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

Phospholipase C-eta enzymes as putative protein kinase C and Ca^{2+} signalling components in neuronal and neuroendocrine tissues

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Running Title: PLCηs 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 (PLCη) has recently been discovered in mammals. Recent research, reviewed here, shows that this class consists of two enzymes PLCη1 and PLCη2. 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. PLCη 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 (PIP_2) and result in the release of 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) from membranes in response to receptor activation. These products trigger the activation of protein kinase C and the release of Ca^{2+} 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 PIP_2 (6-8) suggesting that PLCηs, like other PLCs, are involved in production of the secondary messengers DAG and IP_3.

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 PLCδ1 and PLCδ4 (6,12) but appears to be more important for membrane localization of PLCη2. Approximately 85% of native PLCη2 and 97% of a FLAG-tagged PLCη2 constructs were found to localize to plasma membrane, whereas only 6% of a PLCη2 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 PLCδ1 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 Ca^{2+} 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 Ca^{2+} and mediates Ca^{2+}-dependent interactions with the lipid membrane. It has been speculated that the C2 domain of PLCδ1 may contain as many as four Ca^{2+}-binding sites (15). PLCηs share a close evolutionary relationship with other PLC isozymes and are most closely related to the PLCδ class (5,6). However, unlike PLCδs, the PLCη 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 PLCηs are likely to be of functional yet undefined importance.

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

ΨXΨ-COOH, where Ψ represents a hydrophobic residue) at the C-terminus. PLCβs 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 PLCηs, like PLCβs, play role in the formation of PDZ multi-protein complexes.

Three splice variants of PLCη1 and five splice variants of PLCη2 have been identified, all of which differ in length in the C-terminal region. The three variants of PLCη1, '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 PLCη1a (VQI-COOH) differs from that of PLCη1b and PLCη1c (LRL-COOH). This hints that variant 'a' may function as part of a different PDZ protein-scaffold than the other two variants. The five PLCη2 variants encode human proteins of 1416, 989, 1583, 1156 and 1211 amino acids and have been categorisd 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 PLCη enzymes

Murine PLCη1 expression has been investigated by RT-PCR using primer pairs targeting a common region of the three splice variants and also for the PLCη1a 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. PLCη1a was detected in the brain and lung only. Immunoblotting confirmed expression of PLCη1a 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 PLCη2 gene expression is detectable in both brain and intestine of mice (5,6). In addition, expressed sequence tags (ESTs) corresponding to human PLCη2 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 PLCη2 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 PLCη2 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 PLCη1 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). PLCη 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 PLCη2 increases with post natal age. In addition PLCη2 was found to have a very similar expression pattern to that of neuron marker protein, microtubule-associated protein 2 (MAP2) (6). This suggests that PLCη2 is particularly likely to be involved in some aspect of neural or neuroendocrine functioning.

Further evidence that PLCη2 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 LβT2 and αT3 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 PLCη2 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). LβT2 and αT3 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 PLCη2 in these neuroendocrine cells as well as neurons, a possible role for PLCη2 may affect vesicle exocytosis. This process is not only Ca^{2+}-driven but requires formation of pre-synaptic-like PDZ domain-protein complexes at the surface of the cell membrane (28,29).

Regulation and Differential Ca^{2+} Sensitivity

In the recent study by Zhou et al. (8), co-expression of PLCη2 with the G proteins, $G\beta_1$ and $G\gamma_2$ resulted in elevated PLC activity in COS-7 cells. This suggests that PLCη2 may be activated in response to G protein-coupled receptor activation. Gβγ dimers have also been shown to activate PLCβ1-3 and PLCε (30-32) through

interaction with the PH domain (33). Whether the observed Gβγ-mediated stimulation of PLC activity in PLCη2 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 Gβγ are conserved. These include Arg587 (which corresponds to Arg78 in PLCη2) and is essential for Gβγ-induced activation of this enzyme (34).

All PLC isozymes can be activated by Ca^{2+} *in vitro*, but PLCδ1 is more sensitive to Ca^{2+} compared with the other isozymes and it can be constitutively tethered to PIP_2-containing membranes via its PH domain in the absence of other signals (35). It has therefore been speculated that an increase in the intracellular Ca^{2+} to a level sufficient to fix the C2 domain of PLCδ1 to the membrane, triggers its activation (35). Thus, it has been postulated that activation of PLCδ1 isozymes may occur following receptor-mediated activation of other PLC isozymes (36). Interestingly, PLCη1 and PLCη2 exhibit Ca^{2+}-dependent (PIP_2)-hydrolyzing activity *in vitro* but differ greatly, compared with other PLCs, in their sensitivity toward Ca^{2+}. Both enymes display maximal activity at a Ca^{2+} concentration of ~1μM (6,7), which is at least 10-fold lower than that required by PLCδ1 (6). Increased sensitivity means that PLCηs may catalyse PIP_2-hydrolysis at much lower Ca^{2+} concentrations than PLCδ1 *in vivo* and indicate that like the PLCδ class, PLCη2 may not necessarily be linked to receptor-mediated activation. Alternatively, this enzyme may amplify the signaling events of other PLCs or even Ca^{2+} channels. A speculative representation of PLCη signaling is shown in Figure 2. Kinetic analyses for either of the PLCη enzymes have yet to be performed but it would be interesting to determine whether PIP_2-hydrolysis occurs at a comparable or faster rate than other PLC enzymes under

physiological conditions. If so, PLCη2 may facilitate very rapid communication between cells due to its elevated Ca^{2+} sensitivity.

