The Role of EF-hand Domains and C2 Domain in Regulation of Enzymatic Activity of Phospholipase Cζ*

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Sperm-specific phospholipase Cζ (PLCζ) induces Ca2+-oscillations and egg activation when injected into mouse eggs. PLCζ has such a high Ca2+-sensitivity of PLC activity that the enzyme can be active in resting cells at ~100 nm Ca2+, suitable for a putative sperm factor to be introduced into the egg at fertilization (Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T., and Miyazaki, S. (2004) J. Biol. Chem. 279, 10408–10412). In the present structure-function analysis, deletion of EF1 and EF2 of the N-terminal four EF-hand domains caused marked reduction of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)-hydrolyzing activity in vitro and loss of Ca2+-oscillation-inducing activity in mouse eggs after injection of RNA encoding the mutant. However, deletion of EF1 and EF2 or mutation of EF1 or EF2 at the x and z positions of the putative Ca2+-binding loop little affected the Ca2+-sensitivity of the PLC activity, whereas deletion of EF1 to EF3 caused 12-fold elevation of the EC50 of Ca2+ concentration. Thus, EF1 and EF2 are important for the PLCζ activity, and EF3 is responsible for its high Ca2+-sensitivity. Deletion of four EF-hand domains or the C-terminal C2 domain caused complete loss of PLC activity, indicating that both regions are prerequisites for PLCζ activity. Screening of interactions between the C2 domain and phosphoinositides revealed that C2 has substantial affinity to PI(3)P and, to a lesser extent, to PI(3,5)P2 but not to PI(4,5)P2 or acidic phospholipids. PI(3)P and PI(5)P reduced PLCζ activity in vitro, suggesting that the interaction could play a role for negative regulation of PLCζ.

PLCζ

PLCζ is a novel type of PLC (the enzyme that hydrolyzes membrane PI(4,5)P2 into IP3 and diacylglycerol). PLCζ is strongly noted in developmental biology because it is specifically expressed in mammalian sperm (1) and is capable of inducing repetitive increase in [Ca2+], and subsequent early embryonic development when expressed in mouse eggs by injection of RNA encoding PLCζ (1–3). At fertilization of mammals, evidence indicates that a cytosolic sperm factor is introduced into the ooplasm upon sperm-egg fusion and induces Ca2+-oscillations (4, 5), which are due to Ca2+ release from the endoplasmic reticulum mainly through the IP3 receptor (6) and are a pivotal signal for egg activation (5). Ca2+-oscillations similar to those at fertilization are produced by PLCζ expressed in a mouse egg at an estimated level comparable with the content in a single mouse sperm (1, 2). Injection of recombinant PLCζ into mouse eggs induces Ca2+-oscillations as well (7). The Ca2+-oscillation-inducing activity of sperm extract (4, 8) is lost when the extract is pretreated with an antibody against PLCζ (1). Thus, PLCζ is a strong candidate for the sperm-derived Ca2+-oscillation-inducing protein.

PLCζ possesses biochemically interesting characteristics. It is composed of four EF-hand domains in the N terminal, calyctic X and Y domains, and a C2 domain in the C terminus (1) (Fig. 1A) common to other types of PLC, but PLCζ lacks the N-terminal PH domain found in PLCβ, -γ, -δ, and -ε. PH domain of PLCβ, -γ, or -δ binds to PI(3)P, PI(3,4,5)P3, or PI(4,5)P2, respectively (9–11). The PH and C2 domains of PLCβ also interact with the G protein subunits Gβγ and Goq, respectively, for activation of PLCβ (12, 13). PLCζ is phosphorylated at the tyrosine residues 771, 783, and 1254 in response to growth factor stimulation leading to activation of PLCζ (9–11). As for PLCζ, which lacks the PH domain, there is no prediction about the domain responsible for binding to phosphoinositides and for enzymatic activation.

