PLC γ 2 activates CREB-dependent transcription in PC12 cells through phosphorylation of CREB at Serine 133

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Abstract

The cAMP and Ca²⁺ signaling pathways activate the transcription factor CREB through its phosphorylation at Serine 133. Activation of CREB is involved in the regulation of various biological phenomena. To understand further the mechanisms of the regulation of CREB activity in response to activation of the cAMP and Ca²⁺ signaling pathways, we examined the roles of PLC γ s in CREB activation in PC12 cells. siRNA-mediated reduction of the expression of PLC γ 2, but not PLC γ 1, inhibited both the phosphorylation of CREB at S133 and the activation of CREB-dependent transcription following treatment of cells with forskolin or ionomycin, which increases the intracellular concentrations of cAMP or Ca²⁺, respectively. Importantly, the siRNA targeting PLC γ 2 completely abolished CREB activation by Ca²⁺ signaling but not by cAMP signaling. These results suggest that PLC γ 2 functions as an essential signal transducer leading to CREB activation in response to activation of the Ca²⁺ signaling pathway and that the cAMP signaling pathway might activate CREB through phosphorylation of CREB by PKA and another signaling pathway mediated by PLC γ 2.

Introduction

The transcription factor CREB has been shown to regulate various biological phenomena, including memory consolidation, circadian rhythm, spermatogenesis, gluconeogenesis and cell survival (Waeber et al. 1991; Ginty et al. 1993; Ghosh et al. 1994; Sassone-Corsi 1995; Silva et al. 1998; Herzig et al. 2001; Mayr and Montminy 2001a; Kida et al. 2002). Furthermore, abundant evidence suggests that abnormalities of CREB signaling pathway are involved in the etiology of several diseases, including Rubinstein–Taybi syn-

drome, Alzheimer's disease and Huntington's disease (Petrij et al. 1995; Marambaud et al. 2003; Obrietan and Hoyt 2004). PKA and CaMKs phosphorylate CREB on Serine 133 (S133) in response to an increase in the intracellular concentrations of cAMP and Ca²⁺ (Gonzalez and Montminy 1989; Sheng et al. 1990; Sheng et al. 1991; Brindle and Montminy 1992; Bito et al. 1996; Schonwasser et al. 1998; Shaywitz and Greenberg 1999; Mayr and Montminy 2001a). Phosphorylated CREB interacts with the transcriptional coactivator CBP and activates transcription through the cAMP responsive element

(CRE) within the promoters of target genes (Chrivia et al. 1993; Kwok et al. 1994; Brindle et al. 1995; Mayr et al. 2001b). Recent studies have shown that CREB can be phosphorylated on S133 by multiple kinases, such as PKC, RSKs and p70 S6 kinase (Gonzalez and Montminy 1989; de Groot et al. 1993; Xie and Rothstein 1995; Xing et al. 1996; Sung et al. 2001).

Phosphoinositide-specific phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DG) in response to various extracellular signals (Majerus et al. 1986; Rhee and Choi 1992; Berridge 1993; Rebecchi and Pentyla 2000). IP₃ and DG act as intracellular second messengers, leading to rapid Ca²⁺ release from the endoplasmic reticulum (ER) and activation of protein kinase C (PKC), respectively (Nishizuka 1988; Rhee and Choi 1992; Berridge 1993; Rebecchi and Pentyla 2000). The PLC isozymes have been divided into three families: PLC- β , PLC- γ and PLC- δ . PLC- β is activated by G protein-activated receptors (GPCR), whereas PLC- γ isoforms (PLC- γ 1 and PLC- γ 2) are activated by tyrosine kinase receptors and nonreceptor tyrosine kinases (Rhee and Choi 1992; Berridge 1993; Liao et al. 1993; Park et al. 1993; Rebecchi and Pentyla 2000). Importantly, PLC γ s also potentiate Ca²⁺ responses in a lipase-independent manner (Patterson et al. 2002; Nishida et al. 2003). Additionally, recent studies have demonstrated that PLC γ s function as downstream signal transducers of PKA-induced signaling pathways (Yu et al. 1996).