Analysis of the Ca^{2+}-mediated lipid-binding site in the C2 domain of synaptotagmin I (a Ca^{2+}-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 PLCη enzymes but not in PLCδ1, where Asp172 is equivalent to Asn645 (38). Ca^{2+} ions would therefore be expected to bind more tightly at this site in synaptotagmin I and the PLCηs than to PLCδ1. This may explain the greater Ca^{2+}-sensitivity exhibited by PLCηs. Other PLCs such as the PLCβ and PLCγ enzymes also contain a C2 domain, yet here the key residues involved in Ca^{2+} binding are not conserved (39).

In conclusion, PLCη 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 Ca^{2+}-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 PLCηs. It is hoped that these findings stimulate further research towards elucidating their physiological function.

Acknowledgments

We thank the Medical Research Council (MRC) and Biotechnology and Biological Sciences Research Council (BBSRC) for funding, Dr Pamela Mellon (University of California) for GT1-7, LβT2 and αT3 cell lines and Prof. Kiyoko Fukami (Tokyo University of Pharmacy and Life Science) for providing anti-PLCη2 antibody.

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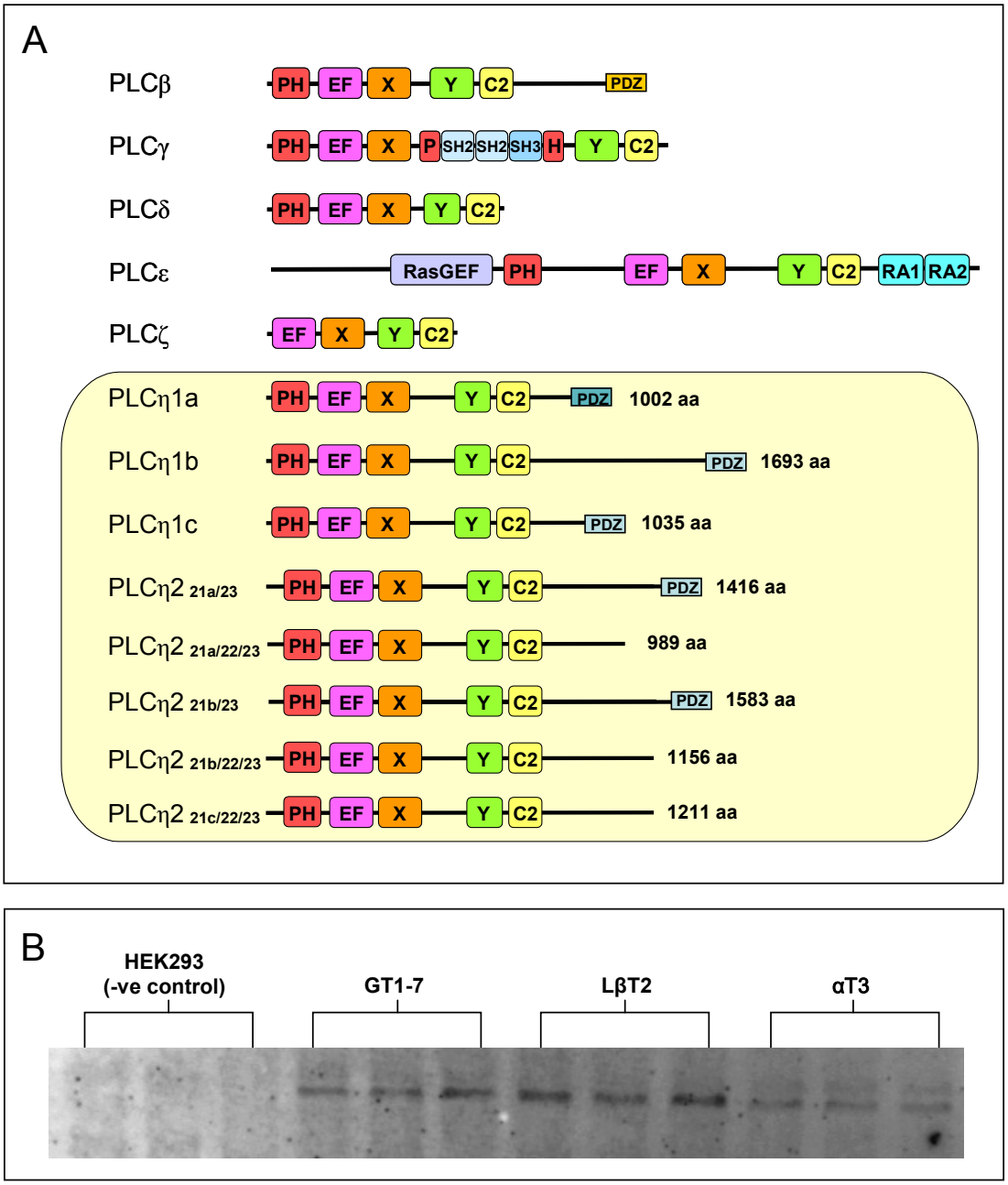
Figure legends

FIG. 1. A. Domain organization in murine PLC-isozymes including all known forms of PLCηs (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 PLCη2 in mouse GT1-7 neuronal, and LβT2 and αT3 neuroendocrine cells.

