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**Phospholipase C-eta enzymes as putative protein kinase C and Ca²⁺
signalling components in neuronal and neuroendocrine tissues**

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Running Title: PLC η s in neuronal and neuroendocrine tissues

Abstract

Phosphoinositol-specific phospholipase C enzymes (PLCs) are central to inositol lipid signaling pathways, facilitating intracellular Ca^{2+} release and protein kinase C activation. A sixth class of Phosphoinositol-specific PLC with a novel domain structure, PLC-eta (PLC η) has recently been discovered in mammals. Recent research, reviewed here, shows that this class consists of two enzymes PLC η 1 and PLC η 2. Both enzymes hydrolyze phosphatidylinositol 4,5-bisphosphate and are more sensitive to Ca^{2+} than other PLC isozymes and are likely to mediate G-protein coupled receptor signaling pathways. Both enzymes are expressed in neuron-enriched regions, being abundant in the brain. We demonstrate that they are also expressed in neuroendocrine cell lines. PLC η enzymes therefore represent novel proteins influencing intracellular Ca^{2+} dynamics and protein kinase C activation in the brain and neuroendocrine systems.

Keywords: Ca^{2+} signaling; protein kinase C; receptor-mediated signaling; neuroendocrine; neuron.

Introduction

Phospholipase C enzymes (PLCs, EC 3.1.4.3) catalyze the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) and result in the release of 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃) from membranes in response to receptor activation. These products trigger the activation of protein kinase C and the release of Ca²⁺ from intracellular stores, respectively (1,2). They are crucial for initiation of cellular activation, proliferation, differentiation and apoptosis. Until recently, five distinct classes of PLCs that specifically react with phosphatidylinositols were known to exist in mammals, the β, γ, δ, ε and ζ-type enzymes (3,4). They have been classified on the basis of amino acid sequence, domain structure and amino acid similarity and by the mechanisms through which they are recruited in response to activated receptors. We, along with several other groups, identified a sixth class that was termed PLCη (5-8). Two putative PLCη enzymes were identified in humans and mice, PLCη1 and PLCη2 (5) and both were confirmed to catalyze hydrolysis of PIP₂ (6-8) suggesting that PLCηs, like other PLCs, are involved in production of the secondary messengers DAG and IP₃.

Domain Structure of PLCη enzymes

Like other mammalian PLCs, the domain structure of PLCηs consists of Pleckstrin homology (PH), EF-hand, catalytic X and Y domains and protein kinase C conserved region 2 (C2). Figure 1A shows the domain organization of all mammalian PLC-isozymes including all known forms of PLCη. Certain isoforms of PLCη1 and PLCη2 possess domain complements similar to PLCβ, which couple to G protein-coupled receptors (GPCRs). The PH domain (absent in PLCζ; ~110 residues) binds polyphosphoinositides (9) and is present in many signaling proteins that associate

with phospholipid membranes (10,11). This domain is not essential for membrane localization of PLC δ 1 and PLC δ 4 (6,12) but appears to be more important for membrane localization of PLC η 2. Approximately 85% of native PLC η 2 and 97% of a FLAG-tagged PLC η 2 constructs were found to localize to plasma membrane, whereas only 6% of a PLC η 2 construct with the PH domain removed was present at the cell surface (6). The role of the EF hand domain (~65 residues) in PLCs is unclear but the X-ray structure of PLC δ 1 shows that it serves as a flexible “hinge-like” link between the PH and the catalytic domains (13). The X and Y domains (~150 and ~115 residues, respectively) fold to form the catalytic site. The X domain is involved in both substrate and Ca²⁺ binding (which is essential for catalysis), while the Y domain primarily interacts with the substrate (13). These regions contain the highest degree of sequence identity (between 60-85%) among different mammalian PLCs (14). The C2 domain is essential for catalytic activity (15) and is often associated with proteins that interact with phospholipids. In some PLCs the C2 domain binds Ca²⁺ and mediates Ca²⁺-dependent interactions with the lipid membrane. It has been speculated that the C2 domain of PLC δ 1 may contain as many as four Ca²⁺-binding sites (15). PLC η s share a close evolutionary relationship with other PLC isozymes and are most closely related to the PLC δ class (5,6). However, unlike PLC δ s, the PLC η enzymes contain an extended loop (~100 residues longer) between the X and Y domains and also include an additional C-terminal region that is rich in serine and proline residues. Serine- and proline-rich regions have proposed roles in protein-protein interactions (16,17). The C-terminal region of PLC η s are likely to be of functional yet undefined importance.