PLCζ has domain features similar to those of PLCδ except for the PH domain (Fig. 1A). PLCδ is suggested to be regulated by Ca2+, because PLCδ1 has Ca2+-binding sites in not only the EF-hand domains but also in the X and Y domains and the C2 domain (11). We have recently shown (7) that the PI(4,5)P2-hydrolyzing activity of recombinant PLCζ is ~100-fold more sensitive to Ca2+ than that of PLCδ1 and is 70% maximal at 100 nM [Ca2+], which is usually the basal [Ca2+]i of cells. Recombinant PLCζ has ~20-fold higher Ca2+-oscillation-inducing activity than PLCδ1 (7). Thus, PLCζ possesses remarkable properties that are favorable for the sperm factor to initiate (7) and maintain (3) Ca2+-oscillations via continuously produced IP3. Using mutational analysis in the present study, we addressed which domains of PLCζ are responsible for the Ca2+-sensitive PLC activity in vitro, Ca2+-oscillation-inducing activity in mouse eggs, and phosphoinositide binding activity on the PLC activity. We focused on four N-terminal EF-hand domains because they are likely to be Ca2+-binding sites and could be critical for the high Ca2+-sensitivity. We also focused on the C2 domain, as three Ca2+-binding sites are predicted in the C2
domain of PLCδ1 (9, 14). The C2 domain of PLCζ could play another role such as interaction with phosphoinositides. Our study revealed that both EF-hand domains and the C2 domain are critical for Ca2+ oscillation-inducing activity as well as the PLC activity of PLCζ. Furthermore, we found that the C2 domain of PLCζ binds to PI(3)P or PI(5)P, and this interaction may negatively regulate the PLC activity.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—cDNAs for mutants of PLCζ, domains of PLCζ and PLCδ1, and a PLCζ/EFδ1 chimera were prepared using PCR techniques. To obtain recombinant PLCζs, mutant DNA were subcloned into pFastBacHT (Invitrogen) or GST-FastBac (provided by Dr. T. Takewana) used in a baculovirus expression system (7). To obtain cDNAs for expression of PLCs in mouse eggs, PLCζ (1), short PLCζ (s-PLCζ) (2, 7), and PLCζ mutants were fused with a fluorescent protein, “Venus” (15), in the N terminus and subcloned into pBluescript II (SK+) containing the T7 promoter (Stratagene). The following proteins were prepared (see Fig. 1A for designation and domain features). 1) For EF-hand domain deletion mutants ΔEF1, ΔEF1–2, and ΔEF1–4, residues Met1–Leu39, Met1–Ile75, and Met1–Gln163 were deleted, respectively. PLCΔEF1–3 (s-PLCζ) that lacks 110 amino acid residues from the N terminus corresponding to three EF-hand domains (GenBankTM accession number AK006672) was cloned as described previously (7, 2). For ΔC2 and ΔE11–647 deletion mutants, Leu152, Thr156, and Leu158, Glu167, were deleted. 3) For PLCζ having EF1AA and EF2AA, Asp20 and Gly57 in EF1 and Asp86 and Gly88 in EF2 were replaced with alanine corresponding to the x and z positions of the predicted EF-hand motif. 4) The C2 domain (C2D) isolated from PLCζ (Val120–Glu464) was prepared with added GST. 5) For PLCζ/EFδ1, the residual region except the EF-hand domains (Asp54–Glu464) of PLCζ was replaced with that of PLCδ1 (Asp54–Glu776). 6) The isolated PH domain (6PHD) of PLCζ and its K30N/K32N mutant (16) were prepared and fused with GST.

**Fig. 1. Domain features and expression of PLCζ and its mutants.** A, schematic illustration of the domain features of PLCζ mutants and the PLCζ/δ1 chimera. B, purified proteins shown by 10% SDS-polyacrylamide gel and silver staining. The number of lanes corresponds to those in A. Asterisks mark the bands of indicated mutant proteins. C, alignment of Ca2+–binding loop sequences of the EF-hand domain of troponin C and calmodulin and predicted EF-hand domains of the PLCζ and PLCζ domains of troponin C and calmodulin and predicted EF-hand domains.
**Structure-Function Analysis of PLC**

### RESULTS

**EF-hand Domains and C2 Domain Are Necessary for Catalytic Activity of PLC**—Table I presents the specific PI(4,5)P$_2$-hydrolyzing activity of recombinant PLC$_\Delta$ protein and its mutants measured in vitro at [Ca$^{2+}$] between 1 and 30 μM, at which a maximal activity was obtained. PLC$_\Delta$EF1–4, which lacks all of the four EF-hand domains, had no PLC activity at 1 and 10 μM [Ca$^{2+}$] (Table I and Fig. 2A). s-PLC$_\Delta$ (PLC$_\Delta$EF1–3), a PLC$_\Delta$ variant that is expressed in the mouse testis and lacks the EF1, EF2, and EF3 domains, had substantial activity (20 nmol/min/mg at 10 μM [Ca$^{2+}$]). Deletion of the C2 domain (PLC$_\Delta$C2) or the 37 amino acid residues in the C-terminal region next to the C2 domain (PLC$_\Delta$611–647) resulted in complete loss of PLC activity (less than 0.1 nmol/min/mg) at 1 and 10 μM [Ca$^{2+}$] (Table I). GST-tagged PLC$_\Delta$EF1–3 serves as a positive control for these inactive mutants that were tagged with GST. These results indicate that whole N-terminal EF-hand domains are critical for PLC activity and that the C2 domain including the C-terminal region is also prerequisite for PLC activity.

