) are involved in regulated interaction of PLCs with membranes and hydrolysis of PIP$_2$. Other modules contribute to receptor G-protein and tyrosine kinase-mediated regulation of hydrolytic activity.
PI-PLC homologs identified in lower eukaryotes including plants, fungi, yeast, Dictyostelium discoideum and higher plants are all δ-isoforms (Essen et al., 1996).

2.3 Structural Organisation of PI-PLCs

2.3.1 Domain Arrangement

Mammalian PI-PLCs, as mentioned previously show a wide sequence divergence and this in turn is reflected in their structural organisation. All PI-PLCs show a modular arrangement of domains and this is represented schematically in Fig. III (Irvine, 1996; Singer et al., 1997; Williams, 1999). As mentioned before, all isoforms have two regions called X and Y that constitute the catalytic domain separated by a linker region. They also have additional domains that either help in gaining access/binding to the substrate at the membrane surface or impart unique regulatory properties. All mammalian PI-PLCs with the exception of PLCε have a Pleckstrin Homology (PH) domain near their amino termini. All mammalian PI-PLCs have a C2 domain near their carboxy terminal end. The region between the amino terminus and the X domain forms a structure with four EF Hands and is seen in the β, γ and δ forms. The β-isoforms contain an extended carboxy terminus consisting of 400-500 amino acids for regulation by trimeric G-proteins. The γ-isozymes have an insertion of about 500 residues between the two halves of the catalytic domain. This inserted region shows the presence of an additional PH domain along with two Src Homology 2 (SH2) and one Src Homology 3 (SH3) domains. The presence of the SH2 and SH3 domains allows for the regulation of the γ-isoforms by receptor and non-receptor tyrosine kinases (Irvine, 1996). The ε-isozymes have the characteristic X, Y and C2 domains (Kelley et al., 2001; Lopez et al., 2001; Song et al., 2001). In addition they have two Ras-binding motifs at the carboxy-terminal end called the RA domain (RA1 and RA2). One of the PLCε reported from humans shows a single RA domain (Song et al., 2001). PLCε also has a CDC25-like domain domain at its N-terminal end (replacing the PH domain) which shows the presence of three conserved stretches seen in the catalytic domains of the GTP/GDP binding proteins acting on Ras-like small GTP binding proteins. The presence of the RA
domains and the CDC25 domains strongly suggests regulation of PLCε by Ras or vice versa.

Members of the δ-subfamily are the simplest in terms of structural organisation as they only have all the sequences common to all PI-PLC subfamilies. Thus rat PLCδ1 shows the presence of X, Y C2, PH and EF hand domains (Essen, et al., 1996). Their widespread appearance in plants, yeast, slime moulds suggests that this class evolved first in eukaryotes and split from the mammalian evolutionary tree at an early point. In all lower eukaryotes and in higher plants the structural organisation of PI-PLCs is even simpler and contain only the X, Y and C2 domains. The PH domain is notably absent while one EF hand motif is sometimes reported to occur before the X domain (Flick and Thorner, 1993; Hirayama et al., 1995; Shi et al, 1995a; Kopka et al.; 1998a,).

2.3.2 Structure of PI-PLC

Although the three dimensional structure has been determined only for the PLCδ1 isozyme, it has served as a valuable guide for understanding the organisation of other enzymes (Essen et al., 1996). Each of the domains present is now described briefly.

2.3.2.1 PH Domain

The N-terminal of PLCδ1 shows the presence of a PH domain. It is a module consisting of about 120 amino acids and was first identified in Pleckstrin, a major substrate for protein kinase C (PKC). It was subsequently found to occur in a large number of proteins involved in cellular signalling or cytoskeletal functions (Lemmon et al., 1996).

Initial characterisation of the amino terminal of PLC δ1 involved studies with a 77 KDa proteolytic fragment that left the X, Y and carboxyl terminal intact but removed the first 60 amino acids. This tryptic fragment was found to exhibit reduced capacity to hydrolyse PIP2 in mixed micelles in a processive manner. The 77 KDa fragment was also shown to have a reduced affinity towards PIP2 (Cifuentes et al., 1993). In a separate study it was demonstrated that the inclusion of PIP2 in mixed micelles of PI: PS: PC (1:5:5) increased the rate of hydrolysis of PI in a dose dependent manner (Lomasney et al., 1996). The incorporation of PIP2 in these vesicles decreased the micellar dissociation constant (Kₛ) almost ten fold while the Michaelis constant (Kₘ) was reduced 3.5 fold. This PIP2-mediated enzyme activation required the presence of certain residues between

6
20-40 at the amino terminus. Thus the amino terminus/PH domain of PLCδ1 was thought to be required for initial PIP$_2$ binding and hydrolysis in a processive manner. The amino terminal region was also shown to be required for binding IP$_3$ (Yagisawa et al., 1994). Deletion of 223 residues from the amino terminus completely abolished IP$_3$ binding while deletion of the X region only partially inhibited the binding while deletion of the Y region did not affect binding. The 77 KDa tryptic fragment was shown to have minimal IP$_3$ binding. Thus the PH domain appeared to be important for binding of both PIP$_2$ and IP$_3$. Competition binding studies and isothermal titration calorimetry (ITC) performed with isolated the PH domain confirmed that it binds with high affinity to IP$_3$ ($K_d = 0.21 \mu$M) and PIP$_2$ ($K_d = 1.7 \mu$M) (Lemmon et al., 1995). This binding specificity of the isolated PH domain was comparable to that seen for the intact PLCδ1. Binding of IP$_3$ to PLCδ1 has been shown to inhibit PLC activity (Lemmon et al., 1996).

The crystal structure of the PLCδ1 PH domain in complex with IP$_3$ consists of seven anti-parallel $\beta$-strands arranged into a barrel-like structure with one half of the barrel consisting of three sheets and the other half consisting of four with a C-terminal $\alpha$-helix at the bottom (Ferguson et al., 1995). The PLCδ1 PH domain shows stereo-specific high affinity lipid headgroup binding that is accomplished by an extensive network of hydrogen bonds. Taken together, all these features are suggestive of a model where the PH domain of PLCδ1 serves to anchor the enzyme to PIP$_2$-rich membranes permitting a processive mode of hydrolysis of substrate (Cifuentes et al., 1993). IP$_3$ binding to the PH domain competes with PIP$_2$ binding (affinity for IP$_3$ is higher) and thus inhibits PLCδ1 thus suggesting a probable feedback regulation of PLCδ1(Lemmon et al., 1996).

The PH domain of PLCδ1 is thought to be involved in membrane binding. Green fluorescent protein (GFP) tagged PLCδ1 or the GFP tagged PH domain were found to be predominantly localised at the plasma membrane in unstimulated MDCK cells. (Fujii et al., 1999) The induction of hyper-osmotic stress in these cells lead to the rapid dissociation of GFP-PLCδ1 from the plasma membrane and this coincided with phosphoinositide breakdown. Inhibitors of PLC hydrolysis, ionophores or replacement with an iso-osmotic buffer blocked the translocation to the membrane. The residues interacting with IP$_3$ in the PLCδ1 are not conserved amongst the various mammalian PI-
PLC classes (Williams, 1999). Hence the mode of recruitment to the membrane may be different in these cases. Also, the PH domain is absent in the δ-isoform reported from lower eukaryotes and higher plants (Kopka et al., 1998a). Despite this membrane recruitment presumably occurs, though no detailed study in this regard is currently available.

2.3.2.2 EF Hand Domain
The EF hand domain in PLCδ1 is a region of the enzyme that is flexibly attached to the PH domain as evidenced by the lack of an ordered organisation in the crystal structure. It consists of four helix-loop-helix motifs that are arranged in two lobes. Although the first two lobes seem to have residues that are required for calcium binding, there is no experimental evidence for the same (Essen et al., 1996). The second lobe has no residues conserved for calcium binding and was in fact not recognised to be an EF hand until the crystal structure was worked out (Essen et al., 1996). This is substantiated by ITC studies of the PH domain deleted PLCδ1 where the stoichiometry of calcium binding to the enzyme was examined (Grobler and Hurley, 1998). The study did not suggest the presence of any site(s) in the EF hand domain that could bind calcium. The second lobe is well ordered and interacts with the C-terminal end of PLCδ1. Deletion variants of PLCδ1 and PLCγ lacking the second lobe were found to be catalytically inactive leading to suggestions that removal of the second lobe destabilised the enzyme structure and that the second EF hand might only play a structural role (Essen et al., 1996, Williams, 1999). Recently, the recognition of a functional nuclear export signal in the EF hand of PLCδ1 suggests that this domain might have additional roles in PI-PLCs that do not involve calcium binding (Yamaga et al., 1999). More detailed studies on the EF hand will help throw light on its importance in mammalian PI-PLCs. PI-PLCs from lower eukaryotes or plants either do not show an EF hand structure or display just one EF hand motif.

2.3.2.3 Catalytic Domain
The designation of the X and Y domains as catalytic regions was confirmed from the crystal structure of PLCδ1 determined at a resolution of 2.4 Å (Essen et al., 1996).
These two regions are separated in the primary structure but come together in a three dimensional structure to form a unit referred to as a "distorted but closed triose-phosphate isomerase TIM barrel". The TIM barrel structure consists of \((\alpha\beta)_8\) repeats \((n = 8)\) and is found in a large number of proteins which are otherwise functionally not related (Heinz et al., 1995). The X domain forms one half of the TIM barrel with a typical \(\alpha\beta\alpha\beta\alpha\beta\alpha\beta\alpha\beta\) motif. The other half is formed by the Y region. Unlike a typical TIM barrel, the \(\alpha\)-helix between the fifth and sixth strands is missing. Interestingly, despite wide sequence divergence from mammalian PI-PLCs, the crystal structure of \(B.\ cereus\) PI-PLC exhibits a similar imperfect TIM barrel organisation suggesting the divergence of PLCs from a common ancestor (Heinz et al., 1995). Instead of eight, six repeating units are seen. Helices between strands IV and V and V and VI are missing. The region between the IV and V strands in the \(B.\ cereus\) PI-PLC is connected by a wide loop and is structurally equivalent to the linker insertion connecting these strands in the mammalian enzymes.