Previous studies have suggested that PLC γ s play important roles in CREB activation (Minichiello et al. 2002). Indeed, PLC- γ 1 mediates the phosphorylation of CREB at S133 in response to the activation of tyrosine kinase receptors (trkB) in hippocampal primary neurons (Minichiello et al. 2002). In this report, to understand the mechanisms of CREB activation by cAMP and Ca²⁺ signaling pathways, we examined the roles of PLC- γ s in cAMP- and Ca²⁺-induced CREB activation in PC12 cells using siRNAs targeting PLC γ 1 and PLC γ 2. We found that reduced expression of PLC- γ 2, but not PLC- γ 1, inhibited both the phosphorylation of CREB at S133 and CREB-dependent transcription in response to activation of cAMP or Ca²⁺ signals. These results indicate a critical role for PLC- γ 2 in CREB activation in PC12 cells.

Materials and methods

Plasmid construction

Full-length cDNAs encoding murine PLC γ 1 were obtained by reverse transcription (RT)-PCR using mouse brain mRNA as a template. The following oligonucleotides were used as primers for PCR: PLC γ 1 forward primer, GGGGGATCCACGC-GTATGGAATTCCGCTTGAAGAC; PLC γ 1 reverse primer, GGGAAGCTTACGCGTCTAGAGGCGGTTGTCTCCAT. The resulting PCR fragment encoding PLC γ 1 was subcloned into the *Bam*HI-*Hind*III sites of pCMV-myc (pmyc; CLONTECH, NJ), generating pmyc-PLC γ 1. To generate GAL4-dependent luciferase reporter plasmid (pGAL4RE-Luc), a 305 bp fragment containing five copies of the GAL4 response element (GAL4RE) and the SV40 minimal promoter was amplified by PCR using pG5CAT vector (CLONTECH, CA) as a template and the following primers: forward primer, GGGCCATGGACTAGTCTGCAGCTATGACCATGATTACGCGC, and reverse primer, GGGACGCGTAGATCTTTTGA GCTTCCTTAGCTCCTG. The resulting PCR fragment was subcloned into the *Mlu*I-*Nco*I sites of pGL3 (Promega, WI). To generate a plasmid expressing GAL4-CREB (pGAL4-CREB), a full-length cDNA encoding rat CREB was amplified by PCR using RSV-CREB as a template, as previously described (Kida et al. 2002), and the resulting PCR fragment encoding CREB was subcloned into the *Hind*III-*Bam*HI sites of pM (CLONTECH, CA). To generate the β -galactosidase expression vector (pRc/RSV- β -gal), a *Bam*HI-*Hind*III fragment encoding β -galactosidase from pCH110 (Amersham, NJ) was subcloned into the corresponding restriction sites in pRc/RSV (Invitrogen, CA). The CRE-dependent luciferase reporter plasmid, pCRE-Luc, was purchased from STRATAGENE (CA).

Cell culture and transient transfection

PC12 cells were maintained in Dulbecco's modified Eagle's (DMEM) medium (NISSUI, Japan) supplemented with 10% fetal bovine serum (JRH BIOSCIENCE, KS), 5% horse serum (JRH BIOSCIENCE, KS), penicillin (100 U/ml), and streptomycin (100 μ g/ml) at 37 °C in 5% CO₂. Cells

were grown in 24-well plates and transiently transfected using the lipofectAMINE2000 reagent (Invitrogen, CA) according to the manufacture's instructions. Empty vector (pcDNA3) was used to standardize the total amount of DNA transfected (250 ng). Solutions of forskolin and ionomycin (Wako, Japan) were prepared in DMSO at 1 or 0.5 mM, respectively.

