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Calmodulin potentiates Gβγ activation of phospholipase C-β3

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ABSTRACT

Phospholipase C-β (PLC-β) isozymes (EC 3.1.4.11) hydrolyze the membrane phospholipid phosphatidylinositol-4,5-bisphosphate to generate intracellular second messenger signaling molecules inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) in response to receptor activation and other cellular stimuli. PLCβ1 and PLCβ3 isozymes were previously demonstrated to bind the calcium-sensitive molecule calmodulin [McCullar JS, Larsen SA, Millimaki RA, Filtz TM. Calmodulin is a phospholipase C-{beta} interacting protein. J Biol Chem 2003;278(36):33708–13]. We have now shown through fluorescence anisotropy that calmodulin/PLCβ3 affinities increase with increasing calcium in a physiologically relevant concentration range. The bimolecular affinity constants for calmodulin interaction with PLCβ1 or PLCβ3 were estimated as 260 and 200 nM, respectively, from fluorescence anisotropy data. There was no effect of calmodulin on basal or Gαq-stimulated catalytic activity for either isozyme. However, the interaction between calmodulin and PLCβ3 leads to potentiation of activation by the G-protein βγ dimer in an in vitro assay. 1321N1 cells treated with calmodulin inhibitors concurrent with and post-stimulation of muscarinic receptors significantly reduced [3*H*]PIP hydrolysis. Together these data are suggestive of cooperative role for calmodulin in the G-protein βγ dimer-stimulated activity of PLCβ3.

1. Introduction

Phosphatidylinositol phospholipid-specific phospholipase C (PLC)[1] is a key intracellular signaling molecule that catalyzes the hydrolysis of PIP2 into IP3, a regulator of cytosolic calcium levels, and diacylglycerol, a well-characterized activator of protein kinase C [2]. The identified PLC isozymes have been classified by sequence homology into six families, β, γ, δ, ε, ζ, and η [3–5]. Each family has unique mechanisms of activation and regulation. The activity of the PLCβ family of isozymes is stimulated by membrane G-protein coupled receptors (GPCR) through heterotrimeric guanine nucleotide binding (G) proteins, which are composed of a GTP/GDP-binding α subunit and a βγ dimer.

There are four identified and characterized isoforms of PLCβ numbered 1–4. PLCβ2 and PLCβ4 have limited tissue

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Abbreviations: PLC, phosphatidylinositol phospholipid-specific phospholipase C; PE, phosphatidylethanolamine; PS, phosphatidylserine; PIP 16:0, synthetic phosphatidylinositol-4-phosphate with symmetric 16:0 saturated fatty acyl chains; PIP, phosphatidylinositol-4-phosphate; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; SDS-PAGE, sodium dodecyl phosphate polyacrylamide gel electrophoresis; W-13, N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; BSA, bovine serum albumin

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distributions, whereas PLCβ1 and PLCβ3 are nearly ubiquitous in human tissues; PLCβ1 being dominant in brain and PLCβ3 being dominant in heart and smooth muscle [2]. PLCβ1 and PLCβ3 are both activated by calcium and Gαq [6–8], but the PLCβ3 isoform is additionally sensitive to activation by Gβγ [9]. The mechanisms of regulation of PLCβ3 are incompletely understood despite the enzyme's importance in a variety of cellular processes [10–14]. Aberrancies in expression of PLCβ3 can lead to tumorigenesis [13,15,16], and PLCβ3 knockout mice show changes in μ-opioid response [12] or early embryonic lethality [17].

Calmodulin is an established, ubiquitous and abundant calcium-sensitive regulatory protein associated with a vast diversity of cellular functions including signal transduction [18,19]. Calmodulin binds four molecules of calcium cooperatively, and undergoes a significant conformational change upon calcium binding that is important for its many calcium-sensitive regulatory functions. Calmodulin binding sites are nearly as diverse as the number of calmodulin binding proteins, but generally are amphipathic α-helices, typically 20–35 amino acids long, with basic and hydrophobic residues sorting to opposite sides on an α-helical projection. The list of various mechanisms by which calmodulin regulates proteins is growing nearly as fast as the list of calmodulin binding proteins [18,20].

We previously reported that calmodulin directly interacts with PLCβ1 and PLCβ3, and that calmodulin inhibitors attenuate inositol phosphate (IP) accumulation in whole cells [1]. To further understand this interaction, we sought to determine the direct effect of calmodulin on PLC-β activity in vitro and to determine the binding affinity and calcium dependence of the PLCβ/calmodulin interaction.