The X and Y regions are capable of forming an independent active unit despite the intervening linker region whose length is highly variable. Prolonged tryptic digestion of PLC81 gives 45 KDa and 32 KDa fragments that co-sediment as an active complex of 70 KDa in a glycerol gradient. Activity studies with the 70 KDa complex have shown that the presence of an intact intervening linker region is not essential for PIP\(_2\) hydrolysis (Cifuentes et al., 1993). This was also confirmed by co-expression of the X and Y polypeptides of PLC\(\gamma\)1 in insect cells, leading to their association with the complex showing 20-100 fold higher specific activity towards PIP\(_2\) as compared to the holozyme (Horstman et al., 1996). This association of the X and Y polypeptide upon co-expression has also been reported for PLC\(\beta\)2 (Zhang and Neer, 2001).

The active site is a broad solvent accessible depression at the C-terminal end of the barrel (Essen et al., 1996; Essen et al., 1997a). All residues at the active site are conserved and this has been confirmed by alignment and mutation studies (Ellis et al., 1998). The inositol ring sits edge-on in the active site cleft. A network of hydrogen bonds and salt bridges between the active site (containing calcium) and the bound inositol phosphate ensures that with the exception of the 6-OH group, all groups of IP\(_3\) are stereospecifically recognised. Further the binding of calcium and IP\(_3\) to the active site is not accompanied by significant structural changes (Essen et al., 1997a).
The preference of mammalian PI-PLCs for \( \text{PIP}_2 \) over \( \text{PIP} \) and \( \text{PI} \) is the direct consequence of various salt bridges between the 4 and 5 phosphate groups and a cluster of basic residues (Lys\textsubscript{438}, Lys\textsubscript{440}, Arg\textsubscript{549}) (Essen et al., 1996). The 3-hydroxyl group of \( \text{IP}_3 \) is hydrogen bonded to Glu\textsubscript{341} and Arg\textsubscript{549} and there is insufficient space for a phosphate at the 3-position in the inositol ring. This explains why PLC\textsubscript{81} does not act on \( \text{PIP}_3 \) as a substrate. The 2-hydroxyl group interacts with calcium which is also coordinated with Asn\textsubscript{312}, Asp\textsubscript{343} and Glu\textsubscript{390}. Substitution of these amino acids results in a shift towards higher calcium concentrations required for \( \text{PIP}_2 \) binding (Ellis et al., 1998).

The function performed by calcium at the active site of mammalian PI-PLCs is replaced by positively charged residues in \textit{B. cereus} PI-PLC (Heinz et al., 1995). The 1-hydroxyl group of \( \text{IP}_3 \) interacts with the imidazole rings of His\textsubscript{311} and His\textsubscript{356}. These two residues are absolutely essential for activity and mutations in these residues results in a 6,000-20,000 fold drop in activity (Ellis et al., 1998). Their structurally equivalent counterparts in \textit{B. cereus} PI-PLC are His\textsubscript{332} and His\textsubscript{382} respectively (Heinz et al., 1995). Naturally occurring PLC-like proteins, PLC-L and PLC-L2 are catalytically inactive and an analysis of their sequence reveals that the His\textsubscript{356} is replaced in these proteins by other amino acids (Otsuki M. et al., 1999). The inositol ring is held in place by van der Waals interactions with the aromatic ring of Tyr\textsubscript{551} (Tyr\textsubscript{200} in \textit{B. cereus} PI-PLC) (Heinz et al., 1995; Essen L. O. et al., 1996).

The structure of PLC\textsubscript{81} has a hydrophobic ridge surrounding one edge of the active site opening (Essen et al., 1996). Based on studies of the enzyme in complex with the detergent CHAPSO, it has been suggested that the hydrophobic ridge of the enzyme is the portion that penetrates the membrane during interfacial catalysis to facilitate entry of phospholipid substrates into the active site. The residues thought to be important at the hydrophobic rim include Leu\textsubscript{320}, Tyr\textsubscript{358}, Phe\textsubscript{360}, Leu\textsubscript{529} and Trp\textsubscript{555} (Ellis et al., 1998). A similar clustering of hydrophobic residues at the rim of the active site has been observed for \textit{B. cereus} PI-PLC with two tryptophans being fully exposed to solvent (Heinz et al., 1995). While the active site governs the stereo-specificity of the binding headgroup, additional non-stereospecific contact(s) with the lipid moiety might contribute towards subtle differences in determining activity. Studies with PI-analogs with varying fatty acyl chain lengths show that there might exist a relationship between acyl chain
length and the affinity of PLC for its substrate (Rebecchi et al., 1993). A very low rate of hydrolysis of di-C₄-PI, a PI analog, suggests that a minimum chain length is required for positioning of the substrate at the active site and is consistent with the fact that deacylated inositol lipids are not hydrolysed by PLC.

2.3.2.4 C2 Domain

C2 domains are 100 amino acid residue β sandwiches found in a large number of proteins that are involved in signalling or membrane traffic (Rizo and Sudhof, 1998). C2 domains ordinarily bind calcium and phospholipids; however all C2 domains are not obligatory calcium and phospholipid binding modules. The calcium dependent phospholipid binding function of the C2 domain has been observed in the PLA₂, synaptotagmin and PKC. These domains are known to bind to phospholipids cooperatively in the presence of calcium, suggesting that they might have a membrane anchoring role (Rizo and Sudhof, 1998). Two crystal structures of C2 domains are reported. The C2 domain in PLC81 and the C2A domain of synaptotagmin I from an anti-parallel eight stranded β-sandwich but are topologically different by a circular permutation (Essen et al., 1996; Grobler et al, 1996; Essen et al., 1997b). Nevertheless, the calcium binding regions (CBRs) in both C2 domains are found to be at the same end of the β-sandwich.

Crystallographic studies on PLC81 with La³⁺ as a calcium analogue revealed that three loops in the β-sandwich are involved in metal binding and two principal metal binding sites were described (Essen et al., 1996). Residues implicated in calcium coordination include: Asn₆₄₅, Asp₆₅₃, Asp₇₀₆ and Asp₇₀₈. A lower resolution study of PLC81 in complex with samarium, another calcium analogue also suggested presence of two binding sites (Grobler et al, 1996). This study claimed that CBR1 and CBR3 undergo a conformational change upon metal binding and this creates a gap allowing for the binding of the phospholipid headgroup. Crystal structures of PLC81 with La³⁺, Ba²⁺ and Ca²⁺ at 3Å₀ resolution also suggested the presence of two binding sites (Essen et al., 1997b). ITC studies with a catalytically inactive mutant of PH domain deleted PLC81 showed that the C2 domain jaws bound three calcium ions (Grobler and Hurley, 1998). The C2 domain has a rigid interface with the catalytic domain, the putative calcium dependent phospholipid binding site being oriented on the same side as the active site and
the CBRs placed at a distance of 40 Å from the active site (Essen et al., 1996; Grobler et al., 1996). A comparison of the activity of wild type PLCδ1 with a mutant in the C2 domain jaws (PLCδ1-134, σJaws) showed that neither the basal enzyme activity nor its calcium dependency was altered (Grobler and Hurley, 1998). Thus calcium binding to C2 domain was not required for basal enzyme activity. Gel filtration and ITC studies with the PH domain deleted catalytically inactive PLCδ1 (PLCδ1-134, H311A) showed only a weak enhancement of binding of PLCδ1 to large unilamellar vesicles in the presence of calcium and ruled out a catalytically productive interaction between the C2 domain jaws and vesicles composed of PC and PIP2. Given its evolutionary conservation across species (in contrast to the PH domain), it is possible that calcium binding at the C2 domain may have an as-of-yet uncharacterised role in regulation of PLCδ activity. Alternatively, the C2 domain may be an evolutionary remnant whose calcium dependent phospholipid binding function in lower eukaryotes has been taken over by additional domains in mammalian PI-PLCs.

2.3.2.5 SH2/SH3 Domains

The SH2 domain plays a vital role in signal transduction processes by binding to sites of tyrosine phosphorylation following growth factor stimulation. These hundred amino acid modules are present in proteins which can be subdivided into two groups; those having enzymatic activity that is linked to downstream signalling events and adaptor molecules lacking associated enzymatic functions but are important for protein-protein interactions. The structure of a complex of the PLCγ1 SH2 domain bound to a phosphotyrosine containing peptide from the PDGF receptor is reported (Pascal et al., 1994). A positively charged pocket to one end of a groove of the surface of the domain accommodates the phosphotyrosine, while the other end of the groove that is lined with a set of hydrophobic residues interacts with hydrophobic positions of the receptor derived peptides. SH2 domains of the PLCγ isozyme have also been shown to interact with PIP3 (Rameh et al., 1998). This high affinity binding of SH2 domain of PLCγ (Kohda et al., 1993) was shown to compete with the binding to tyrosine phosphorylated receptors. Interaction with PIP3 was thought to help in membrane recruitment.
SH3 domains are 50 amino acid modules that consist of antiparallel β-strands forming a barrel-like structure (Kohda et al., 1993). Conserved aliphatic and aromatic residues form a hydrophobic pocket on the molecular surface. This pocket has been implicated in binding to target proteins which are often characterised by the presence of polyproline stretches. A probable role of the SH3 domain in regulating PLCγ activity has been discussed in another section.