To examine the role of the residual region except for the EF-hand domains, a chimera was formed by replacing the region from the X domain to the C terminus of PLC$_\Delta$ with that of PLC$_\Delta$1 (PLC$_\Delta$EF1–61, Fig. 1A). This chimera showed no detectable PLC activity at 1 and 10 μM [Ca$^{2+}$] (Table I) even if four EF-hand domains are normally prepared. Therefore, the enzymatic activity of PLC$_\Delta$ seems to require a specific matching in the PLC$_\Delta$ molecule between the four N-terminal EF-hand domains and the other region containing the catalytic X and Y domains and C-terminal C2 domain.

**Effects of Deletion of EF-hand Domains on PLC Activity and Its Ca$^{2+}$ Sensitivity**—The PLC activity of PLC$_\Delta$ was remarkably reduced when the EF-hand domains were deleted one by one from the N terminus (Table I). The specific activity (nmol/min/mg) assayed at 1 μM [Ca$^{2+}$] was 1300 for PLC$_\Delta$1, 290 (22% activity of PLC$_\Delta$) for deletion of EF1 (PLC$_\Delta$EF1), 40 (3%) for deletion of EF1 and EF2 (PLC$_\Delta$EF1–2), and 70 (5%) for deletion of EF1–EF3 (His-tagged s-PLC$_\Delta$, assayed at 30 μM [Ca$^{2+}$]). Thus, EF1 and EF2 are greatly responsible for the PLC activity.

### Table 1: Specific Pi(4,5)P2-hydrolyzing activity of PLC$_\Delta$ and mutants

| Specific activity | [Ca$^{2+}$] | EC$_{50}$ [Ca$^{2+}$] |
|------------------|-------------|----------------------|
| PLC$_\Delta$     | 1300 ± 110  | 1.3                  |
| PLC$_\Delta$EF1A| 650 ± 60    | 1.3                  |
| PLC$_\Delta$EF2A| 590 ± 90    | 3.9                  |
| PLC$_\Delta$EF1  | 290 ± 10    | 3.9                  |
| PLC$_\Delta$EF1–2| 40 ± 1      | 9.5                  |
| PLC$_\Delta$EF1–3| 70 ± 3      | 30 373               |
| PLC$_\Delta$EF1–3b| 20 ± 3      | 10                   |
| PLC$_\Delta$EF1–4| <0.1        | 1.10                 |
| PLC$_\Delta$EF1–6| <0.1        | 1.10                 |
| PLC$_\Delta$EF1–2| <0.1        | 1.10                 |
| PLC$_\Delta$EF1–4b| <0.1        | 1.10                 |

$^a$ [Ca$^{2+}$] at which each protein (from PLC$_\Delta$ to PLC$_\Delta$EF1–3) showed a maximal activity. The activity of four mutants from the bottom was assayed at 1 and 10 μM [Ca$^{2+}$].

$^b$ GST fusion protein.

