Phosphatidylinositol-specific Phospholipase C in Fetal Membranes and Uterine Decidua

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ABSTRACT An assay procedure was developed in which phosphatidyl[2-^{3}H]inositol was employed as substrate for the measurement of phosphatidylinositol-specific phospholipase C activity. Employing this assay, phosphatidylinositol-specific phospholipase C activity in human fetal membranes and uterine decidua was identified and characterized. The specific activity of this enzyme in amnion (4.4 μmol $\times$ mg^{-1} protein $\times$ h^{-1}) was three times that in uterine decidua and more than five times that in chorion laeve. No difference was found between the specific activity of phosphatidylinositol-specific phospholipase C in placental amnion and that in reflected amnion. The products of phosphatidylinositol hydrolysis in short-term incubations were stoichiometric amounts of diacylglycerol and inositol-1,2-cyclic-phosphate plus inositol-1-phosphate. After longer periods of incubation, monoacylglycerol also was detected. Diacylglycerol lipase activity also was demonstrated in these tissues. More than 90% of phosphatidylinositol-specific phospholipase C activity of amnion tissue was recovered in the 105,000-*g* supernatant fraction, and optimal enzymatic activity in vitro was observed at pH 6.5–7.5 in the presence of Ca^{2+} (8 mM) and mercaptoethanol (4 mM). Phosphatidylinositol-specific phospholipase C activity was stimulated by fatty acids in low concentrations, but was inhibited by lysophosphatidylcholine and a variety of detergents. No effect of labor on the specific activity of phosphatidylinositol-specific phospholipase C in either fetal membranes or uterine decidua could be detected. The finding of an active phosphatidylinositol-specific phospholipase C activity in human fetal membranes and uterine decidua is complementary to our previous finding of a selective loss of arachidonic acid from phosphatidylinositol of human fetal membranes during labor. The action of phosphatidylinositol-specific phospholipase C, coupled to diacylglycerol lipase action, could provide a mechanism for the release of arachidonic acid for prostaglandin biosynthesis during parturition.

INTRODUCTION

Prostaglandins or prostaglandin-related compounds appear to play an important role in the initiation of human parturition (1, 2). The obligate precursor of prostaglandins of the 2-series is free arachidonic acid (3). However, in mammalian tissues arachidonic acid is present predominantly in an esterified form; thus, the enzymatic release of free arachidonic acid is of signal importance in the regulation of prostaglandin biosynthesis (4). Human fetal membranes and uterine decidua contain an unusually high content of esterified arachidonic acid (5, 6); and ~66% of the arachidonic acid of human fetal membranes is present in the glycerophospholipids of these tissues (7). Greater amounts of free fatty acids are found in amniotic fluid obtained during labor than in amniotic fluid obtained before labor, and the concentration of arachidonic acid in amniotic fluid during labor is disproportionately increased compared with that of other fatty acids (6, 8).

In a previous study we found that phospholipase A_2 of human fetal membranes exhibited substrate specificity for the *sn*-2 arachidonoyl esters of phosphatidylethanolamine (9). Other evidence that phosphatidylethanolamine may be a storage form of arachidonic acid for prostaglandin biosynthesis was the finding that, during labor, there is a marked reduction in the

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arachidonic acid content of phosphatidylethanolamine in amnion (10). However, the arachidonic acid content of phosphatidylinositol in amnion also decreases during labor (10). The possibility that phosphatidylinositol also may supply arachidonic acid for prostaglandin biosynthesis during labor is attractive since, in many tissues, 1-stearoyl-2-arachidonoylglycerophosphoinositol is the predominant molecular species of this phospholipid (11). However, a phosphatidylinositol-specific phospholipase A_2 has not been described. Recently, it was shown that when platelets were stimulated with thrombin, phosphatidylinositol was hydrolyzed rapidly to diacylglycerol (12–15), which in turn was hydrolyzed by a diacylglycerol lipase to produce free arachidonic acid (13, 16). The hydrolysis of phosphatidylinositol is catalyzed by a specific phospholipase C (phosphatidylinositol phosphodiesterase, EC 3.1.4.10) in a reaction leading to the formation of diacylglycerol, *myo*-inositol-1-phosphate and *myo*-inositol-1,2-cyclic phosphate. Phosphatidylinositol-specific phospholipase C activity has been demonstrated in several tissues (17–26). The purpose of the present investigation was to identify and to characterize, if present, phosphatidylinositol-specific phospholipase C activity in human fetal membranes and uterine decidua. This research was part of a continuing investigation designed to determine the mechanisms that give rise to the selective release of arachidonic acid during human parturition.