2.4 Catalytic Mechanism

An interesting aspect of mammalian PI-PLCs is their ability to form 1, 2 cyclic inositol phosphates as by-products of phosphoinositide hydrolysis. The ratio of acyclic to cyclic product depends upon the isozyme class (γ > δ > β), substrate (PIP2 > PIP > PI), pH and calcium concentration (Essen et al., 1997a). In contrast to eukaryotic PI-PLCs which predominantly generate acyclic products, B. cereus and other bacterial PI-PLCs form cyclic inositol phosphate (cIP) with the hydrolysis of the cIP proceeding slowly (Heinz et al., 1995). The catalytic mechanism (including transition state, cyclic and acyclic reactions and reaction course) has been elucidated from studies on the crystal structure of PLCδ1 soaked with compounds that mimicked the reaction course by having varying phosphorylation states at the 1 and 2-hydroxyl positions of D-myoinositol (Essen et al., 1997a). The mechanism is evolutionarily conserved in mammals and B. cereus, requires two histidine residues and involves sequential phospho-transferase and phospho-hydrolase activities in which the cIP represents a reaction intermediate (Essen et al., 1996; Essen et al., 1997a). The phospho-transfer reaction includes the following steps:

(a) Deprotonation of the 2-OH group by a base whose assignment is ambiguous due to the presence of several potential candidates in the vicinity of the group but could be Glu390; calcium stabilizes the negative charge of the deprotonated 2-OH group.

(b) Nucleophilic attack by the deprotonated 2-OH group on the 1-phosphoryl group. This is preceded by a change in the C2-C1-O1-P1 torsion angle from −47° to 18°, leading to the O2-P1 distance reducing to less than 2.3 Å.
Fig. IV Reaction scheme for the general acid/general base catalysis by PLCδ1. The proposed reaction mechanism for PIP₂ hydrolysis involves deprotonation by a general base (B) for which His₃₁₁, Glu₃₄₁, and Glu₁₉₀ are candidates. The nucleophilic attack of the 2-hydroxyl group on the 1-phosphoryl group requires a movement of the phosphoryl group towards the 2-hydroxyl group by 1.4 Å. As a consequence, an oxygen atom is transiently coordinated to the catalytic calcium ion. The second step is the cleavage of the enzyme-bound cyclic intermediate by an activated water (adapted from Essen et al., 1997a).
(c) Formation of a highly negatively charged pentavalent transition state where the attacking 2-OH group and the leaving DAG group occupy apical positions in a trigonal bipyramid. The pentavalent transition state is stabilised by various hydrogen bonds between the ciP and His_{311}, Glu_{390} and Asn_{312}.

(d) Protonation of the leaving DAG group with His_{356} acting as a general acid catalyst. Phosphohydrolysis of the enzyme bound ciP passes through a transition state analogous to the phospho-transfer reaction and leads to the protonation of the deprotonated 2-OH group to give acyclic IP_{3}; activated water serves as the proton donor. The roles of the two histidine residues are now reversed with His_{356} acting as the general base and His_{311} acting as the acid catalyst. The reaction mechanism is illustrated in Fig. IV.

2.5 Membrane Association and Catalysis

Membrane structure affects lipid interactions with many proteins including PI-PLCs. The interaction of PLC with its substrate at the membrane surface is a two step process involving an initial binding to the membrane surface followed by association of the substrate with the active site of the enzyme. Thus, partitioning of a soluble enzyme onto the membrane surface leads to a “reduction in dimensionality” and therefore the parameters normally applied to enzyme kinetics do not work (Hurley and Grobler, 1997). Surface concentration terms such as mole ratio are used instead to characterise enzyme activity. Most enzyme assays are carried out in mixed micelles and it has been seen that as the mole ratio of the substrate in a mixed micelle is decreased, there is a decrease in the apparent V_{max} of the reaction. This phenomenon is called surface dilution kinetics and applies to PI-PLC among other lipid-dependent enzymes (Carman et al., 1995). This drop in V_{max} as a function of the decrease in the mole ratio of PIP_{2} has been interpreted as being indicative of a processive mode of catalysis. This dependence of enzyme activity on the mole ratio is true for PLC\_\beta_{1}, PLC\_\gamma_{1} and PLC\_\delta_{1} (Singer et al., 1997). In addition, activity has also been shown to be influenced by monolayer surface pressure and increase in the
monolayer surface pressure has been shown to bring about a decrease in the activity in PLCβ1, PLCγ1 and PLCδ1 (James et al., 1997).

PLCδ1 contains PH and C2 domains that are thought to be important for membrane binding. A "tether and fix" mechanism has been postulated for the binding of PLCδ1 to the membrane surface with the PH domain binding the enzyme to the membrane surface by specific interaction with PIP2 and the C2 domain fixing the catalytic domain in a productive orientation relative to the membrane (Essen et al., 1996). Membrane association in the β, γ and ε-isoforms may be brought about by additional sites. The PH and SH2 domains of PLCγ1 have been implicated in membrane association in interactions mediated by PIP3 while the carboxy-terminal in PLCβ is thought to contribute to association with particulate fractions (Wu et al., 1993; Kim et al., 1996; Rameh et al., 1998). For PLCε, Ras may indirectly contribute to membrane association (Song et al., 2001).

2.6 Regulation of PLC Isoforms

2.6.1 Regulation of PLCβ

Hetero-trimeric G-proteins are known to mediate PIP2 hydrolysis and this effect is specific for the PLCβ isozymes. This was established from studies on action of hormones on G-protein coupled receptors, the action of guanine nucleotides in cell free preparations and the inhibition in some cases of the hydrolysis of PIP2 by pertussis toxin. A special class of the α subunit of the hetero-trimeric G-proteins termed Gq are involved in the activation of PLCβ and form the pertussis toxin-insensitive component of PIP2 hydrolysis observed in certain cell types (Exton, 1997). The demonstration of a direct involvement of the α subunit of Gq in the activation of PLCβ involved reconstitution studies. Thus a 42 KDa activated G-protein α subunit was found to markedly stimulate PIP2 hydrolysis (Taylor et al., 1991). This effect was specific for PLCβ1 because PLCγ1 or PLCδ1 did not show a stimulation of activity. All α subunits of the members of the Gq family (αq, α11, α14-16) possess the ability to activate PLCβ isozymes. Reconstitution of m1 muscarinic receptor, G-
protein (Gq, G11, G14) and PLCβ in lipid vesicles lead to activation of PLCβ upon addition of agonist (carbamylcholine) in the presence of GTPγS or GTP (Nakamura et al., 1995). A comparison among the PLCβ isozymes for activation by Gq showed that the order of responsiveness was PLCβ1 > PLCβ3 > PLCβ4 > PLCβ2 (Exton, 1997).

The βγ subunits of G-proteins have also been shown to modulate PLCβ activity; the efficacy for regulation by βγ dimer differs from that seen for the α subunit and is as follows: PLCβ3 > PLCβ2 > PLCβ1. PLCβ4 is not affected by the βγ complex (Singer et al., 1997). One of the first reports regarding regulation of PLC activity by the βγ dimer involved crude PLC preparations from HL-60 cells (Camps et al., 1992). Activation by bovine retinal transducin (βγt) was found to be additive to activation by GTPγS. Stimulation of HL-60 PLC was not restricted to βγt but was also observed with highly purified βγ subunits from bovine brain. Reconstitution of purified PLCβ isozymes with βγ in lipid vesicles containing PIP2 showed a 2-8.5 fold increase in activity of PLCβ1, PLCβ2 and PLCβ3. These results have also been substantiated by transient expression studies. The ability to activate PLCβ isozymes requires prenylation of the γ subunit. There is a lack of selectivity in the ability of βγ subunits to activate PLCβ as multiple combinations of various β and γ subunits have shown to be equally effective. Given the multiplicity of PLCβ isozymes and the G-protein subunits available in cells, unique responses obtained in cells are a function of the nature of the receptor subtype including its density and distribution, difference in the abundance/type of G-proteins expressed in cells and differences in the tissue distribution of the PLCβ isozymes (Exton, 1997). Thus PLCβ1 is highly expressed in the cerebral cortex of mice while PLCβ4 expression is highest in the cerebellum with PLCβ3 levels being very low throughout the brain (Kim et al., 1997). Studies with PLCβ1−/− and PLCβ4−/− mice show that PLCβ1 is involved in signal transduction in the cerebral cortex by coupling to muscarinic acetylcholine receptors while PLCβ4 works through the metabotropic glutamate receptor.
The site of interaction of Goαq with the PLCβ isoyme has been localised to its carboxyl terminus (Wu et al., 1993; Kim et al., 1996). Deletion of the carboxyl amino acids 903-1142 of PLCβ1 was found to result in the loss of ability to be activated by Goαq though basal activity was not affected (Wu et al., 1993). The carboxyl region (amino acids 903-1030) was subdivided into two segments: one called the P box required for particulate association and for interaction with Goαq and the other region called the G box required only for interaction with Goαq. Calpain cleaved PLCβ1 which gives rise to a 100 KDa fragment and where the first 880 amino acids are left intact also gave identical results (Park et al., 1993). The 100 KDa fragment had basal PIP2 hydrolysing activity comparable to the intact PLCβ1 but lacked complete responsiveness to Goαq. This carboxy-terminal region of PLCβ1 that is removed by calpain action is enriched in basic residues and has been suggested to be important for interaction with Goαq. PLCβ2 also requires an intact carboxy-terminal for activation by Goαq (Zhang and Neer, 2001). The site in PLCβ that is thought to interact with the βγ subunits lies in the first half of the Y domain.