PLC η s also contain a class II PDZ (post synaptic density protein, *Drosophila* disc large tumor suppressor, and zo-1 protein) conserved binding motif (PDZCBM;

$\Psi X \Psi$ -COOH, where Ψ represents a hydrophobic residue) at the C-terminus. PLC β s also contain a PDZCBM albeit a different motif (class I; (S/T)X(L/V)-COOH) and have been shown to be involved in the formation of multi-protein scaffolds including the InaD complex which mediates the assembly of photoreceptors via TRP channel activation (18,19) and the Na⁺/H⁺ exchanger regulatory factor 1-assembled complex in the kidney (20). It is therefore likely that in addition to classical PLC signaling PLC η s, like PLC β s, play role in the formation of PDZ multi-protein complexes.

Three splice variants of PLC η 1 and five splice variants of PLC η 2 have been identified, all of which differ in length in the C-terminal region. The three variants of PLC η 1, 'a', 'b' and 'c' encode human proteins of 1002, 1693 and 1035 amino acids, respectively (7). Interestingly, all three variants contain class II PDZCBM, although the actual sequence of the motif for PLC η 1a (VQI-COOH) differs from that of PLC η 1b and PLC η 1c (LRL-COOH). This hints that variant 'a' may function as part of a different PDZ protein-scaffold than the other two variants. The five PLC η 2 variants encode human proteins of 1416, 989, 1583, 1156 and 1211 amino acids and have been categorised according to the exon structure of the spliced forms: '21a/23', '21a/22/23', '21b/23', '21b/22/23' and '21c/22/23', respectively (8). Of these five variants, only two ('21a/23' and '21b/23') contain a class II PDZCBM (both LRL-COOH), whilst the other three variants do not contain a PDZCBM at all. This again suggests the potential for differing functions between spliced forms *in vivo*.

Expression of PLC η enzymes

Murine PLC η 1 expression has been investigated by RT-PCR using primer pairs targeting a common region of the three splice variants and also for the PLC η 1a variant only (7). In the range of tissues examined the PCR products of the common

region were most abundant in brain and kidney but were also observed in lung, spleen, intestine, thymus and pancreas. PLC η 1a was detected in the brain and lung only. Immunoblotting confirmed expression of PLC η 1a protein in neuronal tissues such as cerebrum, cerebellum and spinal cord. *In situ* hybridization revealed a high level of expression throughout the brain, especially in neuronal cell enriched regions such as the inner layer of the olfactory bulb, the hippocampus, Purkinje layer of cerebellum, cerebral cortex, zona incerta, habenular nuclei and hypothalamus (7). RT-PCR and Northern blot analyses have shown that PLC η 2 gene expression is detectable in both brain and intestine of mice (5,6). In addition, expressed sequence tags (ESTs) corresponding to human PLC η 2 were identified in cDNAs isolated from a range of neuron-rich tissues including anaplastic oligodendroglioma, epithelioid carcinoma, leukopheresis, lymph, nerve tumor, optic nerve, pancreatic islet, pituitary and retinoblastoma cell populations (5). Immunoblot analysis detected PLC η 2 expression in the brain but not in a variety of other murine tissues including small intestine, heart, skeletal muscle, kidney, liver, lung, testis or spleen (6). Expression of PLC η 2 protein in the brain was found to be developmentally dependent, being detectable 1-2 weeks after birth. It was also detected at high levels in neuron-containing primary cultures but not in astrocyte cultures. *In situ* hybridization on murine tissue sections showed gene expression in pyramidal cells of the olfactory bulb, hippocampus and cerebral cortex, three regions where PLC η 1 is also expressed.