RNAi

To reduce the expression of target genes using siRNA, we targeted sequences in the middle of the open reading frames of GFP (AAGCTGACCCTGAAGTTCATC, nucleotides 124 to 144), PLC γ 1 (AAACAACCGGCTCTTCGTCTT, nucleotides 2670–2690) and PLC γ 2 (AAGAATCCAAGGACTTTGAG, nucleotides 229–249). We determined that these sequences are unique to the corresponding genes by a BLAST search of the GenBankTM database. The target sequence for PLC γ 1 was determined in a previous report (Patterson et al. 2002). We constructed pSUPER-GFP, pSUPER-PLC γ 1 and pSUPER-PLC γ 2 using GeneTailorTM Site-Directed Mutagenesis System (Invitrogen, CA). We performed PCR using pSUPER (Brummelkamp et al. 2002) as a template and the following primers: pSUPER-GFP forward primer, GAGAGATGAACTTCAGGGTCAGCTTTTTGGAAAAGCTTATCGATACCG, pSUPER-GFP reverse primer, TTGAAGATGAAGTTCAGGGTCAGCGGGATCTGTGGTCTCATACAG, pSUPER-PLC γ 1 forward primer, GAGAAAGACGAAGAGCCGGTTGTTTTTTGGAAAAGCTTATCGATACCG, pSUPER-PLC γ 1 reverse primer, TTGAAAAGACGAAGAGCCGGTTGTGGGATCTGTGGTCTCATACAG, pSUPER-PLC γ 2 forward primer, GAGACTCAAAGTCCTTGGAGTTCTTTTTGGAAAAGCTTATCGATACCG, pSUPER-PLC γ 2 reverse primer, TTGAACTCAAAGTCCTTGGAGTTCGGGATCTGTGGTCTCATACAG. The resultant PCR fragment was self-ligated to generate a pSUPER vector containing the target sequence of GFP, PLC γ 1 or PLC γ 2. As described above, pSUPER vectors were transiently transfected into cells that were analyzed 72 h later.

Western blotting

Seventy-two hours after the transfection, cells were treated with forskolin (1 μ M) or ionomycin (0.5 μ M). 10 min later, whole cell extracts were prepared in SDS sample buffer (50 mM Tris-HCl (pH 6.8), 2.4% (w/v) SDS, 14.8% (v/v) 2-mercapt ethanol, 1% (w/v) Bromophenol Blue). Proteins were separated by electrophoresis through 5 or 10% SDS-polyacrylamide gels to detect CREB or PLC γ s, respectively. Western blotting using anti-phospho-CREB (Ser133) antibody, anti-CREB antibody (1:1000; Cell Signaling, MA), anti-PLC γ 2 antibody (1:1000; Santa Cruz, CA), anti- α -tubulin antibody (1:1000; Santa Cruz, CA) or anti-myc antibody (1:1000; CLONTECH, CA) were performed as previously described (Hosoda et al. 2004). Following signal development with Amersham's ECL plus systems (Amersham, NJ), membranes were analyzed by the Lumi-imagerTM chemiluminescence detection system (Roche Diagnostics, Basel). The amount of total protein or CREB in each lane was confirmed to be comparable by reprobing with anti- α -tubulin or anti-CREB antibody, respectively.

Reporter assay

Seventy-two hours after transfection, cells were treated with forskolin (1 μ M) or ionomycin (0.5 μ M) 1.5 or 3.0 h prior to the reporter assay, respectively. Luciferase and β -galactosidase activities were then measured as previously described (Hosoda et al. 2004). pRc/RSV- β -gal (100 ng) was co-transfected as a control for transfection efficiency. Luciferase activity was normalized to β -galactosidase activity. All reporter assays were performed in triplicate in three independent experiments. Each value represents the mean $\pm$ SD.

Results

siPLC γ 1 and siPLC γ 2 reduce PLC γ 1 or PLC γ 2 expression, respectively, in PC12 cells

To examine the role of PLC γ in the activation of CREB *in vivo*, we employed RNA interference

(RNAi) to reduce the expression of endogenous PLC γ 1 and 2. To do so, we used vectors (pSUPER-PLC γ 1 and pSUPER-PLC γ 2) that expressed short hairpin RNAs that targeted PLC γ 1 or PLC γ 2 (siPLC γ 1 or siPLC γ 2, respectively) from the human H1 promoter. siPLC γ 1 and siPLC γ 2 targeted nucleotides 2670–2690 or 229–249 3' to the initiation codon of the corresponding mRNA, respectively. We characterized the ability of siPLC γ 1 and siPLC γ 2 to reduce the expression of PLC γ 1 and 2, using siGFP as a negative control. Importantly, a previous report has shown that both PLC γ 1 and PLC γ 2 are expressed and functional in PC12 cells (Patterson et al. 2002). We first transiently transfected pmc-PLC γ 1 with pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2 into PC12 cells and examined the expression level of exogenous PLC γ 1 (myc-PLC γ 1) by Western blot analysis. As shown in Figure 1a, Western analyses using anti-myc antibody revealed the nearly complete suppression of myc-PLC γ 1 expression following transfection of the cells with pSUPER-PLC γ 1, but not with pSUPER, pSUPER-GFP or pSUPER-PLC γ 2. We next transiently transfected pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2 into PC12 cells and examined the expression of