Studies with Goαq/Goαs chimeras and mutated forms of Goαq have defined two sites on Goαq that interact with the carboxyl terminal of PLCβ isoymes (Venkatakrishnan and Exton, 1996). These PLC activating residues in Goαq are located in the third helix and in the linker which connects the third helix to the adjacent β-sheet. This region lies next to a stretch which is known from crystal structure studies of Goαq to be involved in conformational changes upon activation.

**PLC and GTPase activity:** Receptor stimulation by an agonist leads to activation of trimeric G-proteins. The α subunit of G-proteins bind to GTP and deactivation occurs when bound GTP is hydrolysed to GDP. This hydrolytic activity is promoted by GTPase activating proteins (GAPs). PLCβ has the ability to activate intrinsic GTPase activity of Goαq 2000-fold (Berstein et al., 1992). In vitro studies involving monoclonal antibodies raised against PLCβ1 blocked the stimulating activity of Gq as well as the Goαq GAP activity of PLCβ1 proving that the source of GAP activity is PLCβ1 itself (Berstein et al., 1992). The rapid turnover of GTP was thought to
ensure retention of the receptor-G-protein complex which would not be possible if a new receptor had to bind to the G-protein by lateral diffusion prior to each exchange event. While in vitro studies such as the one described above among others have unequivocally demonstrated that the GAP activity of PLCβ isozymes, in vivo demonstration of GAP activity had not been available until recently. The norpA gene of Drosophila encodes a PLCβ that is predominantly expressed in the rhabdomeres of the compound eye (Smith et al., 1991). Photoreceptors in norpA mutants show essentially little no electrophysiological response to light stimuli, and therefore the flies are blind. In addition to the reduced amplitude and initial slope of the electrophysiological response, the light response in norpA mutants is very prolonged relative to wild-type responses. This suggests that the deactivation following the light stimulus is also not operational in these mutants. The severity of the phenotype (the initial response to light and the subsequent deactivation) correlates with the level of PLC expression in mutants with different norpA alleles (Montell, 2000; Cook et al., 2000).

To understand the prolonged deactivation seen in these mutants the light-dependent GTPγS binding activity (representing protein available for activation by light) and GTPase activity (reflecting the amount of activated G-protein that is turned off) was measured. In all mutants the GTPγS binding assay showed similar amounts of G-protein available for activation by light. The light-dependent GTPase activity was however graded and strongly correlated to PLC levels in these mutants. This meant that only one PLC molecule was stimulated by one G-protein molecule and was followed by signal termination due to the GAP activity of the former. Physiologically this was important for the high temporal and intensity resolution in response to light. Thus rapid inactivation of the Gαq-GTP complex by PLCβ GAP activity seems to have different effects depending on the cell type, either contributing to a rapid response or to specificity in signalling. Studies suggest that the GAP activity of PLCβ1 lies in its carboxyl terminal region and is included in the region previously shown to be important for responsiveness to Gq (Paulssen et al., 1996):
2.6.2 Regulation of PLCγ

PLCγ has been identified as one of several effectors of receptors that are protein tyrosine kinases (PDGF, EGF) or are linked to cellular tyrosine kinases (src, syk, lck, fyn). Receptor stimulation brings about auto-phosphorylation which contributes to association with PLCγ with the receptor (as evidenced by co-immunoprecipitation studies) and an increase in the tyrosine phosphate content of PLCγ (Kim et al., 1990). The SH2 domain of PLCγ has been implicated in binding to phosphorylated tyrosine residues on the receptor. Analysis of tryptic peptides of PLCγ1 obtained after in vitro phosphorylation identified four tyrosine residues that were phosphorylated (Tyr472, Tyr771, Tyr783, Tyr1254) while in vivo phosphorylation showed the presence of only two sites (Kim et al., 1990; Wahl et al., 1990). Three of these phosphorylation sites were in the linker region connecting the X and Y domain while the fourth site (Tyr1254) was found to be located at the carboxyl terminal end. The two most readily phosphorylated sites, Tyr771 and Tyr783 are located between the SH2 and SH3 domains.

The role of phosphorylation of PLCγ1 is highly debated. Tyrosine phosphorylation was not accompanied by increase in activity in vitro (Kim et al., 1990). In another study it was demonstrated that mutation of Tyr783 to Phe completely blocked PLC activation by PDGF as evidenced by a complete lack of inositol phosphate production (Kim et al., 1991). This in vivo data conflicted with the in vitro data from the same study which showed that wild type and mutant PLCγ1 in homogenates has comparable activities. It was suggested that phosphorylation relieved a negative inhibition present in vivo. In vivo candidates for negative inhibition include the cytoskeletal protein profilin (Goldschmidt-Clermont et al., 1990; Goldschmidt-Clermont et al., 1991). The activity of the unphosphorylated form of PLC was shown to be inhibited by the PIP2-binding protein profilin while this inhibition was not observed for the phosphorylated form of PLC. It was suggested that the phosphorylated form of PLC might possess a higher affinity for its substrate and could compete effectively with profilin to bind PIP2. Thus phosphorylation of PLCγ1 could either lead to its direct activation or relieve negative
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inhibition. Co-expression of the X and Y polypeptides of PLCγ1 in insect cells led to their physical association and this complex had a 17 fold higher activity at a PIP2 concentration of 400 μM as compared to the holozyme (Horstman et al., 1996). This again suggests a negative regulation which may be acting through the linker region. Phosphorylation has also been suggested to be important for targeting of PLCγ1 to appropriate sites within the cell which in turn could govern its regulation. In this context the SH3 domain is important. Immunofluorescence microscopy studies with micro-injected truncated protein consisting of the SH3 and SH2 domains of PLCγ1 showed co-localisation with the actin cytoskeleton (Bar-Sagi et al., 1993). Micro-injection with the SH3 domain alone showed a distribution pattern similar to that seen for the SH2 and SH3 domains together, however micro-injection with only the SH2 domain resulted in diffuse cytoplasmic distribution. This suggests that the SH3 domain may contribute to cytoskeletal association. In this context the placement of the phosphorylation sites close to the SH3 domain could potentially contribute to change in conformation leading to exposure of this domain and possible interaction with target proteins.

In addition to regulation by receptor and non-receptor tyrosine kinases, PLCγ also appears to be regulated by heptahelical receptors that couple to G-proteins such as the angiotensin II, receptor AT1 in rat aortic smooth muscle and the muscarinic acetylcholine receptor in Chinese hamster ovary cells (Rhee and Bae, 1997). Leukotriene is known to trigger Gβγ association with PLCγ1 and this interaction was shown to be mediated through the PH domain of PLCγ1 (Thodeti et al., 2000). Receptors coupled to PLD, PLA2 or PI-3-kinase are also reported to stimulate PLCγ1 activity indirectly in the absence of tyrosine phosphorylation via generation of second messenger(s) (Jones and Carpenter, 1993; Hwang et al., 1996; Falasca et al., 1998). Thus phosphatidic acid appears to equally activate unphosphorylated and phosphorylated forms of PLCγ1 by an allosteric mechanism. Arachidonic acid has been shown to bring about a 15-20 fold activation of PLCγ1 activity (effect was specific for gamma isozymes) in the presence of submicromolar concentrations of calcium. PI-3-kinase activity generates PIP3 which has been shown to bind to the PH domain of PLCγ1 bringing about its activation and translocation to membranes.
Phosphorylation of PLCγ1 at serine residues in Cu6Bu1 cells has been reported in response to cAMP elevating agents (Kim et al., 1989). No change in activity was seen in vitro and it was suggested that phosphorylation could modify its ability to interact with other proteins.

2.6.3 Regulation of PLCε

Previous studies have suggested that Ras may regulate PLC activity (Smith et al., 1990). Microinjection of Ras or PLC protein purified from bovine brain leads to the transformation of NIH3T3 cells and to DNA synthesis. PLCε has two domains that are potential points for interaction with Ras, an N-terminal CDC25-like guanine nucleotide exchange factor (GEF) domain and a C-terminal RA domain. Three independent studies, one in C. elegans and two in mammalian cells have demonstrated that the RA domain has the ability to bind to Ras (Shibatohge et al., 1998; Lopez et al., 2001; Song et al., 2001). H-Ras binding to the RA2 domain of rat PLCε in a GTP-dependent manner has been shown (Kelley et al., 2001). This binding was abolished by the mutation of a critical lysine residue in the RA domain that is conserved in other RA domains also. Further mutations in Ras which have been reported to affect its binding to effectors was also shown to inhibit binding to PLCε. The interaction between the RA domain of PLCε and Ras has also been confirmed by yeast two hybrid studies and by assays involving inhibition of the activation of adenylyl cyclase by Ras (Shibatohge et al., 1998; Song et al., 2001). Ras is an activator of adenylyl cyclase and addition of PLCε inhibited Ras activity in a dose dependent manner. The $K_D$ value for H-Ras binding was 40 nM, comparable to those of other Ras effectors (Song et al., 2001).

Association with Ras was not shown to increase PLCε activity in vitro (Shibatohge et al., 1998; Song et al., 2001). Constitutively active Ras, when co-expressed in COS-7 cells with PLCε showed a > 5.5 fold stimulation in PLCε basal activity suggesting that additional factors might be required for PLCε activation (Kelley et al., 2001; Song et al., 2001). Farnesylation of H-Ras is important for membrane localisation and a mutant of H-Ras at this site is unable to stimulate PLCε
activity (Kelley et al., 2001). Thus PLC is a possible effector for Ras. Localisation studies with PLCε fusions with GFP expressed in COS-7 cells shows that it is evenly distributed in to cytosol when expressed alone in serum starved cells (Song et al., 2001). Upon co-expression with activated Ras, there is a large amount of PLCε associated with the membrane surface.