To analyze the Ca$^{2+}$ sensitivity, the PLC activity was assayed at [Ca$^{2+}$] between 10$^{-8}$ and 3 × 10$^{-3}$ μM, and presented as the percentage relative to the maximal activity (Fig. 2A). As reported previously (7), the PLC activity of PLC$_\Delta$ was significantly recognized at [Ca$^{2+}$] as low as 10 nm and reached a maximum at 1 μM [Ca$^{2+}$]. There was ~80% maximal activity at 100 μM, which is the resting [Ca$^{2+}$] level in mouse eggs (8) as well as somatic cells (22). The [Ca$^{2+}$] for giving a half-maximal PLC activity, EC$_{50}$, was obtained by fitting a curve to the data using the Hill equation. The EC$_{50}$ of PLC$_\Delta$ was calculated as 32 nm. Deletion of EF1 and EF2 did not cause a marked decrease in the Ca$^{2+}$ sensitivity (Fig. 2A). The EC$_{50}$ was 98 μM for PLC$_\Delta$EF1–2 and 93 μM for PLC$_\Delta$EF1–2 (Table I). In contrast, the PLC activity of PLC$_\Delta$EF1–3 was about 15% maximal at 100 μM [Ca$^{2+}$] and reached the maximum at 30 μM [Ca$^{2+}$] (Fig. 2A). The EC$_{50}$ was 373 μM, 12-fold higher than that of PLC$_\Delta$ (Table I). These results indicate that the region containing EF3 contributes to the high Ca$^{2+}$ dependence of the PLC activity. However, the EC$_{50}$ of PLC$_\Delta$EF1–3 is still 15-fold lower than that of PLC$_\Delta$ (5.7 μM) (7). The Hill constant obtained from curves in Fig. 2A was around 1.0 for EF-hand domain deletion mutants as well as for PLC$_\Delta$. There may be a coordinating structural determinant(s) other than EF1-EF3 for the highly Ca$^{2+}$-sensitive enzymatic activity.

**Effects of Point Mutation in EF1 and EF2 on PLC Activity and Its Ca$^{2+}$ Sensitivity**—According to the ProDom EF-hand pattern (23), EF1 and EF2 of PLC$_\Delta$ as well as those of PLC$_\Delta$ contain the Ca$^{2+}$-binding loop sequence homologous to a well conserved Ca$^{2+}$-binding site of calmodulin or troponin C (25, 26) (Fig. 1C). Of the Ca$^{2+}$-coordinating residues at the x, y, and z positions of troponin C, the oxygen-containing side chains of amino acids at x, z, and −y play a critical role in Ca$^{2+}$ binding (25, 26). To analyze the function of EF1 or EF2, two point mutations to alanine were introduced at
Structure-Function Analysis of PLCζ

The specific activity of PLCζEF1AA and PLCζEF2AA was 650 and 590 nmol/min/mg at 1 μM [Ca^{2+}], respectively (Table I); the point mutation reduced the PLC activity to about half. The EC_{50} was 66 nM for EF1AA and 64 nM for EF2AA. The values ranged between the EC_{50} of PLCζ and those of PLCζEF1 and PLCζEF1–2 (Table I). The Ca^{2+} dependence of the PLC activity of these mutants was thought to be approximately similar to that of PLCζ (Fig. 2B). Ca^{2+} binding, if any, to the putative Ca^{2+} binding loop in EF1 and EF2 does not play a significant role in the regulation of the Ca^{2+} sensitivity of PLCζ, consistent with the deletion mutant study of EF1 and EF2.

**EF-hand Domains and C2 Domain Are Necessary for Ca^{2+} Oscillation-inducing Activity**—To analyze whether the enzymatic property of PLCζ mutants is correlated to the Ca^{2+} oscillation-inducing activity, PLCζ mutant proteins were expressed in mouse eggs by injection of 50 ng/μL RNA encoding the respective proteins as described previously (1, 2). Fusion of Venus to the N or C terminus of PLCζ did not affect the Ca^{2+} oscillation-inducing activity (2). Changes in [Ca^{2+}], were recorded for 3 h, and fluorescence intensity (F) of the Venus-PLCζ mutant was measured at 3 h after RNA injection to estimate the expressed amount of the protein. Venus-PLCζ induced fertilization-like Ca^{2+} oscillations starting from 25–30 min after RNA injection (Fig. 3A). PLCζEF1AA (Fig. 3B) and PLCζEF2AA also produced repetitive Ca^{2+} spikes in a similar pattern (Table II) and induced egg activation as indicated by the formation of the second polar body and the (female) pronucleus (not shown). In contrast, none of the PLCζEF deletion mutants caused any detectable [Ca^{2+}], rise for 3 h after RNA injection (Fig. 3C and Table II). Both PLCζΔ611–647 and PLCζΔC2 (Fig. 3D) failed to induce any Ca^{2+} spike (Table II).