The hippocampus and cerebral cortex are involved in memory and learning (21,22). The olfactory bulb functions in odor and pheromone perception and is also involved in neuro-hormonal programming of the hypothalamo-pituitary axis (23,24). PLC η enzymes may therefore play a vital role in neural signaling pertaining to memory and learning or neuron-hormonal regulation. This hypothesis is consistent

with the observation that PLC η 2 increases with post natal age. In addition PLC η 2 was found to have a very similar expression pattern to that of neuron marker protein, microtubule-associated protein 2 (MAP2) (6). This suggests that PLC η 2 is particularly likely to be involved in some aspect of neural or neuroendocrine functioning.

Further evidence that PLC η 2 may play a role in the hypothalamo-pituitary axis has come from recent work in our laboratory where we have found this protein to be present in the GnRH neuronal cell line, GT1-7 and in the L β T2 and α T3 pituitary cell lines but not in HEK293 cells (Figure 1B). HEK293 cells were used as a negative control due to the absence of product when mRNA isolated from these cells was assayed by RT-PCR using primers able to detect human PLC η 2 transcript. The GT1-7 cell line is a well-characterized model of the hypothalamic neuron able to secrete gonadotropin-releasing hormone (GnRH) in culture (25). L β T2 and α T3 cells are pituitary gonadotrope-like cells, which express GnRH receptor and can be stimulated to release the pituitary hormones luteinizing hormone and follicle stimulating hormone (26,27). Given the presence of PLC η 2 in these neuroendocrine cells as well as neurons, a possible role for PLC η 2 may affect vesicle exocytosis. This process is not only Ca²⁺-driven but requires formation of pre-synaptic-like PDZ domain-protein complexes at the surface of the cell membrane (28,29).

Regulation and Differential Ca²⁺ Sensitivity

In the recent study by Zhou et al. (8), co-expression of PLC η 2 with the G proteins, G β ₁ and G γ ₂ resulted in elevated PLC activity in COS-7 cells. This suggests that PLC η 2 may be activated in response to G protein-coupled receptor activation. G $\beta\gamma$ dimers have also been shown to activate PLC β 1-3 and PLC ϵ (30-32) through

interaction with the PH domain (33). Whether the observed G $\beta\gamma$ -mediated stimulation of PLC activity in PLC η 2 is a direct or indirect effect remains to be examined. However, sequence analysis reveals that several key residues in the PH domain of bovine GSK2 that are known to directly bind G $\beta\gamma$ are conserved. These include Arg587 (which corresponds to Arg78 in PLC η 2) and is essential for G $\beta\gamma$ -induced activation of this enzyme (34).

All PLC isozymes can be activated by Ca²⁺ *in vitro*, but PLC δ 1 is more sensitive to Ca²⁺ compared with the other isozymes and it can be constitutively tethered to PIP₂-containing membranes via its PH domain in the absence of other signals (35). It has therefore been speculated that an increase in the intracellular Ca²⁺ to a level sufficient to fix the C2 domain of PLC δ 1 to the membrane, triggers its activation (35). Thus, it has been postulated that activation of PLC δ 1 isozymes may occur following receptor-mediated activation of other PLC isozymes (36). Interestingly, PLC η 1 and PLC η 2 exhibit Ca²⁺-dependent (PIP₂)-hydrolyzing activity *in vitro* but differ greatly, compared with other PLCs, in their sensitivity toward Ca²⁺. Both enzymes display maximal activity at a Ca²⁺ concentration of ~1 μ M (6,7), which is at least 10-fold lower than that required by PLC δ 1 (6). Increased sensitivity means that PLC η s may catalyse PIP₂-hydrolysis at much lower Ca²⁺ concentrations than PLC δ 1 *in vivo* and indicate that like the PLC δ class, PLC η 2 may not necessarily be linked to receptor-mediated activation. Alternatively, this enzyme may amplify the signaling events of other PLCs or even Ca²⁺ channels. A speculative representation of PLC η signaling is shown in Figure 2. Kinetic analyses for either of the PLC η enzymes have yet to be performed but it would be interesting to determine whether PIP₂-hydrolysis occurs at a comparable or faster rate than other PLC enzymes under