The upstream regulator for Ras activation of PLCε has not been defined but two reports suggest possible regulation by EGF (Kelley et al., 2001; Song et al., 2001). EGF stimulation of COS-7 cells expressing PLCε shows its membrane localisation which is reversed by co-expression of a dominant negative mutant of H-Ras. Another report implicates Go12 as an activator of PLCε and suggests that the CDC25-like domain at N-terminus of PLCε functions as a GEF and stimulates Ras activity (Lopez et al., 2001). This in turn has been shown to activate the MAP kinase pathway. The PIP2 hydrolysing activity of PLCε is stimulated by Go12 but not by Goq. This apparent lack of stimulation of PLCε by Goq has also been confirmed by Kelley et al. (2001). The ability to activate Ras (as judged by increase in phosphorylated MAP kinase in co-expression studies) was independent of PLCε activity. This study suggests that Ras might be an effector of PLCε and not the other way round. In all studies so far the role of either the RA domain or the CDC25-like domain have been examined. A more detailed study where effects of deletions/mutations in both domains is examined will throw more light on this issue.

2.6.4 Regulation of PLCδ

In spite of extensive characterisation of PLCδ, not much is known about its mode of regulation in mammalian cells. G-protein mediated mechanisms are reported have been proposed for the regulation of PLCδ that involve both trimeric and monomeric forms. α1B adrenoreceptors are known to couple to the α subunit of a special class of GTP-binding proteins called transglutaminases II (Gαh) which have been shown to stimulate a membrane bound 69 KDa PLC. Studies involving in vitro reconstitution of the α1B adrenoreceptors, Gαh and the 69 KDa PLC have confirmed these results.
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(Baeck et al., 1993; Das et al., 1993). Subsequent characterisations revealed that the 69 KDa PLC was actually a proteolytic fragment derived from PLCδ1 (Feng et al., 1996). The site for interaction on the Gαh has been mapped by deletion, competitive inhibition use of synthetic peptides with sequence stretches identical with residues of Gαh and chimeras in which residues of human heart Gαh were substituted with residues from human coagulation Factor XII. These studies have demonstrated that an interaction site lies at the carboxyl terminal and spans the residues Val66-Lys6n of human heart Gαh (Hwang et al., 1995; Feng et al., 1996).

A novel 122 Kda protein with limited homology to RhoGAP (p122) has been isolated in a screen using anti-PLCδ1 antibodies and was found to activate PLCδ1 but not PLCβ1 or PLCγ1 (Homma and Emori, 1995). PLCδ1 activity was potentiated by alterations in the Km and Vmax values without affecting its calcium dependency. The region implicated in GAP activity was also suggested to be involved in modulating PLCδ1 activity. This is physiologically relevant because Rho proteins and PIP2 have been shown to influence processes such as membrane ruffling which are under the control of cytoskeletal elements (Singer et al., 1997). That it is indeed the GAP domain that is responsible for this effect is demonstrated by the deletion and mutation studies (Sekimata et al., 1999). Over-expression of p122 was not stable and microinjection was shown to inhibit formation of stress fibres and focal adhesions. Fluo3 based calcium microscopy has shown that over-expression of p122 resulted in a rapid elevation of intracellular calcium levels suggesting indirectly that p122 stimulates PIP2 - hydrolysing activity of PLCδ1.

Finally, calcium in itself may be a modulator of PLCδ1 activity by virtue of its ability to enhance activity by interacting with the site on the enzyme other than the catalytic site. This is consistent with a comparative study of the PLC isoforms, where it was observed that the δ-isoforms have a 5 fold higher requirement for calcium concentration than β or γ isoforms for optimal PIP2 hydrolysis (Takenawa et al., 1991). The activation of δ-isoforms by calcium has been examined in permeabilized cell systems (Allen et al., 1997). HL-60 cells, upon extended permeabilization, are depleted of endogenous proteins including PLCs. Extracts from COS cells expressing
PLCβ1, PLCγ1 or PLCδ1 (normalized for PLC activity) were added to permeabilized HL-60 cells and inositol phosphate production was measured at different calcium concentrations within the physiological range. In such a system it was seen that increase in calcium concentration on its own was insufficient to activate PLCβ1 or PLCγ1. In contrast, the activity of PLCδ1 was approximately 10-fold higher when the calcium concentration was increased to 1-10 μM. The activation of PLC hydrolysis by the δ-isoforms has been suggested to be mediated by the C2 domain. Despite extensive evidence that the C2 domain has calcium binding activity, there is no data available to suggest that this domain is involved in calcium dependent activation of PLCδ. In fact, the $K_{act}$ due to calcium is identical for PLCδ1 and PLCδ1-134, ε jaws, strongly suggesting that their calcium activation mechanisms are identical and that the calcium binding to the C2 domain jaws does not contribute to activation of PLCδ1 (Grobler et al., 1998). PLCδ might also be secondarily activated to receptor mediated activation of other PLC isozymes or calcium channels but this remains to be proved.

2.6.5 Additional Modes of Regulation

Several lines of evidence suggest that the events leading to the synthesis or degradation of PIP2 are also important in modulating PLC activity. Thus, permeabilized HL60 cells in which the phosphatidylinositol transfer protein (PITP) was depleted were found to show a rapid decline in inositol phosphate production due to PLCβ activity (Cunningham et al., 1995). This activity was restored upon addition of exogenous PITP. PITP is required for the transfer of PI from the endoplasmic reticulum to the membrane surface. Depletion of PIP2 due to PLCβ activity is thought to be replenished by the sequential action of PIP2 synthesizing enzymes, the process being mediated by PITP. A similar requirement for PITP in the replenishment of the substrate pool and hence activity has been shown for rat PLCδ1 (Allen et al., 1997). Stimulation of PLCδ1 activity by PITP was shown to require an intact PH domain

CDP-Diacylglycerol Synthase (CDS) is an enzyme that is required for the generation of PIP2 from phosphatidic acid (Wu et al., 1995). An eye-specific
isoforms of CDS is a key regulator of *Drosophila* phototransduction. Cds mutants of *Drosophila* show severe defects in signalling in the eye which can be pharmacologically rescued by addition of PIP$_2$. Thus PIP$_2$ availability is a limiting factor in these mutants. Given that norpA, which encodes a PLC$_{\beta}$ in *Drosophila* functions upstream of the cds gene in the signalling pathway and that addition of PIP$_2$ can rescue this effect, is suggestive of the fact that PIP$_2$ regeneration may also be an important event in controlling PLC signalling.

### 2.7 Nuclear PI-PLC

The envelope of the eukaryotic nucleus is a double membrane consisting of phospholipid and protein. Several lines of evidence that a phosphoinositide signalling pathway analogous to that scene in the cytoplasm is also present in the nucleus. The first components of this pathway that were identified in the nucleus were PIP$_2$ and its precursors (Irvine, 2000). Treatment of intact Swiss 3T3 cells with Insulin Growth Factor-I (IGF-I) induces activation of PI-PLC and is accompanied by a decrease in the levels of PIP and PIP$_2$ in the nucleus and a parallel increase in the nuclear DAG levels. These nuclear changes were detectable only when highly purified nuclei were used. Nuclear fractionation studies have shown that PI-4-5-kinase, DAG kinase and PI-PLC activities are associated with the internal matrix while PI kinase was found to be exclusively localised in the peripheral matrix (Payrastre et al., 1992). This suggests that the former enzymes are mostly intranuclear and are not normally associated with the nuclear membrane. Most of the literature pertaining to nuclear localisation of PI-PLC involves 3 isozymes: PLC$_{\beta}1$, PLC$_{\delta}1$ and PLC$_{\delta}4$. PLC$_{\beta}1$ exists in two alternatively spliced forms - PLC$_{\beta}1$a and PLC$_{\beta}1$b (Bahk et al., 1994). The two forms differ from each another in their molecular weights and in the presence of a 1738 nucleotide extension at the 3' end of PLC$_{\beta}1$b. Expression of the PLC$_{\beta}1$a form in CV-1, Rat-2, Swiss 3T3 cells as analysed by immunoblotting showed that the enzyme in Rat 2 and Swiss 3T3 cells is localised in the nuclear fraction and the cytosolic fraction with an approximately equal distribution (Kim et al., 1996). Mutations in Lys$_{1055}$-Lys$_{1066}$ that are present in the carboxyl terminal of PLC$_{\beta}1$a reduced the nuclear localisation to 17%. These basic residues were suggested to be involved in
binding to nuclear components and their mutation was therefore suggested to abrogate nuclear localisation. In this study the distribution of PLCβ1b was not examined. In contrast, in another study where localisation of both PLCβ1a and PLCβ1b was examined in wild type erythroleukemic Friend cells PLCβ1a was localised nearly equally in both cytosolic and nuclear compartments while PLCβ1b was almost exclusively present in the nucleus. Overexpression of these isoforms in the Friend cells confirmed these results. Mutants for the nuclear localization signal (M2b) present at the carboxyl terminal end (Lys1056, Lys1063 and Lys1070) abolished localisation to the nucleus (Faenza et al., 2000). Overexpression of both forms of PLCβ1 in turn lead to overexpression of endogenous cyclin D3 and associated cdk4 in the absence of serum stimulation and hence progression into G1 phase. This progression into the G1 phase was not seen upon overexpression of the M2b forms of PLCβ1. This suggests a direct role of PLCβ1 signalling in progression through G1.