METHODS

Materials. All chemicals and solvents used in this study were reagent and/or analytical grade. *Myo*-[2-^{3}H]inositol (12.5 Ci/mmol) and [1-^{14}C]oleic acid (55 mCi/mmol) were purchased from New England Nuclear, Boston, Mass. Pig liver phosphatidylinositol, CDP-diacylglycerol (prepared from egg lecithin), phosphatidylserine (from beef brain), phosphatidylethanolamine (from egg), phosphatidylglycerol (from egg lecithin), phosphatidylcholine (dioleoyl), 1-oleoyl-*sn*-glycero-3-phosphocholine (oleoyl lysophosphatidylcholine), 1-palmitoyl-*sn*-glycero-3-phosphocholine and 1-oleoylglycerol were obtained from Serdary Research Laboratories, Ontario, Canada. Phosphatidylinositol was analyzed by two-dimensional thin-layer chromatography (27), and on the basis of phosphate determination (28) was found to be >94% pure. *Rac*-1,2-dioleoylglycerol and 1,3-dioleoylglycerol were gifts from Dr. F. H. Mattson of Proctor and Gamble, Cincinnati, Ohio. Oleic and arachidonic acids were obtained from Nu Check Prep., Elysian, Minn. Heptadecanoic acid was obtained from Analabs, Inc., North Haven, Conn. Triton X-100 (scintillar grade) was obtained from Mallinckrodt, Inc., St. Louis, Mo. Miranol H2M was obtained from Miranol Chemical Co., Inc., Irvington, N. J. Cetylpyridinium chloride, adenosine monophosphate, bovine serum albumin, *myo*-inositol and *myo*-inositol-2-phosphate were purchased from Sigma Chemical Co., St. Louis, Mo. Precoated thin-layer silica gel G and high resolution plates were obtained from Analtech Inc., Newark, Del.

Preparation of fetal membranes. Human fetal membranes, which were not contaminated with meconium or antiseptics, were removed from term gestation placentae at the time of spontaneous vaginal delivery or at the time of elective cesarean section performed before the onset of labor. The membranes were washed several times with ice-cold NaCl (0.15 M). The amnion tissue was peeled away from chorion laeve, and for some experiments the amnion was divided into placental and reflected parts. The uterine decidua vera was removed from the chorion laeve by sharp dissection. Employing this technique, it was found by histologic examination that the chorion laeve after dissection was not contaminated by decidual tissue. The separated fetal membranes and the uterine decidua were washed with ice-cold sucrose solution (0.32 M), blotted, and weighed. A portion (2 g) of each tissue was cut into small pieces, placed in 5 ml of ice-cold sucrose (0.32 M), and homogenized for 30 s, employing a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.) with the small blade and a dial setting of 6. The whole homogenate was centrifuged at 750 *g* for 10-min (Sorvall-Superspeed RC2B centrifuge, type SS-34 rotor; DuPont Instruments—Sorvall Biomedical Div., Newtown, Conn.), and the resulting supernatant fluid was removed and passed through four layers of cheesecloth. In some experiments, an aliquot of this extract ("homogenate") was employed as the enzyme source. The homogenate was centrifuged at 10,000 *g* for 10 min. The 10,000-*g* pellet (mitochondria-enriched fraction) was resuspended in sucrose (0.32 M) and the 10,000-*g* supernatant fraction was centrifuged at 105,000 *g* for 60 min (Beckman L2-65 centrifuge, type Ti50 rotor; Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The 105,000-*g* pellet (microsome-enriched fraction) was resuspended in sucrose (0.32 M) and centrifuged at 105,000 *g* for 60 min. The resulting pellet was resuspended in sucrose (0.32 M) by hand homogenization with a Potter-Elvehjem teflon-glass homogenizer. Each of the subcellular fractions was assayed immediately after preparation, since loss of phospholipase C activity was observed when these preparations were stored at −70°C for >24 h. After 48 h at −70°C, ~25% of the activity was lost.

Preparation of substrates. Phosphatidyl[2-^{3}H]inositol was prepared by a modification of the method of Bleasdale et al. (29). Microsomes (~9 mg of protein) were isolated from lung tissue of female New Zealand white rabbits (29) and were incubated in Tris-HCl buffer (175 mM, pH 8.5) containing [2-^{3}H]*myo*-inositol (0.35 mM, 100 μCi/μmol), CDP-diacylglycerol from egg lecithin (0.5 mM), AMP (5 mM), mercaptoethanol (5 mM), and $MnCl_2$ (3.8 mM) in a total volume of 10 ml. The incubation was conducted at 37°C in a shaking water bath. After 20 min, and again after 40 min, 2 ml of CDP-diacylglycerol (2.5 mM) was added to the incubation mixture. After 60 min, the reaction was stopped by the addition of 42 ml of methanolic HCl (0.1 M) followed by 84 ml of chloroform. After mixing, 28 ml of KCl (1.5 M) containing *myo*-inositol (0.2 M) was added, and the mixture was centrifuged at 750 *g* for 10 min. The lower layer was removed and retained; the upper layer was extracted twice more with 42 ml of chloroform. The pooled lower layers (three) were washed twice with 30 ml of methanol/KCl (1.5 M) containing *myo*-inositol (0.2 M) (1:1, vol/vol). The washed extract was taken to dryness in vacuo, and the residue was dissolved in 5 ml of chloroform and stored at −20°C. The radiochemical homogeneity of the substrate was evaluated by thin-layer chromatography as described (29). More than 97% of the incorporated radioactivity cochromatographed with authentic phosphatidylinositol. This extract (hereafter called "radiolabeled total lipid extract") was used, without further purification, in the assay procedure described below.