The expression level of these five inactive mutants was higher than that of PLCζ at 3 h after RNA injection (Table II). However, as shown in Fig. 3E, there was a large (~40-fold) difference between F of Venus-PLCζ at 25–30 min (the time when the first Ca^{2+} spike appeared, arrow 1) and F of Venus-s-PLCζ at 3 h (up to the time Ca^{2+} spike had never occurred, arrow 2). The results characterize an all-or-none mode of the Ca^{2+} oscillation-inducing activity of these mutants in this series of assays. The PLCζ mutants showing the specific PLC activity lower than one-quarter of the activity of PLCζ (Table I) are incapable of inducing Ca^{2+} oscillations (Table II).

**Binding of C2 Domain to Phosphoinositides**—The C2 domain of PLCζ1 (14), cytosolic phospholipase A2 (27), or protein kinase C (28) has been shown to interact with phospholipids and induce the selective translocation and activation of these enzymes. PLCζ is devoid of the PH domain that is found in other PLC isoforms and is known to interact with phosphoinositides. Therefore, interaction of the C2 domain of PLCζ with phosphoinositides was examined by preparing the C2 domain with the neighboring C-terminal region added and with GST in the N terminus (ζC2D). The protein-lipid overlay assay showed that ζC2D bound clearly to PI(3)P and weakly to PI(5)P but not to PI(4)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, and PI(3,4,5)P3 (Fig. 4A). 2xFYVE (18) and 61PHD (16, 29) have been used for specific probes for PI(3)P and PI(4,5)P2, respectively. In this assay, 2xFYVE and PLCΔ1PHD specifically bound to PI(3)P and PI(4,5)P2, respectively (Fig. 4A).

The binding ability of ζC2D to phosphoinositides and other phospholipids was also assayed using liposomes containing the respective lipid at increasing percentages (Fig. 4B). ζC2D-PI(3)P binding was detected in the pellet of liposomes containing 10 or 20% PI(3)P (Fig. 4B, 1), although higher levels of PI(3)P content in liposomes were required than those for 2xFYVE (Fig. 4B, 7). On the other hand, binding of ζC2D to PI(4,5)P2 was not detected (Fig. 4B, 6), although it was clearly
to the PLC...

In the co-existence of micelles containing 40% of PLC... 13%... percentage, and the total lipid content was kept constant by changing the amount of PC and PE. The ratio of PC/PE was 1:1 in experiments 1–5, 1:4 in experiments 6, 8, and 9, and 4:1 in experiment 7. Both supernatant (s) and pellet (p) were subjected to SDS-PAGE and Coomassie Brilliant Blue staining. PA, phosphatidic acid detected for δPHD even at a much lower content of PI(4,5)P (Fig. 4B, 8) but not seen for δPHDK30N/K32N (replacement of both Lys30 and Lys32 with Asn) (Fig. 4B, 9). The liposome assay failed to show the binding of ζC2D to PI(5)P (Fig. 4B, 2). Binding to PI (Fig. 4B, 3), PS (Fig. 4B, 4), or phosphatidic acid (Fig. 4B, 5), negatively charged phospholipids like PI(3)P or PI(5)P, was not detected. Thus, ζC2D has substantial affinity to PI(3)P and slight affinity to PI(5)P but not to other phosphoinositides or acidic lipids.

Effect of Phosphoinositides on PI(4,5)P2-hydrolyzing Activity of PLCζ in Vitro—The effects of PI(3)P and PI(5)P, which bind to the PLCζ C2 domain, on the PI(4,5)P2-hydrolyzing activity of PLCζ were examined. The activity of PLCζ was reduced to 0 or 13% in the co-existence of micelles containing 40 μM PI(4,5)P2 as the substrate and another micelle containing 200 μM PI(3)P or PI(5)P at 1 μM [Ca2+] (Fig. 5A). The activity was not affected by PC, PE, or PS (Fig. 5A). When assayed in the same micelles containing both PI(4,5)P2 and PI(3)P or PI(5)P at the ratio of 1:1, the activity of PLCζ was reduced to 40 or 50%, respectively (Fig. 5B). The value was 20 or 30% when the ratio was 1:5. No reduction of the PLC activity occurred in the presence of 5-fold higher PC or PS. These results suggest that PLCζ as well as ζC2D has a specific affinity to PI(3)P or PI(5)P and binds preferentially to PI(3)P or PI(5)P possibly via the C2 domain, resulting in reduced access of PLCζ to its substrate. The magnitude of this effect was not markedly different at 100 nM, 1 μM, and 10 μM [Ca2+] (Fig. 5C), suggesting that the suppressing effect of the PI(3)P- or PI(5)P-PLCζ interaction on the PI(4,5)P2-hydrolyzing activity operates independently of the Ca2+ -regulated activation process of PLCζ.

