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High-performance liquid chromatographic assay with ultraviolet spectrometric detection for the evaluation of inhibitors of phosphatidylinositol-specific phospholipase C

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Abstract

A nonradioactive spectrometric assay for the evaluation of inhibitors of phosphatidylinositol-specific phospholipase C (PI–PLC) is described. L-α-Phosphatidylinositol from bovine liver was used as substrate in the presence of the micelle-forming detergent deoxycholic acid. PI–PLC isolated from *Bacillus cereus* and crude cytosol fractions from porcine brain were used as enzyme sources. PI–PLC activity was determined by measuring the release of 1-stearoyl-2-arachidonoyl-*sn*-glycerol with reversed-phase HPLC and UV detection at 200 nm. PI–PLC from *B. cereus* was not inhibited by the putative PI–PLC inhibitors U-73122 and ET-18-OCH_3 at 100 μM, whereas the isobenzofuranone derivative **5** blocked the enzyme with an IC_{50} of 75 μM. PI–PLC activity present in porcine brain cytosol was decreased by all three test compounds at 100 μM to approximately 30 to 50%.

Keywords: Phosphatidylinositol; Phospholipase C; *Bacillus cereus*; Porcine brain cytosol; Inhibitors; 1,2-Diacylglycerol; 1,3-Diacylglycerol; HPLC; UV detection; MS; APCI

Certain growth factors, neurotransmitters, and mitogens bind to G protein-coupled receptors or tyrosine kinase receptors on the cell surface, initiating the activation of phosphatidylinositol-specific phospholipases C (PI–PLC)[1] on the internal face of the cell membrane. PI–PLC catalyzes the hydrolysis of phosphatidylinositols to yield two second-messenger molecules: diacylglycerol and inositol phosphates (Fig. 1) [1,2]. These two molecules initiate a series of events that result in profound cellular changes such as cell proliferation and neuronal activity. To date, 13 different PI–PLC have been identified in mammals: PLCβ(1–4), PLCγ (1 and 2), PLCδ(1, 3, and 4), PLCε(1), PLCζ(1), and PLCη(1 and 2) [1–3]. Despite the prominent role of PI–PLC in physiological and pathophysiological processes, few inhibitors of these enzymes have been described until now [4,5].

For PI–PLC inhibitor screening and characterization, often PI–PLC from *Bacillus cereus* is used [6–9] because the catalytic domain of this commercially available bacterial enzyme shows the same architecture as the catalytic domain of certain mammalian PI–PLC [10,11]. Besides, mammalian enzymes such as PLCβ, PLCγ, and PLCδ are applied [12–22].

The traditional assay of PI–PLC activity involves the determination of water-soluble inositol phosphates from radiolabeled phosphatidylinositols [6,12–22]. Alternatively, the amount of inositol phosphates released by PI–PLC is monitored with ^{31}P NMR [8,9] or by photometric phosphate analysis after decomposition of organic phosphate [23]. Other assays apply artificial chromogenic

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[1] *Abbreviations used:* PI–PLC, phosphatidylinositol-specific phospholipase C; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; NDGA, nordihydroguaiaretic acid; DMSO, dimethyl sulfoxide; IS, internal standard; BSA, bovine serum albumin; APCI, atmospheric pressure chemical ionization.

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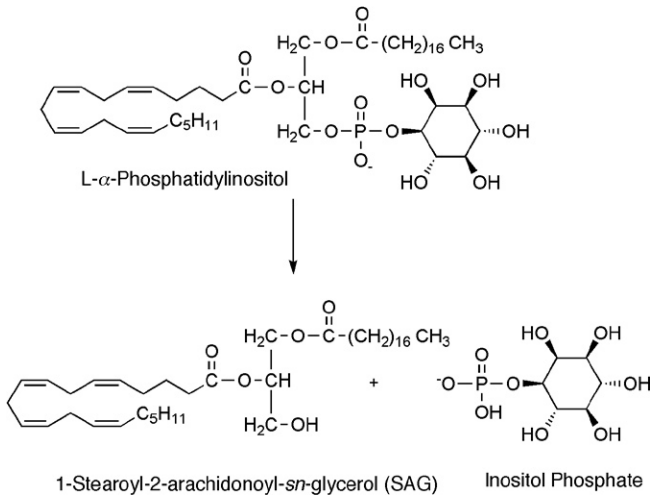

Fig. 1. Cleavage of L-α-phosphatidylinositol by phosphatidylinositol-specific PLC.

and fluorogenic substrates [7,8,24–32]. Furthermore, PI–PLC activity is determined by measuring diacylglycerol liberation from natural phosphatidylinositols by capillary electrophoresis with UV detection [33] or by the use of turbidimetry [34].

We have developed a PI–PLC assay that monitors the liberation of diacylglycerols from bovine liver phosphatidylinositols by reversed-phase HPLC and UV detection at 200 nm. As enzyme sources, purified PI–PLC from *B. cereus* and crude porcine brain cytosol were used.