The diacylglycerol substrate (1-palmitoyl-2-oleoyl-*sn*-glycerol) used for the assay of diacylglycerol lipase activity was prepared immediately before use from 1-palmitoyl-2-

[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine as described by Bell et al. (13). The 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-3-phosphocholine was prepared from 1-palmitoyl-*sn*-glycero-3-phosphocholine and [1-^{14}C]oleic acid employing rat liver microsomes by the procedure of Waite and van Deenen (30).

Assay of phospholipase C activity. For the standard assay of phospholipase C activity, a stock solution of phosphatidyl[2-^{3}H]inositol (5.0 mM) was prepared as follows. An aliquot (2.5 ml, containing 24 μmol) of phosphatidylinositol from pig liver in chloroform (10 mg/ml) was mixed with 0.2 ml of radiolabeled total lipid extract (equivalent to ~0.3 μmol of total lipid phosphorus and 0.02 μmol of phosphatidylinositol) and taken to dryness under nitrogen. To the dried lipid residue, 5.0 ml of Tris-HCl buffer (20 mM, pH 7.0) was added, followed by sonication for 60 s at 125 W in a Bransonic 220 bath sonicator (Bransonic Cleaning Company, Shelton, Conn.). The standard assay mixture consisted of Tris-HCl (20 mM, pH 7.0), phosphatidyl[2-^{3}H]inositol (2 mM, 0.05 μCi/μmol), mercaptoethanol (4 mM), $CaCl_2$ (4 mM), and enzyme (~80 μg protein), in a total volume of 0.25 ml. When other components were added to the incubation mixture, these were dissolved in Tris-HCl buffer. The incubations were conducted at 37°C in a shaking water bath. After incubation no change in pH was detected. After 30 min, the reaction was terminated by the addition of 1.0 ml of chloroform/methanol (1:2, vol/vol). After mixing, 0.3 ml of chloroform and 0.3 ml of KCl (2 M) were added and mixed, and phases were separated by centrifugation at 750 *g* for 10 min. An aliquot (0.75 ml) of the upper phase (1.2 ml total) was dried in a scintillation vial and assayed for radioactivity employing a Triton X-100 scintillation mixture (29). All determinations were made in duplicate and were corrected for blank values, i.e., the amount of water-soluble radiolabeled products recovered from incubation mixtures in which the enzyme was added after the addition of 1.0 ml of chloroform/methanol (1:2, vol/vol). Blank values were never >30% of experimentally determined values of enzymatic activity.

Analysis of reaction products. The lipid-soluble products were isolated by thin-layer chromatography on silica gel G plates with hexane/diethyl ether/acetic acid (105:45:4.5, by vol) as the developing solvent for separation of mono- and diacylglycerols and chloroform/methanol/acetic acid/H_2O (100:50:14:6, by vol) as the developing solvent for separation of phospholipids. When lipid-soluble products were analyzed for fatty acid composition, the mono- and diacylglycerols were separated by thin-layer chromatography on silica gel high resolution plates with hexane/diethyl ether/methanol/acetic acid (80:21:3:2) as the developing solvent. After chromatography, areas of the chromatogram containing diacylglycerol were removed separately and added to 0.5 ml of spectrograde benzene, and 2.0 ml of boron trifluoride in methanol was then added. The mixture, in an atmosphere of N_2, was heated in a boiling water bath for 30 min. After cooling, 20 ml of distilled H_2O was added, and the sample was extracted three times with hexane (2.5 ml). The upper layers were pooled and evaporated to dryness under nitrogen, and the residue was redissolved in carbon disulfide. The fatty acid methyl esters in this solution were separated by gas chromatography, using a Hewlett-Packard model 5830 A gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) using columns of 10% Sp-2330 on 100/200 chromosorb W (Supelco Inc., Bellefonte, Pa.) at 200°C. Using heptadecanoic acid as internal standard, the amounts of the eluted fatty acid methyl esters were computed.