endogenous PLC γ 2 using anti-PLC γ 2 antibody. As shown in Figure 1b, Western analyses also revealed the nearly complete suppression of PLC γ 2 expression following transfection of the cells with pSUPER-PLC γ 2, but not with pSUPER, pSUPER-GFP or pSUPER-PLC γ 1. Importantly, probing of the same Western blots with control anti- α -tubulin antibody revealed the presence of comparable amounts of extracted proteins in each lane (Figure 1a and b). These data indicate that siPLC γ 1 and siPLC γ 2 reduce the expression of PLC γ 1 or PLC γ 2, respectively, in PC12 cells.

PLC γ 2 but not PLC γ 1 is required for phosphorylation of CREB at S133

We next examined the effects of knockdown of PLC γ 1 or PLC γ 2 expression by RNAi on the phosphorylation of CREB at S133 following the activation of cAMP or Ca²⁺ signaling pathways. We transiently transfected pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2 into PC12 cells and examined the levels of total CREB and S133-phosphorylated CREB using anti-CREB or phospho-CREB antibody, respectively. Treatment of the non-transfected cells with

Figure 1. siPLC γ 1 and siPLC γ 2 reduced PLC γ 1 or PLC γ 2 expression, respectively, in PC12 cells. (a) Cells were transiently transfected with either pmc or pmc-PLC γ 1 (10 ng), in the absence or presence of the individual plasmids pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2 (5 ng), as indicated. (b) Cells were transiently transfected with or without pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2 (5 ng), as indicated. Total extracts from mouse hippocampus (25 μ g) were loaded as a positive control to identify the band corresponding to the PLC γ 2 protein (lane1). The arrow (upper band) indicates the location of the PLC γ 2 protein. The lower band indicates the non-specific binding of anti-PLC γ 2 bodies. Western blot analysis was performed using anti-myc or anti-PLC γ 2 antibodies, as indicated. The amount of protein in the lanes was confirmed to be comparable by reprobing with anti- α -tubulin antibody. Western blot assays were performed in three independent experiments.

forskolin, which stimulates the cAMP signaling pathway, led to an increase in the level of phospho-CREB without affecting the level of total CREB (Figure 2a). Transfection of cells with pSUPER, pSUPER-GFP or pSUPER-PLC γ 1 had no effect upon the phosphorylation of CREB mediated by forskolin treatment. In contrast, the transfection with pSUPER-PLC γ 2 inhibited forskolin-induced phosphorylation of CREB without affecting the level of total CREB. Figure 2b represents the relative CREB activation (ratio of phospho-CREB to total CREB), and confirms the results observed in Figure 2a. These results suggest that PLC γ 2 mediates the phosphorylation of CREB at S133 in response to activation of the cAMP signaling pathway.

We next examined the effects of knockdown of PLC γ s on the phosphorylation of CREB mediated by activation of the Ca $^{2+}$ signaling pathway. Treatment of non-transfected cells with ionomycin, which increases the intracellular Ca $^{2+}$ concentration, led to an increase in CREB phosphorylation

(Figure 2c, d). Interestingly, transfection with pSUPER-PLC γ 2 but not pSUPER, pSUPER-GFP or pSUPER-PLC γ 1, completely abolished ionomycin-induced phosphorylation of CREB at S133 (Figure 2c and d). These results indicate that PLC γ 2 is essential for the ionomycin-induced phosphorylation of CREB at S133.

PLC γ 2, but not PLC γ 1, is required for the activation of CRE-mediated transcription

Previous reports have indicated that the activation of cAMP or Ca $^{2+}$ signaling pathways induces CRE-mediated transcription in PC12 cells (Sheng et al. 1991; Mark et al. 1995; Thompson et al. 1995; Impey et al. 1998). To probe the role of PLC γ s in CRE-mediated transcription induced by activation of the cAMP and Ca $^{2+}$ signaling pathways, we examined the effects of knockdown of the expression of PLC γ 1 and γ 2 in PC12 cells on this process. Cells were transiently transfected with