Materials and methods

Materials

L-α-Phosphatidylinositol ammonium salt from bovine liver primarily containing stearic and arachidonic acids (cat. no. P8443), 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG), deoxycholic acid sodium salt, nordihydroguaiaretic acid (NDGA), arachidonic acid (Sigma); PI–PLC from *B. cereus* (100 U/ml, Invitrogen); dimethyl sulfoxide (DMSO), Tris (Merck); HPLC-grade methanol (Acros); HPLC-grade acetonitrile (VWR); U-73122, ET-18-OCH_3 (Calbiochem); 3-stearoyl-*sn*-glycerol (Bachem); *N*,*N*′-dicyclohexylcarbodiimide (Fluka); and 4-dimethylaminopyridine (Aldrich). Internal standard (IS) 1-phenylhexadecan-1-one was synthesized according to a published procedure [35] and purified by silica gel chromatography eluting with petroleum ether/ethyl acetate (9:1, v/v). 4-(2,4-Dioxothiazolidin-5-ylidenemethyl)-*N*-{4-[1-(4-fluorophenyl)-3-oxo-1*H*-isobenzofuran-1-yl]-2,5-dimethyl-1-phenylpyrrol-3-ylmethyl}benzamide (**5**) was prepared as described recently [36]. Porcine brain was obtained from a regional slaughterhouse.

Preparation of porcine brain cytosol

Each step of the preparation of the brain cytosol was performed at 4 °C. Gray matter of one-half of a porcine brain was homogenized with a fivefold volume of EGTA (1 mM) containing Tris–HCl buffer (20 mM, pH 7.4 at 20 °C) with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 1000*g* for 10 min, and the resulting supernatant was centrifuged again at 10,000*g* for 30 min. Finally, the supernatant was centrifuged at 100,000*g* for 60 min. The new supernatant was concentrated at 4 °C to 1/10 volume by using 2-ml Vivaspin centrifugal filter devices with a molecular weight cutoff of 50 kDa (Vivascience). The concentrate was diluted with one-half of its volume glycerol and stored at −20 °C until used. Applying the method of Bradford with bovine serum albumin (BSA) as standard, a protein concentration of 3.7 mg/ml was determined for the final solution.

Incubation procedure for measuring PI–PLC activity from B. cereus

Here 88 µl of a solution of L-α-phosphatidylinositol ammonium salt (0.57 mM) and deoxycholic acid (3 mM) in Tris–HCl buffer (0.1 M, pH 7.4 at 20 °C) were added to 2 µl of a DMSO solution of the test compound or, in the case of controls and in the kinetic experiments, to 2 µl of DMSO. The mixture was incubated for 10 min at 37 °C. Then the enzyme reaction was started by adding 10 µl of a freshly prepared 1:200 dilution of the PI–PLC stock solution in Tris–HCl buffer (0.1 M, pH 7.4 at 20 °C) containing deoxycholic acid (3 mM). The final reaction mixture (100 µl) included 0.5 mM L-α-phosphatidylinositol, 3 mM deoxycholic acid, and 0.05 U/ml PI–PLC. The incubation was performed at 37 °C for 10 min. In the case of kinetic tests, the incubation time was variable. The enzyme reaction was terminated by the addition of 300 µl of acetonitrile containing NDGA as oxygen scavenger (0.9 µg/300 µl) and 1-phenylhexadecan-1-one as IS (2.5 µg/300 µl). The obtained samples were cooled in an ice bath for 15 min and then centrifuged at 1000*g* and 4 °C for 5 min. The supernatants were subjected to HPLC. Control incubations in the absence of the enzyme were carried out in parallel and used to calculate the specific hydrolysis.

Incubation procedure for measuring PI–PLC activity from porcine brain cytosol

To 2 µl of a DMSO solution of the test compound, or to 2 µl of DMSO (in the case of controls and in the kinetic experiments), was added 68 µl of a solution of L-α-phosphatidylinositol ammonium salt (0.73 mM) and deoxycholic acid (4.4 mM) in Tris-HCl buffer (0.1 M, pH 7.4 at 20 °C) and 20 µl of a solution of $CaCl_2$ (5.5 mM), EGTA (0.5 mM), and dithiothreitol (0.5 mM) in Tris–HCl buffer (0.1 M, pH 7.4 at 20 °C). The mixture was incubated for 10 min at 37 °C. Then the enzyme reaction was started by adding 10 µl of porcine brain cytosol concentrate. The incubation was performed at 37 °C for 30 min. In the case of kinetic tests, the incubation time was variable. The enzyme reaction was terminated by the addition of 300 µl of acetonitrile containing NDGA as oxygen scavenger (0.9 µg/300 µl) and 1-phenylhexadecan-1-one as IS (2.5 µg/300 µl). The obtained samples were cooled