In experiments where water-soluble products were isolated and identified, the assay procedure was modified to accommodate a total volume of 0.1 ml, and in the extraction procedure KCl (2 M) was replaced by water. All other conditions of the assay procedure were unchanged. Aliquots (0.02 ml) of the upper phases were treated by descending paper chromatography on Whatman No. 1 paper (Whatman Inc., Clifton, N. J.) developed in ethanol/ammonia (13.5 M) (3:2, vol/vol) as described by Dawson and Clarke (19). This chromatographic procedure does not separate completely inositol from inositol-1,2-cyclic phosphate. To ensure that the radioactive product recovered in inositol-1,2-cyclic phosphate was not contaminated by [^{3}H]inositol, samples of the upper phases were subjected to acid hydrolysis (1 M HCl, 90°C for 3 min) before chromatography. Employing this procedure, essentially all inositol-1,2-cyclic phosphate is converted to inositol-1-phosphate (31), which can be separated readily from inositol by this chromatographic procedure. Glycerophosphoinositol, although not a product of the enzymatic reaction measured here, was shown to be separated from inositol-1,2-cyclic phosphate by this chromatographic procedure. The inositol-1,2-cyclic phosphate used as a chromatographic standard was prepared as described by Pizer and Ballou (31).

Assay of diacylglycerol lipase activity. Diacylglycerol lipase activity was assayed employing the procedure described by Rittenhouse-Simmons (16).

Other methods. Protein concentration was measured by the method of Lowry et al. (32), using bovine serum albumin as the reference standard.

RESULTS

For the characterization of phospholipase C activity in human amnion, the 105,000-*g* supernatant fraction of this tissue was employed. However, essentially the same characteristics were observed when the corresponding subcellular fraction from chorion, decidua, or the homogenate fraction from all three tissues was used. The data reported here are either the results of typical experiments or are the mean values from the number of experiments indicated in the figure legends.

Identification of phospholipase C activity. Upon incubation of phosphatidyl[2-^{3}H]inositol with subcellular fractions of either amnion, chorion, or decidua, water-soluble tritium-labeled products were released and were analyzed by liquid scintillation spectrometry. After 30 min of incubation when the water-soluble products were analyzed as above, these were found to be inositol-1,2-cyclic phosphate (55%), inositol-1-phosphate (36%), and inositol (9%). The lipid-soluble products from phosphatidylinositol breakdown catalyzed by all sources of enzyme used (i.e., amnion, chorion, and decidua homogenates, the 105,000-*g* pellets, or the 105,000-*g* supernatant fractions) also were analyzed by thin-layer chromatography (Figs. 1A and 1B). As the incubation time increased, there was a greater loss of substrate, and after 3 h of incubation the hydrolysis of phosphatidylinositol was ~95% complete (Fig. 1A). The nonpolar lipids released during the incubation period were largely 1,2-diacylglycerols (Fig. 1B), with small amounts of 1,3-diacylglycerols (probably derived from 1,2-diacylglycerols by isomerization), monoacylglycerols, and traces of unesterified fatty acids. The diacylglycerols were quantified by gas chromatography of their fatty acid sub-

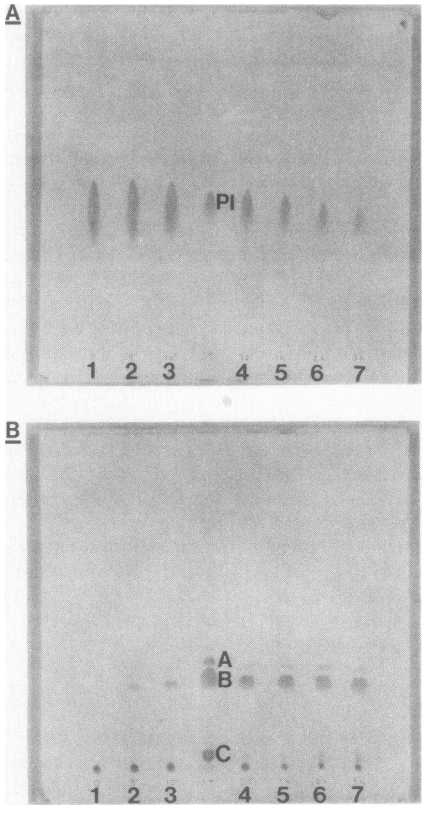

FIGURE 1 Time-course of phosphatidylinositol degradation and lipid product accumulation. Phospholipase C activity was measured in the 105,000-*g* supernatant fraction prepared from amnion tissue. The assay mixture was composed of Tris-HCl buffer (20 mM, pH 7.0), mercaptoethanol (4 mM), $CaCl_2$ (4 mM), phosphatidyl[2-^{3}H]inositol (2 mM, 0.05 μCi/μmol) and the 105,000-*g* supernatant fraction from amnion (80 μg protein) in a total volume of 0.25 ml. After incubation at 37°C for various times, reactions were terminated by the addition of 1.0 ml of chloroform/methanol (1:2, vol/vol), and lipids were extracted (Methods). Lipid extracts were analyzed by two different thin-layer chromatographic procedures: (A) For isolation of phosphatidyl[2-^{3}H]inositol remaining after incubation, lipid extracts were chromatographed on silica gel G plates developed in chloroform/methanol/acetic acid/H_2O (100:50:14:6, by vol); PI, phosphatidylinositol. (B) For isolation of the lipid products of phospholipase C action, lipid extracts were chromatographed on silica gel G plates developed in hexane/diethyl ether/acetic acid (105:45:4.5, by vol); A, 1,3-diacylglycerol; B, 1,2-diacylglycerol; C, monoacylglycerol. Lipid spots on chromatograms were developed by exposure to iodine vapor. Lane 1, no incubation; 2, 5 min; 3, 10 min; 4, 30 min; 5, 60 min; 6, 120 min, and 7, 180 min.