physiological conditions. If so, PLC η 2 may facilitate very rapid communication between cells due to its elevated Ca²⁺ sensitivity.

Analysis of the Ca²⁺-mediated lipid-binding site in the C2 domain of synaptotagmin I (a Ca²⁺-activated vesicle protein) shows that it consists of four aspartate residues, Asp172, Asp178, Asp230 and Asp232 (37). All four of these residues are conserved in the C2 domain of the PLC η enzymes but not in PLC δ 1, where Asp172 is equivalent to Asn645 (38). Ca²⁺ ions would therefore be expected to bind more tightly at this site in synaptotagmin I and the PLC η s than to PLC δ 1. This may explain the greater Ca²⁺-sensitivity exhibited by PLC η s. Other PLCs such as the PLC β and PLC γ enzymes also contain a C2 domain, yet here the key residues involved in Ca²⁺ binding are not conserved (39).

In conclusion, PLC η enzymes represent an exciting new discovery in the field of neurophysiology and molecular data suggests the basis for their involvement in novel protein networks relaying Ca²⁺-signaling and protein kinase C activation. Such networks are likely to be of great importance in the brain and neuroendocrine tissues. Our recent data suggest that neuroendocrine cell lines may be appropriate tools to begin examining the cell biology of PLC η s. It is hoped that these findings stimulate further research towards elucidating their physiological function.

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Figure legends

FIG. 1. A. Domain organization in murine PLC-isozymes including all known forms of PLC η s (yellow box). PH, Pleckstrin homology domain; EF, EF-hand domain; X, catalytic X domain; Y, catalytic Y domain; C2, C2 domain; SH, Src homology domain; RasGEF, guanine nucleotide exchange factor domain for Ras-like small GTPases; RA, Ras association domain; PDZ, post synaptic density protein, *Drosophila* disc large tumor suppressor, and zo-1 protein C-terminal binding motif. B. Western blot showing presence of PLC η 2 in mouse GT1-7 neuronal, and L β T2 and α T3 neuroendocrine cells.

FIG. 2. Schematic representation of putative PLC η signaling. PLC η catalyzed cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) results in the generation of 1,2-diacylglycerol and inositol 1,4,5-triphosphate (IP₃). These products stimulate Ca²⁺ release and protein kinase C (PKC) activation. *In vivo* PLC η 2 may undergo receptor-mediated activation via interaction with G β γ or in response to a small elevation in levels of cytoplasmic Ca²⁺. PLC η s may also be involved in vesicle exocytosis through association with PDZ protein scaffolds.

Figure 1.