Figure 2. PLC γ 2 but not PLC γ 1 is required for phosphorylation of CREB at S133 following activation of cAMP and Ca $^{2+}$ signaling pathway in PC12 cells. Cells were transiently transfected with the plasmids pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2 (5 ng), as indicated. Western blot analysis was performed using anti-phospho-CREB (Ser133) antibody. The amount of total CREB in each lane was confirmed to be comparable by reprobing with anti-CREB antibody. Cells were treated with forskolin (a, b) or ionomycin (c, d), as indicated. (b, d) The bar graphs show the ratio of phosphorylated CREB to total CREB, as estimated by densitometry from the Western blot of (a) and (c), respectively. Western blot assays were performed in four independent experiments. Each value represents the mean $\pm$ SD.

a CRE-luciferase reporter construct in the absence or presence of individual plasmids pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2. Consistent with previous reports, the treatment of non-transfected cells with forskolin or ionomycin led to the activation of the CRE-dependent promoter (Figure 3a, b). Cotransfection with pSUPER-PLC γ 2, but not pSUPER, pSUPER-GFP or pSUPER-PLC γ 1, reduced the activation of CRE-mediated transcription induced by treatment of the cells with forskolin or ionomycin. These results indicate that PLC γ 2, but not PLC γ 1, is necessary for maximal activation of CRE-mediated transcription induced by cAMP or Ca²⁺ signaling pathways in PC12 cells.

PLC γ 2 but not PLC γ 1 is required for the activation of CREB-dependent transcription

PC12 cells express other members of the CREB/ATF family in addition to CREB (D'Arcangelo et al. 1996; Ryu et al. 2002; Suzuki et al. 2002). These factors bind to CRE and regulate CRE-mediated transcription in response to the modu-

lation of various signal transduction cascades (Brindle and Montminy 1992; Livingstone et al. 1995; Chen et al. 1996; Shimomura et al. 1996; Yoshida et al. 2001; Yang et al. 2004). To further understand the roles of PLC γ s in the activation of CREB, we created a fusion of CREB with the GAL4-DNA binding domain (GAL4-CREB). A plasmid expressing GAL4-CREB was transiently cotransfected with the GAL4 reporter construct, in the presence or absence of individual plasmids pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2. Treatment of cells with forskolin or ionomycin led to CREB-dependent activation of transcription from the GAL4-dependent promoter (Figure 4a, b). Consistent with the results of Figure 3a, cotransfection with pSUPER-PLC γ 2, but not pSUPER, pSUPER-GFP or pSUPER-PLC γ 1, inhibited CREB-dependent activation of the GAL4 promoter mediated by forskolin, indicating that PLC γ 2 is required for maximal activation of CREB-dependent transcription induced by cAMP signaling in PC12 cells. Interestingly, siPLC γ 2 completely suppressed CREB-dependent transcription induced by treatment of cells with ionomycin, suggesting that PLC γ 2 plays an

Figure 3. PLC γ 2 but not PLC γ 1 is required for the activation of CRE-mediated transcription following the activation of cAMP or Ca²⁺ signaling pathways in PC12 cells. (a, b) Cells were transiently transfected with pCRE-Luc (100 ng), in the absence or presence of the individual plasmids pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2 (5 ng), as indicated. Cells were treated with forskolin (a) or ionomycin (b), as indicated.

Figure 4. PLC γ 2 but not PLC γ 1 is required for the activation of CREB-dependent transcription following the activation of cAMP or Ca²⁺ signaling pathways in PC12 cells. (a, b) Cells were transiently transfected with pGAL4RE-Luc (100 ng), together with either pM or pGAL4-CREB (45 ng) in the absence or presence of the individual plasmids pSUPER, pSUPER-GFP, pSUPER-PLC γ 1 or pSUPER-PLC γ 2 (5 ng), as indicated. Cells were treated with forskolin (a) or ionomycin (b), as indicated.

essential role in the activation of CREB-dependent transcription in response to Ca²⁺ signaling (Figure 4b). These results suggest that PLC γ 2, but not PLC γ 1, mediates CREB activation in response to cAMP and Ca²⁺ signaling in PC12 cells.