stituents, and it was found that there was a 1:1 molar stoichiometry between the amount of diacylglycerols (1,2- plus 1,3-) formed and the quantity of radiolabeled water-soluble products released at early time periods. For instance, when the 105,000-*g* supernatant fraction of amnion (168 μg protein) was incubated under standard assay conditions, the extent of phosphatidyl[2-^{3}H]inositol hydrolysis after 6 min was 11.8% when assessed by measuring total radiolabeled water-soluble products, and 12.2% when assessed by measuring total diacylglycerol production. Furthermore, after 6 min of incubation the fatty acid composition of the diacylglycerol produced was not significantly different from that of the substrate, phosphatidyl-[2-^{3}H]inositol. Expressed as mole percent, the fatty acid compositions of phosphatidyl[2-^{3}H]inositol and diacylglycerol were, respectively, palmitate 5.2 and 4.5%, stearate 49.5 and 50.4%, oleate 5.4 and 5.4%, linoleate 5.3 and 3.3%, arachidonate 34.6 and 36.3%. After 30 min of incubation, the molar stoichiometry between diacylglycerol recovered and total radiolabeled water-soluble products was <1:1, since by this time appreciable quantities of monoacylglycerols had begun to accumulate (Fig. 1B).

Specificity of phospholipase C. When either phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylglycerol was substituted for phosphatidylinositol, no diacylglycerol formation was detectable under the standard assay conditions (Fig. 2).

Kinetics of phospholipase C activity. The rate of hydrolysis of phosphatidylinositol was constant for 30 min using the assay procedure as described, and also

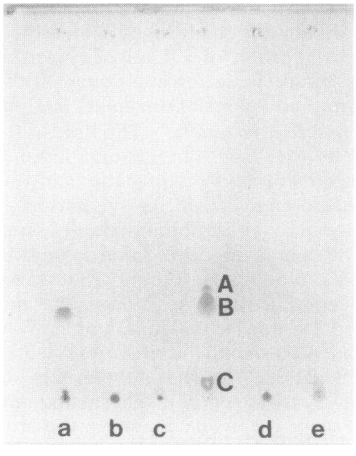

FIGURE 2 Specificity of phospholipase C activity of amnion. Aliquots (80 μg protein) of the 105,000-*g* supernatant fraction of amnion were incubated for 30 min as described in the legend of Fig. 1, except that phosphatidyl[2-^{3}H]inositol (2 mM) was replaced by (*a*) nonradiolabeled phosphatidylinositol (1 mM); (*b*) phosphatidylcholine (dioleoyl, 1 mM); (*c*) phosphatidylglycerol (1 mM); (*d*) phosphatidylethanolamine (1 mM); or (*e*) phosphatidylserine (1 mM). Following incubation, lipids were extracted from the reaction mixture and separated by thin-layer chromatography on silica gel G plates developed in hexane/diethyl ether/acetic acid (105:45:4.5, by vol). The nondesignated lane of the chromatogram contained a mixture of known lipids: A, 1,3-diacylglycerol; B, 1,2-diacylglycerol; C, monoacylglycerol. Lipid spots were rendered by exposure to iodine vapor. It was estimated that hydrolysis of as little as 2.5 nmol of added lipid could be detected by this procedure.