PLCδ4 expression has also been correlated to cell proliferation and was first isolated from a cDNA library prepared from regenerating rat liver. Its expression is high not only in regenerating liver but also in intestine, hepatoma and src-transformed 3Y1 cells (Liu et al., 1996). Immunofluorescence microscopy using anti-PLCδ4 antibodies showed that eight hours after serum stimulation, the nuclear membrane was stained, the density of staining increasing till 16 hours. This period coincides with the S-phase. This strong staining continued to the end of the M-phase, disappeared at 28 hours and reappeared at the beginning of the next S-Phase. Nuclear staining studies were confirmed by immunoblotting of the nuclear fraction at various time points after serum stimulation. It was suggested that the increased PI-PLC levels in the nucleus at the beginning of the S-phase was related to the requirement for increased DAG levels within the nucleus. This DAG was proposed to activate PKC which in turn lead to phosphorylation of β-lamin, leading to nuclear envelope breakdown. No parallel study was done to show that the increase in PI-PLC levels in the nucleus was accompanied by an increase in nuclear PIP2 hydrolysing activity.

As mentioned earlier, PLCδ1 has been shown to possess a functional nuclear export signal (NES) sequence in amino acid residues 164-177 of the EF hand domain (Yamaga et al., 1999). This sequence has also been shown to be present in PLCδ3 by
sequence alignment predictions. The NES is a short leucine-rich sequence motif that has been implicated in mediating active nuclear export. The fluorescence of NES-disrupted GFP-PLC81 expressed in MDCK cells was present not only at the plasma membrane and cytosol but also in the nucleus. This was mimicked by treatment of MDCK cells expressing GFP-PLC81 by leptomycin B which is an inhibitor of NES dependent nuclear export. In this context it must be mentioned that PI-PLC was identified in a screen as a factor required for nuclear export of messenger RNA in yeast and provides indirect evidence for the role of PLC in the nucleus (York et al., 1999). The PLC1 gene in *S. cerevisiae* is required for the production of IP3 which is sequentially phosphorylated to give IP6. IP6 appears to have an as-of-yet undefined role in the nuclear export. PLC deleted mutants show no detectable amounts of IP6 in the cell while PLC1 overexpressing cells show 25 times more IP6 than wild type cells. The final step in the production of IP6 is catalysed by IP5 2 kinase that is encoded by GSL1. Localisation studies with the GSL1 gene product have shown its punctate distribution at the nuclear periphery and is typical for yeast nucleoprotein complexes and nuclear export proteins. If the sites of production of IP3 and IP6 are not spatially distinct and IP3 production occurs in the nucleus, PLC1 in yeast may also show nuclear localisation. This remains to be demonstrated. More recently Lin et al. (2000) have suggested that PLC1 in yeast has a more direct role in controlling the cell division process by interacting with the kinetochore complex PLC1 in yeast was identified in a genetic screen for mutants with increased frequencies of chromosome misaggregation (Lin et al., 2000). The *plc1-l* mutant displayed a 32-fold increase in chromosome misaggregation events and was suggested to a role in controlling cell cycle events.
2.8 Plant PI-PLCs

2.8.1 Biochemical Properties of Plant PI-PLCs

The identification of a calcium stimulated PLC in highly purified plasma membrane preparations of root and shoot tissues of wheat seedlings was one of the first reports of a biochemical characterisation of PI-PLCs in plants (Melin et al., 1987). The study recognised the presence of two distinct categories of PI-PLCs in plants – soluble PI-PLCs that preferred PI as substrate and membrane bound forms that preferred PIP_2 and PIP as substrates. Both forms required calcium for activity; very little activity was detected in the absence of calcium and a calcium concentration of 0.3 μM was found to elevate the activity to 70% of that seen at a calcium concentration of 10 μM. That the reaction was due to PLC activity was confirmed by chromatography of the water soluble hydrolytic product on Dowex AG-1-X8 resin. The non-hydrolysable analog of GTP, GTP-γ-S did not affect activity. In addition, PLC activity in the membrane fraction was stimulated by 1-2 mM Mg^{2+} only in the presence of Ca^{2+} (Melin et al., 1992). This stimulation was not observed for the soluble forms of PI-PLC. PLC activity was also stimulated 3-5 fold by 0.025% - 0.02% deoxycholate (DOC) (Melin et al., 1992; Pical et al., 1992). Activity was also a function of the sidedness of the membrane with right side out vesicles showing 3-5 fold stimulation in the presence of DOC as compared to inside out vesicles in the presence of the same detergent (Pical et al., 1992). Labelling studies in the green alga Dunaliella salina confirmed the presence of a PIP_2-hydrolysing activity in the plasma membrane that was sensitive to calcium (maximal activity at 10 μM concentration) and GTP-γ-S (Einspahr et al., 1989). Another study with suspension cultured rice cells suggested that a PI-dependent PLC activity in the plasma membrane corresponded with a 42 KDa protein (Yotsushima et al., 1993). The PIP_2-hydrolysing activity of this protein was lost upon chromatographic purification, leading to the suggestion that an additional, heat labile factor (possibly a protein) was required for the PIP_2-hydrolysing ability of the 42 KDa protein. Addition of specific chromatographic fractions to the 42 KDa protein resulted in reconstitution of PLC activity.
Detailed kinetic analyses of plant PLCs as observed for mammalian isoforms was not available until recently. Kinetic analysis, vesicle binding studies and monolayer assays using a semi-purified PLC from *Catharanthus roseus* have shown that while plant PLCs are in many ways similar to their mammalian counterparts, there are also significant differences (Hernandez-Sotomayor et al., 1999). Thus an initial increase in monolayer surface pressure was accompanied by an increase in enzyme activity and above an optimum pressure (20N/m), PLC activity was reduced, suggesting that the initial surface pressure was a crucial determinant of PLC penetration into monolayers. Data from vesicle binding assays implies association of plant PLCs with membrane interfaces through substrate as sucrose loaded PS-PC vesicles lacking did not show measurable vesicle binding activity. Finally, kinetic data suggested that the curve for binding to the interface as well as binding to the substrate was linear with a Hill coefficient of one, indicating a single binding site. In mammalian cells the initial binding to the interface for PLC81 was via the PH domain and was dependent on PIP2. Given that this domain is absent in plant PI-PLCs, the authors suggest that the enzyme might first bind non-catalytically to PIP2 at the same site where catalysis occurs.

*Arabidopsis* PI-PLC expressed in *E. coli* has an absolute dependence on calcium for activity. At lower calcium concentrations (1 μM) PIP and PIP2 are the preferred substrates while at higher concentrations (> 0.1 mM) PI was the preferred substrate (Hirayama et al., 1995). Thus, *Arabidopsis* PLC (AtPLC1) exhibits properties of both membrane bound and soluble PI-PLCs in terms of its substrate preference. This dependence of substrate preference on calcium concentration was also observed for soybean PLC, *Nicotiana rustica* PLC and three isoforms of PI-PLC reported form potato (Shi et al., 1995a; Kopka et al., 1998b; Staxen et al., 1999). Interestingly, within the micromolar concentration range of calcium examined, all three isoforms of potato PI-PLC exhibited different activities towards the substrate for the same calcium concentration (Kopka et al., 1998b). This could be due to subtle differences in the properties of the enzymes. Competition experiments with increasing amounts of Mg2+ and Al3+ in the presence of calcium was also checked for the potato PI-PLC isoforms. Al3+ was found to inhibit the activity of potato PI-PLCs.
while Mg$^{2+}$ did not stimulate PLC hydrolytic activity. This contrasts with the observation that PLC activity from roots and shoots of wheat seedlings was stimulated by Mg$^{2+}$ (Melin et al., 1992). Finally, recombinant purified PI-PLC from *N. rustica* was found to be inhibited by U73122, an inhibitor of PLC dependent processes in animal cells (IC$_{50}$=23 μM) but activity was not affected by its inactive analogue U73343 (Staxen et al., 1999).

2.8.2 Cloning of Plant PI-PLCs

The first PI-PLC in plants was cloned from *Arabidopsis thaliana* (Hirayama et al., 1995) and was called AtPLC1. The 2.0 Kb clone encoding a protein of molecular weight 64.3 KDa was isolated by screening of a cDNA library prepared from dehydration stressed plants. The overall structure showed close homology to δ-isoforms of mammalian PI-PLCs and had conserved X, Y and C2 domains. A single EF hand motif was located at the N-terminus of the protein. The PIP$_2$-hydrolysing activity of this protein has been discussed in the previous section. Simultaneously, another PI-PLC was cloned from *Arabidopsis* shoots with developing flower primordial and was called AtPLC1F to distinguish it from the dehydration stress inducible AtPLC1 (now named AtPLC1S) (Yamamoto et al., 1995). Subsequently, a cDNA clone for PI-PLC was isolated from soybean (PLC1) by screening an expression library using anti-(plasma membrane) serum (Shi et al., 1995a). The open reading frame encoded a PI-PLC which was similar in its domain organisation to the *Arabidopsis* clone with the only difference being in the location of the EF hand motif (Shi et al., 1995b). The soybean PI-PLC was localised to the cytosol and plasma membrane. Expression in yeast complemented the lethality phenotype of haploid PI-PLC disruptants. The presence of PI-PLCs in plants is now firmly established with reports of its cloning from Arabidopsis, soybean, potato and *N. rustica* (Pical et al., 1997; Kopka et al., 1998b) All plant PI-PLCs cloned so far show closest homology to δ-isoforms of mammalian PI-PLCs (Essen et al., 1996).

The PI-PLCs are multigene families in *Arabidopsis*, soybean and potato (Hartweck et al., 1997; Hirayama et al., 1997; Kopka et al., 1998b). Southern hybridisation of digested soybean genomic DNA with soybean PLC1 showed a
pattern of strong bands, suggesting that soybean contained additional PI-PLC sequences that shared high homology with soybean PLC1. This was confirmed with the subsequent Genbank deposition reports of eight PI-PLCs from soybean. Three PI-PLCs have been reported from potato (Kopka et al., 1998b). An analysis of the sequence of the *Arabidopsis* genome (The Arabidopsis Information Resource; TAIR) reveals the presence of eight putative PI-PLCs. Chromosome localization studies have shown AtPLC1S to be located on chromosome 5 and AtPLC2 to be located on chromosome 3 (Hirayama et al., 1997). This has been confirmed subsequently by sequencing of the *Arabidopsis* genome. All sequences in Arabidopsis are δ-isoforms and are distributed over chromosomes 3, 4 and 5. Thus four sequences are present on chromosome 3, one on chromosome 4 and three on chromosome 5 (At3g08510; At3g55940; At3g47220; At3g47290; At4g38530; At5g58670; At5g58700; At5g58690).