DISCUSSION

The present study demonstrated the significant role of the EF-hand and C2 domains of PLCζ in the highly Ca2+-sensitive PI(4,5)P2-hydrolyzing activity in vitro and Ca2+ oscillation-inducing activity in the mouse egg. Our study also demonstrated that the C2 domain of PLCζ interacts with PI(3)P or PI(5)P, and this association may cause an inhibitory regulation of PLC activity.

The Role of EF-hand Domains of PLCζ in Regulation of Its Ca2+ Sensitivity—Deletion of EF1 and EF2 remarkably reduced the specific activity of PLCζ, indicating that the two N-terminal EF-hand domains are obligatory for enzymatically active conformation. PLCζ contains the putative Ca2+-binding loop in EF1 and EF2 in which an Asp residue at the x position and a Gly between the z and y positions are conserved. Point mutation of EF1 or EF2 at the x and z positions, which are critical for Ca2+ binding in EF-hand proteins such as troponin C (25, 26), reduced the PLCζ activity to half, suggesting that...
Ca\(^{2+}\) binding to EF1 and EF2 might play a substantial role in the PLC activity. However, the point mutation in EF1 or EF2 did not cause a marked decrease in the Ca\(^{2+}\) sensitivity of the PI(4,5)P\(_2\)-hydrolyzing activity. The two N-terminal EF-hand domains, therefore, are considered to play a structural role to form the active conformation rather than a Ca\(^{2+}\)-binding site for activation of the catalytic activity. Similarly, in PLC\(\alpha\), deletion of the N-terminal EF-hand domain markedly reduces the PLC activity, but point mutation of EF1 at the x, z, and \(-z\) positions does not affect the PLC activity and the Ca\(^{2+}\) sensitivity (24).

Deletion of the EF1, EF2, and EF3 remarkably reduced the Ca\(^{2+}\) sensitivity of PLC\(\alpha\) as indicated by the 12-fold higher EC\(_{50}\) of (Ca\(^{2+}\))\(^{2}\) (Fig. 2A). The result indicates that EF3 is an important domain necessary for the high Ca\(^{2+}\) sensitivity. EF3 could serve as a high affinity Ca\(^{2+}\)-binding site for activation of PLC\(\alpha\). However, the putative Ca\(^{2+}\)-binding loop is less conserved in EF3 than in EF1 or EF2. In addition, PLC\(\alpha\)EF1–1 still has more than 10-fold higher Ca\(^{2+}\) sensitivity than that of PLC\(\alpha\) (7). We performed direct measurement of Ca\(^{2+}\) binding to PLC\(\alpha\) and its variants by a Ca\(^{2+}\)-overlay assay. The Ca\(^{2+}\) binding activity of PLC\(\alpha\)EF1–3 tended to be reduced compared with full-length PLC\(\alpha\) or PLC\(\alpha\)EF1–2 (data not shown). However, the difference was not statistically significant because of fluctuation in the obtained values. The Ca\(^{2+}\) binding level to the EF-hand region of PLC\(\alpha\) and mutants was much lower than that of calmodulin. Taken together, it seems that the high Ca\(^{2+}\) sensitivity of PLC\(\alpha\) may be derived from the highly coordinated structure of the EF-hand region rather than the primary sequence in the Ca\(^{2+}\)-binding loop. It is considered that the PLC\(\alpha\) is folded at the X-Y region in such a way that the N-terminal domains and C-terminal structure are closely apposed and form the catalytic core composed of the catalytic domain, EF-hand domain, and C2 domain (30–32). Replacement of the residual region except for the EF-hand domains with that of PLC\(\delta\) (PLC\(\alpha\)/EF\(\delta\)) could not conserve the PLC activity. A close apposition of the N-terminal EF-hand domains and a specific C-terminal structure might determine the enzymatic activity.