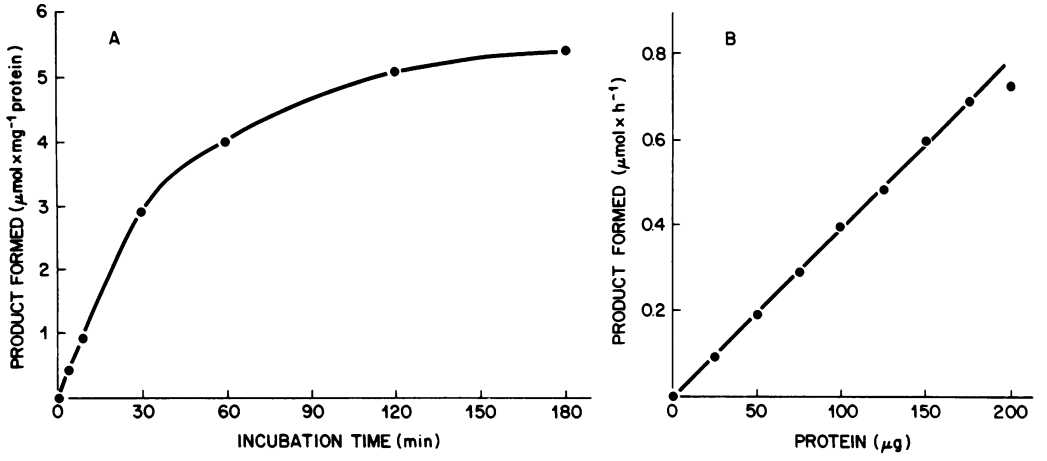

FIGURE 3 Dependence of phosphatidylinositol-specific phospholipase C activity on time and protein concentration. Phosphatidylinositol-specific phospholipase C activity in the 105,000-*g* supernatant fraction of amnion was measured as described in the legend of Fig. 1, except that radiolabeled water-soluble products were measured (Methods). (A) Time-dependence of phospholipase C activity. After 180 min of incubation, hydrolysis of phosphatidylinositol was ~95% complete. (Average of the results of two experiments). (B) Dependence of phospholipase C activity on protein concentration.

was proportional to enzyme concentration up to 200 μg protein in an assay volume of 0.25 ml (Figs. 3A and 3B).

The effect of substrate concentration on the rate of product formation is depicted in Fig. 4. The rate of product formation was independent of substrate concentration between 0.5 and 4 mM phosphatidylinositol.

Effect of pH and various buffers on phospholipase C activity. The optimum pH for phosphatidylinositol-specific phospholipase C activity in the presence of Tris-HCl buffer (20 mM) was 7.0 (Fig. 5). Using Tris-maleate buffer (20 mM), the phosphatidylinositol appeared to be better dispersed (less turbid suspension), but enzymatic activity decreased at pH values >5.0. Maleate might be expected to inhibit enzymatic activity

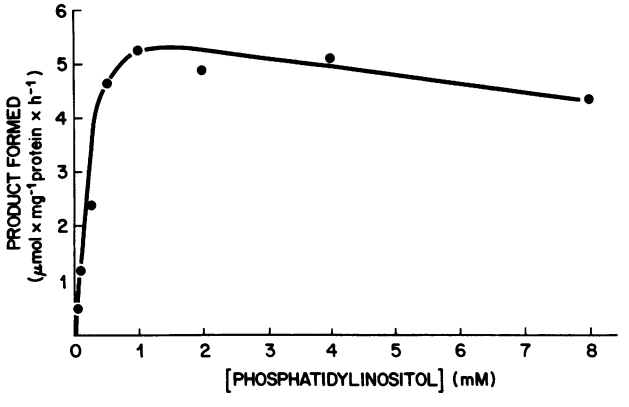

FIGURE 4 Dependence of phosphatidylinositol-specific phospholipase C activity on phosphatidylinositol concentration. Aliquots (80 μg protein) of the 105,000-*g* supernatant fraction of amnion were incubated as described in the legend of Fig. 1 at 37°C for 30 min with phosphatidyl[2-^{3}H]inositol at different concentrations. Suspensions of phosphatidyl[2-^{3}H]-inositol were prepared (Methods) in Tris-HCl buffer (20 mM, pH 7.0) at 2.5 times the desired final concentration in the incubation mixture. An identical dependence of phospholipase C activity on phosphatidyl[2-^{3}H]inositol concentration was observed when incubations were conducted at either pH 5.5 (Tris-maleate, 130 mM) or pH 8.5 (Tris-maleate, 130 mM).

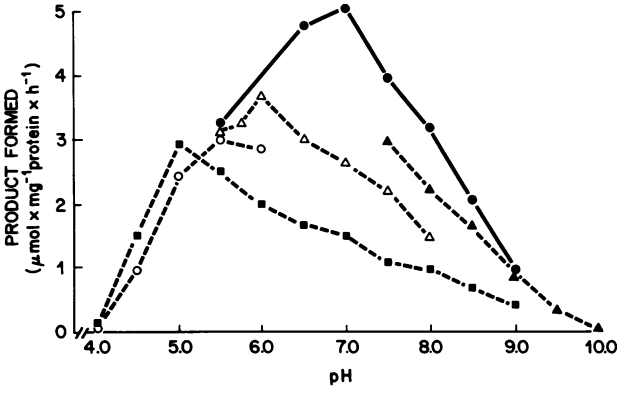

FIGURE 5 The pH dependence of phosphatidylinositol-specific phospholipase C activity. Aliquots (80 μg protein) of the 105,000-g supernatant fraction from amnion tissue were incubated under standard assay conditions (Methods) except that Tris-HCl (20 mM, pH 7.0) was replaced by (■) Tris-maleate (20 mM, pH 4.1–9.0); (○) sodium acetate-acetic acid (100 mM, pH 4.1–6.0); (△) Clark and Lubs buffer (KH_2PO_4–NaOH, 35 mM, pH 5.5–8.0); (●) Tris-HCl (20 mM, pH 5.5–9.0); or (▲) 2-amino-2-methyl-propan-1-ol-HCl (50 mM, pH 7.5–10.0). Incubations were terminated after 30 min at 37°C and water-soluble products were measured as described in Methods. Mean of the results of five experiments.