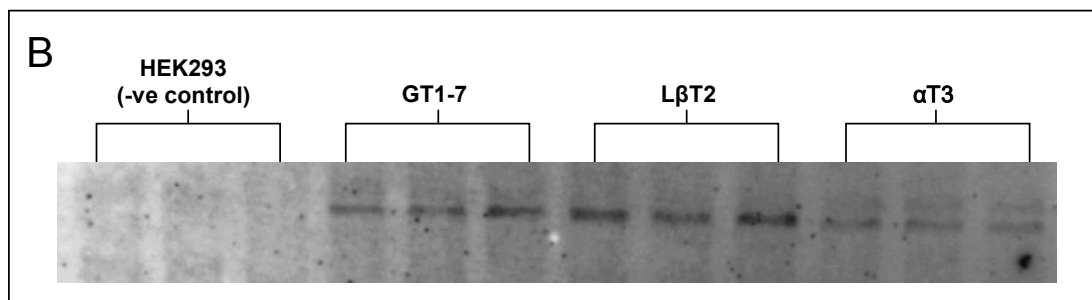
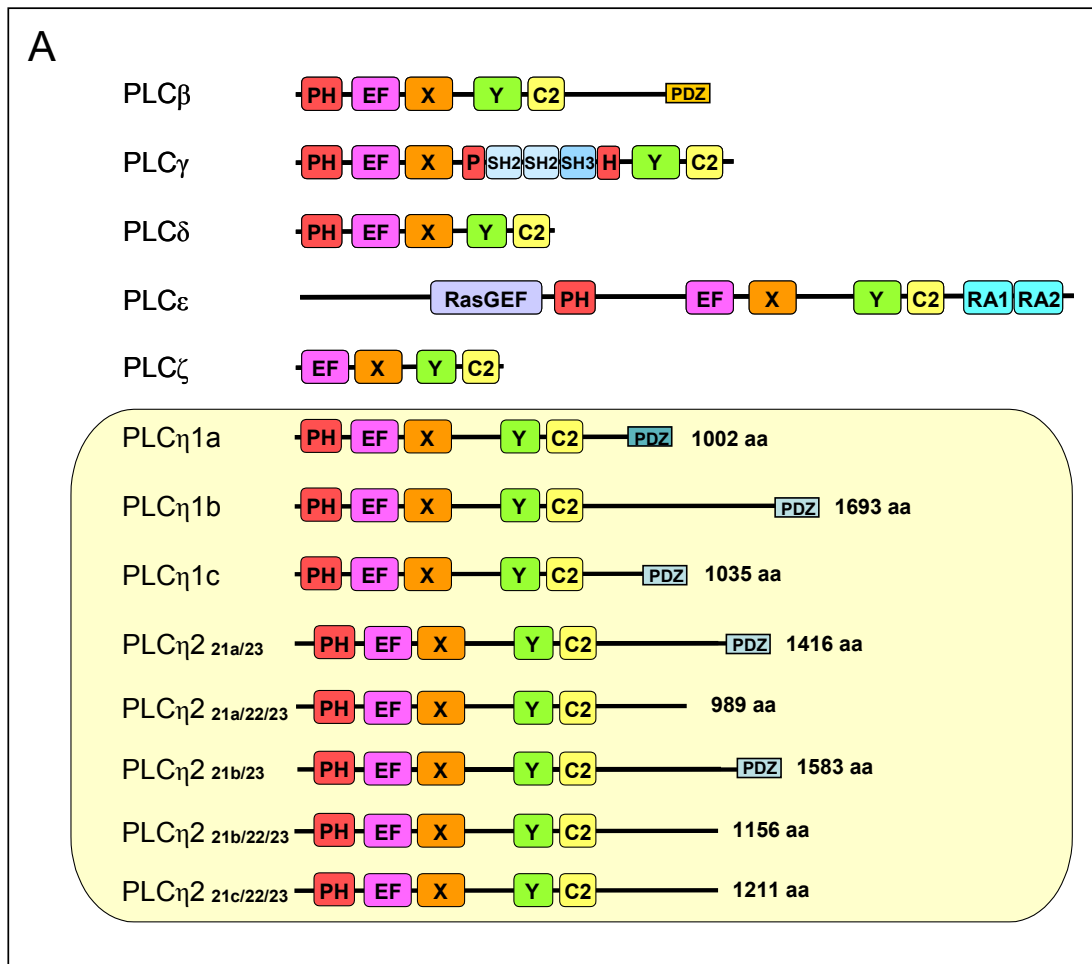


Figure 2.