FIG. 2. Schematic representation of putative PLCη signaling. PLCη catalyzed cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) results in the generation of 1,2-diacylglycerol and inositol 1,4,5-triphosphate (IP_3). These products stimulate Ca^{2+} release and protein kinase C (PKC) activation. *In vivo* PLCη2 may undergo receptor-mediated activation via interaction with Gβγ or in response to a small elevation in levels of cytoplasmic Ca^{2+}. PLCηs may also be involved in vesicle exocytosis through association with PDZ protein scaffolds.

Figure 1.


A
PLCβ
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PLCγ
PH EF X P SH2 SH2 SH3 H Y C2
PLCδ
PH EF X Y C2
PLCε
RasGEF PH EF X Y C2 RA1 RA2
PLCζ
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PLCη2 21a/23
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PLCη2 21a/22/23
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PLCη2 21b/23
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PLCη2 21b/22/23
PH EF X Y C2 1156 aa
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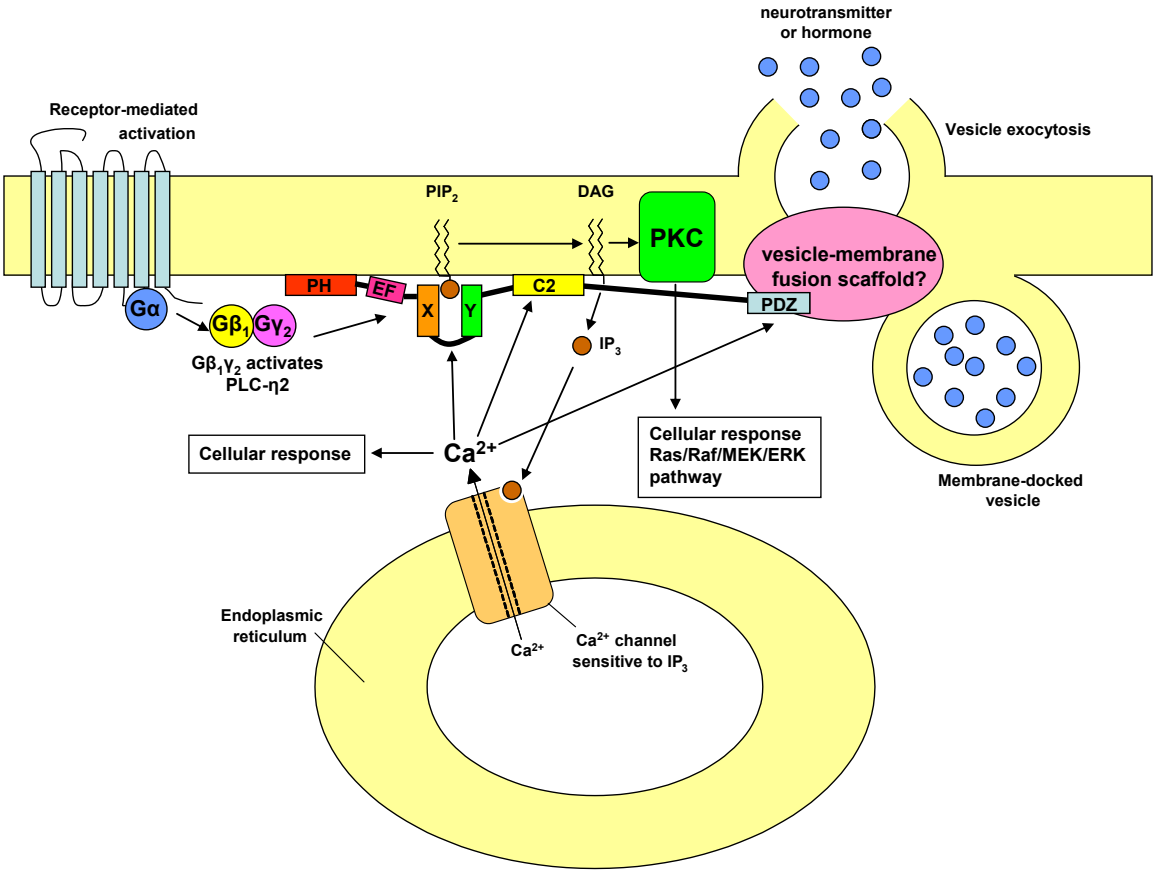
Figure 2.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C2
PDZ
Gα
Gβ1
Gγ2
Gβ1γ2 activates
PLC-η2
IP3
Cellular response
Ca2+
Cellular response
Ras/Raf/MEK/ERK
pathway
Membrane-docked
vesicle
Endoplasmic
reticulum
Ca2+
Ca2+ channel
sensitive to IP3