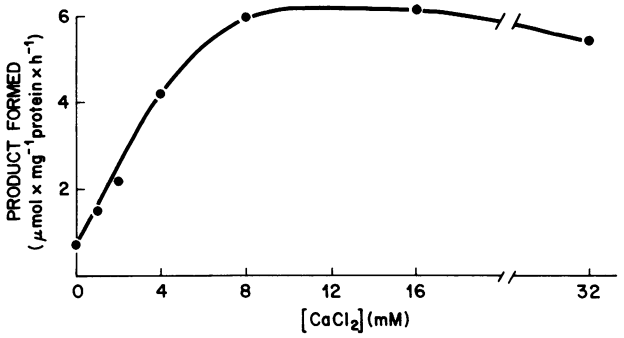

FIGURE 6 The effect of Ca^{2+} ions on phosphatidylinositol-specific phospholipase C activity. Standard assays of phospholipase C activity (Methods) were conducted employing the 105,000-*g* supernatant fraction of amnion tissue (80 μg protein) in the presence of $CaCl_2$ at various concentrations (average of the results of two experiments). A similar dependence of phosphatidylinositol-specific phospholipase C on Ca^{2+} ions was observed when assays were conducted at pH 5.5 (Tris-maleate, 130 mM).

by removing Ca^{2+} in the form of the poorly dissociated calcium salt, or else by reacting with essential thiol groups in the phospholipase C (18). Similarly, enzymatic activity in the presence of phosphate buffer (pH 7.0) was less than that in Tris-HCl buffer (pH 7.0), presumably due to formation of insoluble calcium phosphate. The other two buffer systems used (sodium acetate-acetic acid and 2-amino-2-methyl-propan-1-ol-HCl) have little or no buffering capacity in the pH range that is optimal for enzymatic activity.

Effect of cations on phospholipase C activity. In the absence of added Ca^{2+}, enzymatic activity was only 15% of that observed in the presence of $CaCl_2$ (4 mM). Furthermore, the small amount of enzymatic activity detected in the absence of added Ca^{2+} could be inhibited completely by EDTA (1.0 mM). Maximum phospholipase C activity was observed in the presence of Ca^{2+} (8 mM) (Fig. 6). When other cations were tested alone, none could substitute for Ca^{2+} ions, and in the presence of $CaCl_2$ (4 mM), all inhibited enzymatic activity (Table I). It would appear that the phosphatidylinositol-specific phospholipase C of amnion tissue has an absolute requirement for Ca^{2+}.

Effect of detergents on phospholipase C activity. In an attempt to optimize in vitro conditions for the assay of phospholipase C activity, the effects of five detergents on phospholipase C activity were investigated. These were sodium deoxycholate (anionic), cetylpyridinium chloride (cationic), Triton X-100 (nonionic), Miranol H2M (zwitterionic), and lysophosphatidylcholine, at various concentrations ranging from 0.01 to 8 mg/ml. In the presence of Ca^{2+} (4 mM), all detergents except cetylpyridinium chloride (at low concentrations) were inhibitory (Table II). Deoxycholate, at concentrations within the range employed

TABLE I
Effect of Cations on Phosphatidylinositol-specific Phospholipase C Activity

Cation added	Concentration	Phospholipase C specific activity	
		With $CaCl_2$ (4 mM)	Without $CaCl_2$
	mM	*% control*	
None	—	100.0	15.0
Mg^{2+}	1.0	75.0	15.0
	4.0	91.0	12.0
Mn^{2+}	1.0	69.0	11.0
	4.0	68.0	7.0
Fe^{2+}	1.0	53.0	4.0
	4.0	5.0	0.0
Co^{2+}	1.0	53.0	5.0
	4.0	24.0	5.0
Cd^{2+}	1.0	44.0	ND
	4.0	0.0	ND
Na^{+}	50.0	52.0	20.0
	100.0	64.0	17.0
K^{+}	50.0	52.0	15.0
	100.0	64.0	12.0
Cu^{2+}	0.1	15.0	10.0
	1.0	9.0	9.0
Hg^{2+}	0.1	9.0	11.0
	1.0	3.0	0.0

Standard assays were conducted employing the 105,000-*g* supernatant fraction of amnion tissue (80 μg protein) as described (Methods), except that mercaptoethanol was omitted from incubations. Various cations (as their chlorides) were added to incubation mixtures in the presence or absence of $CaCl_2$ (4 mM). The values given are the phospholipase C specific activities as percentages of the specific activity measured in the presence of $CaCl_2$ (4 mM) with no other cation additions. ND, not determined.

in this study, has been found to stimulate phosphatidylinositol-specific phospholipase C activity in rat cerebral cortex (20). Free oleic and arachidonic acids, at low concentrations, caused a slight increase in phospholipase C activity of amnion tissue. At concentrations between 20 and 40 μM, free arachidonic acid increase enzymatic activity by ~30–40% (Table II).