Discussion

Previous reports have suggested that PLC γ s play important roles in the activation of CREB (Minichiello et al. 2002). In this report, we have shown that siRNA-mediated knockdown of the expression of PLC γ 2, but not PLC γ 1, inhibited both the phosphorylation of CREB and the activation of CREB-dependent transcription. The phosphorylation of CREB and the induction of CREB-dependent transcription are both mediated by the activation of cAMP or Ca²⁺ signaling pathways in PC12 cells. These results suggest that PLC γ 2 activates CREB through the phosphorylation of S133

in response to an increase in intracellular concentrations of cAMP or Ca²⁺ in PC12 cells. Interestingly, siPLC γ 2 completely abolished the activation of CREB induced by treatment of cells with ionomycin, indicating that PLC γ 2 is essential for the activation of CREB by Ca²⁺ signaling in PC12 cells. A previous report has demonstrated that PLC γ 2 amplifies the Ca²⁺ signaling (Patterson et al. 2002; Nishida et al. 2003). Additionally, numerous studies have indicated that an increase in the intracellular Ca²⁺ concentration leads to activation of CaMKs and PKCs (Nishizuka 1988; Schulman et al. 1992; Hook and Means 2001; Elgersma et al. 2004; Griffith 2004). Several CaMKs, including CaMKIV and CaMKII, are known to function as CREB kinases, whereas PKCs activate MAPK, whose activation in turn leads to the activation of CREB kinases (Sheng et al. 1991; Brindle and Montminy 1992; Sassone-Corsi 1995; Xie and Rothstein 1995; Bito et al. 1996; Schonwasser et al. 1998; Shaywitz and Greenberg 1999). These previ-

ous findings suggest that PLC γ 2 potentiates the Ca²⁺ response, resulting in an increase in the phosphorylation of CREB by downstream CREB kinases in the Ca²⁺ signaling pathway.

siPLC γ 2 did not completely abolish the activation of CREB mediated by cAMP signaling. cAMP directly activates PKA, which is known to function as a CREB kinase (Gonzalez and Montminy 1989; Brindle and Montminy 1992; Sassone-Corsi 1995). Importantly, a recent study has shown that PKA stimulates PLC γ 2 activity, suggesting that PLC γ 2 functions as a downstream signal transducer of the cAMP signaling pathway (Yu et al. 1996). Therefore, PKA is thought to activate CREB through both direct and indirect mechanisms: directly via phosphorylation of CREB by PKA itself and indirectly via the activation of PLC γ 2.

Activation of the lipase activity of PLC γ 2 leads to an increase in the IP₃ concentration, leading subsequently to an increase in the Ca²⁺ concentration (Berridge 1993; Carpenter and Ji 1999; Patterson et al. 2002; Nishida et al. 2003). However, the treatment of PC12 cells with forskolin increases the intracellular concentration of cAMP but not Ca²⁺ (data not shown), suggesting that PLC γ 2 mediates the phosphorylation of CREB in a Ca²⁺-independent manner following the activation of the cAMP signaling pathway. Importantly, PLC γ s contain functional domains that interact with other signal transducers (Berridge 1993; Carpenter and Ji 1999; Patterson et al. 2002). Indeed, previous studies have suggested that PLC γ s function in both a lipase-dependent and lipase-independent manner (Smith et al. 1994; Patterson et al. 2002; Nishida et al. 2003). Therefore, in response to the activation of cAMP signaling, PLC γ 2 might lead to the phosphorylation of CREB at S133 in a lipase-independent manner, through the activation of signaling pathways by distinct mechanisms such as the modulation of protein–protein interaction.

A previous report has shown that both PLC γ 1 and PLC γ 2 are expressed in PC12 cells and function as modulators of the Ca²⁺ response (Patterson et al. 2002). In addition, numerous studies have demonstrated that PLC γ 2 exhibits comparable function as a signal transducer with PLC γ 1 (Emori et al. 1989; Sultzman et al. 1991; Rhee and Choi 1992; Carpenter and Ji 1999; Rebecchi and Pentylala 2000; Patterson et al. 2002). However, we have here demonstrated distinct roles for PLC γ 2

and PLC γ 1 in the activation of CREB in PC12 cells. Interestingly, several reports have shown that PLC γ 1 and PLC γ 2 differ in subcellular localization in RBL-2H3 tumor mast cells and that collagen signal transduction stimulates tyrosine phosphorylation of PLC γ 2, but not PLC γ 1, in human platelets (Blake et al. 1994; Barker et al. 1998). These previous findings suggest that PLC γ 2 is activated by cAMP or Ca²⁺ signaling or activates its downstream signaling cascades, in PC12 cells in a different manner from PLC γ 1.

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