### 2.8.3 Involvement of PLC in Stimulus-Response Coupling

Several reports indicate that signals such as hormone, light, elicitors and stress are capable of generating inositol phosphates and that PLC may play a role in transmembrane signalling in plants.

#### 2.8.3.1 Osmoregulation

Increased calcium levels have been implicated in maintaining volume homeostasis associated with osmotic stress and osmo-regulation in plants. One of the first studies in this regard was with the green alga *D. salina*. Hypotonic stress in *D. salina* has been shown to bring about an increase in IP$_3$ levels and this was paralleled by a decrease in PIP and PIP$_2$ levels (Einspahr et al., 1989). The PLC activity in the plasma membranes of this alga was activated by GTP-γ-S over a range of calcium concentrations tested, suggesting the possible involvement of G-proteins in PLC activation. The involvement of phosphoinositides in stress responses in *Arabidopsis* has been confirmed by the cloning of salt stress and dehydration inducible PI-PLC (AtPLC1S) and PI-4-P-5-kinase (Chapman, 1998). Treatment with water was found to slightly induce the AtPLC1 gene expression, suggesting that low osmotic stress
might function as a trigger (Hirayama et al., 1995). Dehydration was found to rapidly induce AtPLC1 mRNA which reached maximum levels within two hours and then declined gradually thereafter. In contrast, accumulation of AtPLC1S mRNA began two hours after salt treatment and reached a maximum at 5-10 hours. Under high salt stress the AtPLC1S expression was ten times that seen in normal tissues Arabidopsis plants grown in liquid media have been shown to rapidly increase PIP2 synthesis in response to treatment with sodium chloride, potassium chloride and sorbitol (DeWald et al., 2001). The data obtained also suggested that the majority of PIP2 synthesized in response to salt and osmotic stress may be utilised for cellular signalling events distinct from the IP3 signalling pathway. Thus there may exist signalling pathways that utilise phosphoinositides in addition to those that lead to IP3 synthesis and degradation in plants. In addition, whole plant IP3 levels were found to increase significantly within a minute of stress application and this was seen to increase further for the next 30 minutes. Studies with calcium indicators have shown that root intracellular concentrations of calcium increased in response to salt and osmotic stress. The accumulation of IP3 occurred in a time frame similar to that observed for stress-induced calcium mobilisation. Induction of potato PI-PLCs has been reported in response to drought stress (Kopka et al., 1998b). Thus StPLC1 and StPLC2 were induced upon both short and long term drought stress while StPLC3 was induced only under long term drought stress.

Processes such as stomatal closure and leaf movement are the direct consequences of changes in osmolarity in specialised cells; guard cells regulate the former while extensor and flexor cells in the pulvini of leaves regulate the latter (Cote et al., 1996). Stomatal closure is brought about by a reduction in turgor pressure within the guard cells. Important clues to the involvement of the phosphoinositide pathway in stomatal closure have come from studies using "caged" calcium and IP3. Introduction of "caged" calcium in Commelina communis guard cells was shown to lead to stomatal closure upon photo-activation (Gilroy et al., 1990). Introduction of photolabile, "caged" IP3 into these cells and its subsequent photo-activation was followed by increase in cytosolic calcium and then stomatal closure. Another study, again based on "caged" IP3 in guard cells of Vicia faba, demonstrated that photo-
activation was followed by inhibition of an inward rectifying K⁺ current (Blatt et al., 1990). The involvement of ABA in activating the IP₃-mediated stomatal closure was also shown in *Vicia faba* guard cells. Metabolic labelling studies showed that a 10s treatment with ABA was followed by a 90% increase in levels of IP₃ (Lee et al., 1996). Concomitantly, ³²P-labelled PIP₂ and PIP levels were found to be decreased by 20% compared to control cells. The involvement of PI-PLC in guard cell signalling has also been indirectly demonstrated by the use of the PI-PLC inhibitor U73122 and its inactive analogue U73343 in *N. rustica* (Staxen et al., 1999). U73122 was found to inhibit ABA-induced increase in cytosolic calcium and stomatal closure while the inactive analogue did not affect this process. Taken together, this suggests a model for ABA-induced stomatal closure wherein, by an unknown mechanism, ABA stimulates a PI-PLC which in turn leads to an increase in IP₃ levels. This in turn leads to elevation in cytosolic calcium levels which causes inhibition of K⁺ channels, a loss in turgor pressure and stomatal closure. The mechanism whereby an increase in calcium leads to closure of potassium channels also needs to be elucidated.

### 2.8.3.2 Hormones

The auxin, 2,4 D has been shown to generate rapid, transient changes in IP₃ and IP₂ within minutes in *Catharanthus roseus* cells arrested in G₁ phase (Ettlinger and Lehle, 1988). The time course of changes in polyphosphoinositides was characterised by a decrease in radioactivity due to PIP and PIP₂ followed by a recovery and an apparent doubling. The inactive analog 3,5 D did not trigger the hydrolysis of phosphoinositides. This suggests the existence of a PLC activity in *C. roseus* cells in the G₁ phase that is activated in response to auxin.

A possible involvement of phosphoinositide-dependent calcium signalling in the regulation of α-amylase expression and in the germination of rice seeds has been suggested (Kashem et al., 2000). This was based on studies with neomycin, an aminoglycoside that is known to bind to PIP₂ and inhibit PLC activity. Neomycin was shown to markedly reduce PIP₂-hydrolysing activity in rice membrane fractions. Neomycin also inhibited germination, growth of seedlings and gibberelin (GA₃)-induced expression of α-amylase in the scutellar and aleurone layers and this was
reversed by supplementation with exogenous calcium. IP3 levels in both tissues were found to increase in response to GA3 and this was reversed by neomycin. The expression of a 55 KDa protein that was recognised by polyclonal antibodies raised against a peptide sequence found to be conserved in plant PLCs was also stimulated by GA3 treatment.

ABA has been implicated in the induction of many genes under dehydration and salt stress conditions. ABA treatment was found to induce accumulation of AtPLC1 mRNA suggesting possible involvement of phosphoinositides in ABA-mediated stress responses in Arabidopsis (Hirayama et al., 1995). Transgenic lines expressing AtPLC1S (antisense) under the control of an inducible promoter showed reduced sensitivity in growth and germination in the presence of ABA as well as reduced IP3 levels (Sanchez and Chua, 2001) in the presence of inducer. The induction of ABA responsive genes such as RD22, RD29 and KIN2 in these lines was also decreased in the presence of the inducer. This suggests that AtPLC1S functions downstream of the ABA signalling pathway in Arabidopsis. In contrast, AtPLC1S sense lines did not show a decrease in IP3 levels or an increase in the activation of the ABA-responsive genes though PLC expression was detectable in vitro in the presence of the inducer. It was therefore suggested that PLC expression was by itself not sufficient to activate ABA induced gene expression in Arabidopsis. The promoter for the AtPLC1S gene has been cloned and shows the presence of a single ABA responsive element at the −245 position and is similar to the CCACGTGG G-box footprint involved in ABA responses (Hartweck et al., 1997). This correlates with the ABA responsiveness of AtPLC1S (Hirayama et al., 1995). The osmo-regulatory signal transduction cascade controlled by ABA has been discussed in the previous section.

2.8.3.3 Plant-Microbe Interactions
The hypersensitive response of plants to pathogen invasion includes localised necrosis, formation of H2O2 leading to the production of an oxidative burst and synthesis of specialised anti-microbial compounds called phytoalexins (Cote et al., 1996). The hypersensitive response is triggered by elicitors, substances derived from
the breakdown of the cell wall of an invading pathogen or the cell walls of the host tissue.

Both short and long term responses that involve PI-PLC in plant-microbe interactions are known. Fungal elicitors were found to stimulate the turnover of PIP and PIP₂ in plasma membranes isolated from pea epicotyls while fungal suppressors of these elicitors reversed this response (Toyoda et al., 1992; Toyoda et al., 1993). Application of an elicitor preparation from the fungus Mycosphaerella pinodes resulted in a neomycin-sensitive accumulation of IP₃ in pea epicotyls within ten minutes and a neomycin-sensitive accumulation of pisatin, a phytoalexin. Neomycin rendered pea leaves sensitive to infection by a fungal strain against which resistance was normally observed. This suggested the activation of a PI-PLC in response to fungal infection in pea. Phospholipase C activation was also observed during elicitation of oxidative burst in suspension cultures of soybean cells (Legendre et al., 1993). Both polygalactouronide elicitor and the G-protein activator, mastoparan were found to promote a transient increase in IP₃ content that exceeded the basal level by 2-7 fold. This increase in IP₃ level was seen within one minute post-stimulation and was blocked by neomycin sulfate. Increase in IP₃ preceded H₂O₂ production. The inactive analogue of mastoparan, mas-17 had no effect on IP₃ levels. Thin layer chromatography of lipid extracts revealed a rapid decrease in PIP and PIP₂ levels following elicitor and mastoparan treatment. The ability of mastoparan to replace the effect of the elicitor was interpreted as being indicative of IP₃ production being under the control of G-proteins in soybean. An opposite response was seen in soybean cells challenged with the bacterial pathogen, Pseudomonas syringae pv glycinea where IP₃ content was found to decrease in both compatible and incompatible interactions and was suggested to be due to reduced PLC activity (Shigaki and Bhattacharyya, 2000). Reduced PLC activity was attributed to suppression of housekeeping activities of cells and a diversion of cellular resources to the production of defense-related compounds and/or growth of pathogens. This illustrates that within the same system distinct pathways exist which might utilise similar signal transduction components to generate opposite responses.
Rhizobium bacteria are able to invade the roots of leguminous hosts and trigger the formation of root nodules, the first visible effect of which is root deformation. Purified rhizobium-secreted nodulation (Nod) factors that are lipochito-oligosaccharides can mimic this effect. Nod factor-induced transcription of ENOD12 expression in *Medicago* could be replaced by mastoparan and was found to be blocked by neomycin and U73122, suggesting the involvement of PI-PLC in this response (Pingret et al., 1998). Treatment of *Vicia sativa* with Nod factors also leads to root hair deformation (den Hartog et al., 2001). The requirement for Nod factors could be replaced by mastoparan and was inhibited by neomycin sulfate in a dose dependent manner. Neomycin treatment was also shown to inhibit Nod factor induced increase in PA in metabolically labelled roots of *Vicia* while mastoparan had an opposite effect, suggesting the involvement of PI-PLC and G-proteins in this response. PIP\(_2\)-dependent PLD activity has been reported from plants and neomycin is known to bind directly to PIP\(_2\) but the decrease in PA was not due to indirect effects of neomycin on PLD signalling pathways (den Hartog et al., 2001). Neomycin-inhibited root hair deformation occurred within a few minutes of treatment.