2. Materials and methods

2.1. Reagents

PLCβ-selective polyclonal rabbit antisera and alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). SuperSignal™ chemiluminescent substrate was purchased from Pierce (Rockford, IL). Fatty acid-free bovine serum albumin (FAF-BSA), W-13 and carbamylcholine chloride (carbachol) were obtained from Calbiochem (San Diego, California). PIP Strip® phospholipid blots were obtained from Echelon Biosciences (Salt Lake City, UT). Synthetic and purified bovine brain PIP, PIP2, PS and PE were purchased from Avanti Polar Lipids (Alabaster, AL). Calmodulin was purified in the presence of calcium from bovine brain as previously described [21]. PLCβ1, PLCβ3, Gαq, Gβγ, and [3*H*]PIP2 were purified as previously described [22–24]. Alexa Fluor® 488 was acquired from Molecular Probes (Eugene, OR) and was conjugated to calmodulin according to the manufacturer's recommended procedure.

2.2. Fluorescence anisotropy

Fluorescence anisotropy and fluorescence emission spectra were recorded with a Perkin-Elmer LS-50 Luminescence Spectrophotometer maintaining constant temperature (20 °C). Measurements of fluorescence anisotropy for PLCβ1 and PLCβ3 with Alexa-calmodulin in buffer containing 0.1 M KCl and 30 mM MOPS, pH 7.2, were performed at an excitation wavelength of 490 nm (band-pass 5 mm) using a linear polarizer, and the fluorescence emission intensities at 525 nm (band-pass 20 mm) were monitored through a second linear polarizer. Anisotropy, *r*, was calculated according to the equation, $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} and I_{VH} are the intensity of vertically or horizontally polarized emitted light, respectively, obtained with vertically polarized exciting light. Four readings were taken for each measurement and averages calculated and recorded. The value of *G*, 0.982, which corrects for unequal transmission of vertically and horizontally polarized emitted light, was obtained from tables prepared by the Anderson laboratory specific for their instrument (personal communication).

2.3. Reconstitution assay

The catalytic activity of PLCβ1 and PLCβ3 was quantitated using [3*H*]PIP2 substrate as described previously [25]. Briefly, 45 ng of purified PLCβ3 or 15 ng of PLCβ1 in 20 μl of 50 mM HEPES pH 7.2, 3 mM EGTA, 80 mM KCl (Buffer 1) and 1 mg/ml fatty acid-free BSA was added to 20 μl of Buffer 1 containing 15 μM PIP2, 135 μM phosphatidylethanolamine and 6–10,000 cpm [3*H*]PIP2. Ten microliters of 50 mM HEPES pH 7.2, 1 mM EDTA, 3 mM EGTA, 5 mM $MgCl_2$, 100 mM NaCl, 1% cholate (Buffer 2) was added to each reaction containing either 30 ng Gαq or 50 ng Gβγ for G-protein-stimulated activity of PLC. Basal PLC activity was quantitated with the same buffer with no added G-protein. Buffer 1 with 9 mM $CaCl_2$ (10 μl) was added to yield a final assay volume of 60 μl. The reaction proceeded at 30 °C for 10 min and was terminated by the addition of 375 μl of ice cold mixture of chloroform, methanol and hydrochloric acid in a ratio of 80:40:1, followed by addition of 125 μl chloroform and 125 μl 0.1N hydrochloric acid with vigorous mixing. The aqueous and organic phases were separated by centrifugation for 5 min at 2000 × *g*. [3*H*]IP3 product release was quantitated by scintillation counting of 400 μl of the upper phase. Triplicate samples were run in three separate experiments.

2.4. Protein lipid overlay assays

Protein lipid overlay assays were conducted using either commercial phospholipid membrane arrays containing synthetic phospholipids (PIP-Strips®) or nitrocellulose membranes spotted in our laboratory with mammalian-derived brain phospholipids. Nitrocellulose membrane phospholipid blots were prepared by spotting with chloroform containing 0, 50 or 100 pmol of phosphatidylserine, phosphatidylethanolamine or PIP2 in 1 μl and allowed to dry. Both purchased and nitrocellulose membranes were blocked with 3% (w/v) fatty acid-free BSA in TBST buffer (150 mM NaCl, 10 mM Tris·HCl, pH 8.0, and 0.1% (v/v) Tween-20) for 1 h at room temperature. Blocked membranes were incubated overnight at 4 °C with 0.5 μg/ml purified PLCβ3 or PLCβ1 in the presence or absence of 0.5 μg/ml calmodulin. The membranes were then washed three times for 10 min in TBST with 3% fatty acid-free BSA,