PLC\(\alpha\)EF1AA and PLC\(\alpha\)EF2AA were capable of inducing Ca\(^{2+}\) oscillations in the mouse egg at about 30 min after RNA injection when expression of the protein was still at a low level (Fig. 3). In contrast, PLC\(\alpha\)EF1, PLC\(\alpha\)EF1–2, and PLC\(\alpha\)EF1–3 were incapable of inducing any Ca\(^{2+}\) spike within 3 h even if expression of the protein reached a much higher level than the level of wild type PLC\(\alpha\) critical for initiation of Ca\(^{2+}\) oscillations. In our experimental condition, PLC\(\alpha\) mutants showed an all-or-none mode of Ca\(^{2+}\) oscillation-inducing ability. Furthermore, all of the PLC\(\alpha\) mutants that induced Ca\(^{2+}\) spikes caused formation of a second polar body and pronucleus. Thus, the N-terminal half of the EF-hand structure is critical for the physiological function of PLC\(\alpha\). The Ca\(^{2+}\) oscillation-inducing ability was lost by deletion of EF1 and EF2 but not by point mutation in the putative Ca\(^{2+}\)-binding loop of EF1 and EF2. The ability may be correlated to the specific activity and overall structure of the mutant.

The Role of C2 Domain of PLC\(\alpha\)—The C2 domain, including 37 external amino acids in the C terminus of PLC\(\alpha\), is necessary for the catalytic activity in vitro and the Ca\(^{2+}\) oscillation-inducing activity in the egg. This region could be essential for the positioning of the enzyme in an active conformation or for the membrane targeting of the enzyme. The C2 domain is known to play a significant role in the Ca\(^{2+}\)-dependent subcellular membrane targeting of several lipid-metabolizing enzymes such as PLC\(\delta\) and cytosolic phospholipase A\(_2\) (27, 30–33). In our previous study (2), Venus-PLC\(\delta\) expressed in the mouse egg was located on the surface in association with the plasma membrane, but this was not detected for Venus-PLC\(\alpha\). Therefore, targeting of PLC\(\alpha\) to phospholipid is unknown. Screening of C2 domain-interacting phosphoinositides revealed that the C2 domain binds to PI(3)P and, to a lesser extent, to PI(5)P. To our knowledge, this is the first example of the binding of the C2 domain to PI(3)P, although the C2 domain of synaptotagmin or JFC1 has been shown to interact mainly with PI(3,4,5)P\(_3\) and to a lesser extent, with other 3'-phosphoinositides (34, 35).

The presence of PI(3)P or PI(5)P remarkably reduced the PLC\(\alpha\)-mediated hydrolysis of PI(4,5)P\(_2\) in micelles. It is likely that the accession of PLC\(\alpha\) to its substrate is perturbed simply because PLC\(\alpha\) binds preferentially to PI(3)P or PI(5)P. Alternatively, the association of the C2 domain with PI(3)P or PI(5)P could interfere with the suitable positioning of PLC\(\alpha\) on the lipid membrane to hydrolyze the substrate. This perturbation of the PLC\(\alpha\) activity was Ca\(^{2+}\)-independent, suggesting that the binding of PI(3)P or PI(5)P to PLC\(\alpha\) occurs irrespective of changes in [Ca\(^{2+}\)]. The physiological significance of the suppressing effect of PI(3)P or PI(5)P on PLC\(\alpha\) remains to be elucidated.

Mammalian PLCs are activated by agonist-induced anchoring to the membrane (11). The PH and C2 domains of PLC\(\beta\) bind to G\(\beta\)Y and Goq, in the plasma membrane, respectively, upon stimulation of the G protein-coupled receptor (12, 13). The PH domain of PLC\(\gamma\) binds to PI(3,4,5)P\(_3\) with a high affinity upon agonist stimulation (36, 37). PLC\(\gamma\) is a Ras effector and localized to the plasma membrane or the perinuclear region upon growth factor stimulation when coexpressed with activated Ha-Ras or Rap1 mutant (38–40). PLC\(\delta\) also associates tightly with PI(4,5)P\(_2\) at the PH domain and is recruited to the plasma membrane (41). In the case of PLC\(\gamma\), activation of the enzyme accompanied by membrane association is not found at present. What is known is that PLC\(\gamma\) is Ca\(^{2+}\)-sensitive in the PLC activity that it could be active at the resting state of the cell and cause repetitive [Ca\(^{2+}\)] \textsuperscript{r} rises continuously. This situation seems to be unfavorable for resting cells. The interaction between the C2 domain and PI(3)P or PI(5)P might play a role in the inhibitory regulation of the enzymatic activity of PLC\(\alpha\), for example in the sperm before fertilization. Further studies on other targets of the C2 domain of PLC\(\alpha\) together with the Ca\(^{2+}\) dependence of the binding are required to reveal the physiological significance of the C2 domain.

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