Long term activation of PI-PLC expression has been demonstrated in both susceptible and resistant interactions between *Xanthomonas oryzae* pv *oryzae* and rice (Young et al., 1996). PLC mRNA levels were found to increase in rice leaves within a day after inoculation. PLC transcript accumulation peaked at two days for resistant interactions, declining thereafter while in susceptible interactions the peak levels reached in resistant interactions was never attained. Thus, as with osmotic stress, plant-microbe interactions may also involve both long and short term effects of PI-PLC.

**2.8.3.4 Light**

Brief irradiation of *Samanea saman* pulvini with light was shown to decrease PIP and PIP\(_2\) levels and a concomitant increase in IP\(_2\) and IP\(_3\) levels by \(^3\)H-inositol labelling of inositol phosphates was observed (Sopory and Chandok, 1996). Differences in PI-PLC activity were also observed in wheat plasma membranes obtained from light and
dark grown shoots (Melin et al., 1987). PLC activity was higher in light grown shoots as compared to dark grown ones while no difference was seen between plasma membranes from light and dark grown roots. The involvement of inositol phospholipid turnover in light-dependent gravity response in roots of maize has also been shown (Reddy et al., 1987). Light treatment of dark-grown roots of maize were shown to stimulate a gravitropic response and an increase in levels IP, IP₂ and IP₃. A more detailed review of the role of light-mediated turnover of phosphoinositides in plants has been presented in Sopory and Chandok (1996). A recent study has implicated a PI-PLC-based signalling system in the control of phosphorylation state of C₄ phosphoenolpyruvate carboxylase (PEPC) in Digitaria sanguinalis (Coursol et al., 2000). D. sanguinalis mesophyll protoplasts were shown to have PIP₂-dependent PLC activity. IP₃ levels were shown to increase 2-fold and peak approximately 3-5 minutes after the beginning of in situ induction of C₄ (PEPC) phosphorylation by illumination. In situ, U73122, but not U73343, abolished increases in IP₃ levels, C₄ PEPC kinase activity and C₄ PEPC phosphorylation.

2.8.3.5 Other Responses

A signalling role for cytosolic calcium has been demonstrated in regulating Papaver rhoeas pollen tube growth during the self-incompatibility response. A calcium-dependent PI-PLC activity was demonstrated in P. rhoeas pollen microsomes (Franklin-Tong et al., 1996). Mastoparan at a concentration of 25 μM inhibited pollen tube growth and induced a biphasic increase in IP₃ levels. This consisted of an initial rapid increase in IP3 levels within seconds, a drop, followed by a more sustained increase lasting for a few minutes. Treatment with mastoparan also resulted in increase in cytosolic calcium. Neomycin was found to block the calcium release induced by mastoparan. This data suggests the involvement of a functional phosphoinositide system in the regulation of pollen tube growth.

Deflagellation in Chlamydomonas seen in response to osmotic or temperature shock, alkalinisation or treatment with mastoparan or calcium is caused by a contraction of centrin at the transition zone that ruptures the microtubular cytoskeleton (Kuin et al., 2000). A rise in intracellular concentration of calcium is
believed to mediate this response. Treatment of cells with mastoparan was found to bring about $^{45}\text{Ca}^{2+}$ release from pre-loaded cells. Mastoparan was also found to activate PLC as demonstrated by the breakdown of $^{32}\text{P}$-labelled PIP$_2$ and the production of diacylglycerol while its inactive analog did not. The production of IP$_3$ was however not shown in this study.

Soybean cell suspensions show a rapid elevation in IP$_3$ levels (as evidenced by HPLC and radio-receptor assays) upon replenishment of growing medium with Murashige-Skoog (MS) salts, indicating an involvement of PI-PLC (Shigaki and Bhattacharyya, 1999). This renewed growth of cells as determined by DNA synthesis was reduced in the presence of U73122; its inactive analog U73343 did not affect growth. The inhibitory effect of U73122 treatment was abolished upon treatment with MS salts. This suggests the involvement of PI-PLC on DNA synthesis and overall growth in soybean. The probable involvement of PI-PLC from *Arabidopsis* in cell division and/or cell wall synthesis was also suggested (Mikami et al., 1997). A PLC-interacting protein (PLC1F) has been isolated from *Arabidopsis* in a two hybrid screen using AtPLC1 as a bait. The PLC1F protein did not show any homology with any known protein in the database and caused aggregation of yeast cells expressing both AtPLC1 and PLC1F, suggesting abnormality in cell division and/or cell wall synthesis. The co-expression of AtPLC1 and PLC1F in could restore growth of glucan-defective yeast on plates containing 0.004% SDS. Interestingly, disruption of the yeast PI-PLC leads to a pleiotropic phenotype characterised by reduced growth at higher temperatures, multi-budded appearance, defects in nutritional pathways, cytokinesis and kinetochore function (Flick and Thorner, 1993; Yoko-O et al., 1993; Flick and Thorner, 1998; Lin et al., 2000). A similar role for PI-PLC in plants is thus possible.

### 2.8.4 Regulation of PI-PLCs in Plants

All PI-PLCs reported so far from plants are homologous to mammalian δ-isofoms. All plant PI-PLCs contain only an additional regulatory domain, the C2 domain besides the X and Y catalytic domains. A probable involvement of the C2 domain in a calcium dependent regulation of plant PI-PLCs cannot be ruled out.

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Pharmacological activation of PI-PLC-dependent pathways by mastoparan in plants described above is suggestive of G-protein mediation (Einspahr et al., 1989; Legendre et al., 1993; Franklin-Tong, et al., 1996; Kuin et al., 2000; den Hartog et al., 2001), however no direct demonstration of G-protein involvement in activating PI-PLC is available in plants. In this context it must be mentioned that a novel PLC Associated Protein (PLAP) was isolated in a yeast two-hybrid screen using soybean PLC1 as bait that showed three transmembrane domains (Dammann and Bhattacharyya, 1999). A search in the “PRINTS” database revealed six footprints with homology to the rhodopsin family of G-protein-coupled receptors. Interestingly, the δ-isoforms of mammalian PI-PLCs have been shown to couple with α1-adrenergic receptors which in turn also belong to the rhodopsin family of G-protein-coupled receptors. Preliminary studies had shown that PI-PLC activity could influence PLAP transcription and suggests the existence of a regulatory feedback mechanism that could control PI-PLC activity in cells. In this connection mention must be made of the isolation of a receptor-like GPR1 protein that was shown to bind to yeast phospholipase C in a two hybrid screen (Ansari, et al., 1999). GPR1 is a member of the hepta-helical family of transmembrane receptors known to activate G-protein coupled pathways. The study suggested (on the basis of two hybrid interactions and generation of null mutations) that phospholipase C modulated the interaction of the GPR1 protein with the Gα protein encoded by GPA2 in yeast as a downstream effect of filamentation control. GPR1 has been earlier shown to couple to the gene GPA2 in two hybrid screens. Given that S. cerevisiae PLC is a δ-isoform, its interaction with the GPR1 protein could have potential implications for the existence of a similar mode of regulation in plants. The interaction of PLAP with PLC in soybean is significant in this regard. More work will establish the role of PLAP in interaction with PLC and has implications for a possible G-protein mediated regulation of PLC in plants.

Finally, calcium may also play an important role in regulating PLC activity in plants. The calcium dependence of the basal activity of plant PLCs is well established (Melin et al., 1987; Melin et al., 1992; Hirayama et al., 1995; Shi et al., 1995a; Kopka et al., 1998b; Staxen et al., 1999). The region between the X and Y
domains in certain plant PI-PLCs has a stretch of acidic amino acid residues (Shi et al., 1995a; see Sopory et al., 2001). These residues may contribute to calcium binding and could lead to modulation of PI-PLC activity. Additionally, calcium binding to the C2 domain could lead to activation; it is also possible that the C2 domain, as in mammalian cells has only a structural role and/or functions only in membrane binding. The C2 domain could also function as an adaptor molecule that interacts with other proteins in a calcium dependent/independent manner, leading to PI-PLC activation. The existence of C2 domains that are known to function in a calcium independent manner and bind to inositol phosphates, calcium channels, AP2 and β-SNAP supports this hypothesis (Rizo and Sudhof, 1998).