Other effectors of phospholipase C activity. Mercaptoethanol (4 mM) increased phospholipase C activity ~25%. On the other hand, Hg^{2+} ions (Table I) reduced drastically enzymatic activity, a finding that supports the importance of a sulfhydryl group for activity of this enzyme, as reported by others (18). The inclusion of bovine serum albumin (5 mg/ml) in the standard assay mixture increased enzymatic activity by ~50%.

TABLE II
Effect of Surfactants on Phosphatidylinositol-specific Phospholipase C Activity

Surfactant	Concentration	Phospholipase C specific activity
	mg/ml	*% control*
None	—	100
Deoxycholate	0.5	40
	2.0	39
	4.0	34
	8.0	15
Triton X-100	0.3	80
	3.0	54
Cetylpyridinium chloride	0.01	130
	0.1	95
	1.0	2
Miranol	0.1	96
	1.0	60
1-Oleoyl-glycero-phosphocholine	0.05	100
	0.5	9
Arachidonic acid	0.0012 (4 μM)	110
	0.006	140
	0.012	130
	0.049	120
Oleic acid	0.011 (40 μM)	105
	0.044	115

Portions (80 μg protein) of the 105,000-*g* supernatant fraction of amnion tissue were incubated under standard assay conditions in the presence of different surfactants. The values given are the phospholipase C specific activities as percentages of specific activity measured in the absence of added surfactant. Fatty acids and deoxycholate were added as the sodium salts.

Tissue and subcellular distribution of phospholipase C. The specific activity of phospholipase C in homogenates of amnion (4.4±0.7 μmol × mg^{-1} protein × h^{-1} [mean±SE, n=11]) was three times that found in decidua (1.5±0.2 μmol × mg^{-1} protein × h^{-1} [mean ±SE, n=4]) and more than five times that found in chorion laeve (0.8±0.2 μmol × mg^{-1} protein × h^{-1} [mean±SE, n=4]). No difference in phospholipase C activity was detected between placental and reflected amnion. Furthermore, the phospholipase C activities in fetal membranes and uterine decidua obtained during or after labor were not significantly different from the activities in the corresponding tissues obtained before labor (but at the equivalent time in gestation). For example, the homogenate fraction of amnion tissue obtained by elective cesarean section before the onset of labor was found to have a phosphatidylinositol-specific phospholipase C activity of 4.0±0.5 μmol × mg^{-1} protein × h^{-1} (mean±SE, n=4). Amnion tissue of an equivalent gestational age, but obtained at the time of vaginal delivery, exhibited a phosphatidylinositol-specific phospholipase C activity of 3.8±0.4 μmol × mg^{-1} protein × h^{-1} (mean±SE, n=4).

When subcellular fractions were prepared from amnion, >90% of the phospholipase C activity was recovered in the 105,000-*g* supernatant fraction (Table III). Presumably, because of the harsh procedure necessary to homogenize amnion, the 105,000-*g* supernatant fraction contained activities of marker enzymes for different subcellular organelles. In particular, 65% of acid phosphatase activity of the homogenate was recovered in the 105,000-*g* supernatant fraction, a finding that indicates lysosome disruption. However, because of the neutral pH optimum of the phospholipase C activity, it seems unlikely that the enzyme in the 105,000-*g* supernatant fraction originated from lysosomes.

Diacylglycerol lipase activity in fetal membranes and uterine decidua. Two observations indicated that the amnion, chorion laeve, and decidua vera tissues contained diacylglycerol lipase activity. First, when phosphatidylinositol was incubated with these tissues for longer time periods, the formation of monoacylglycerol was observed. Second, the 1:1 stoichiometry between inositol phosphate and diacylglycerol formation was found only during the first 15 min of incubation; after this time the rate of accumulation

TABLE III
Subcellular Distribution of Phosphatidylinositol-specific Phospholipase C Activity of Amnion Tissue

Subcellular fraction	Total protein	Phospholipase C activity	
		Total activity	Specific activity
	mg	*μmol × h^{-1}*	*μmol × mg^{-1} protein × h^{-1}*
Homogenate	73.2	267.2	3.65
10,000-*g* pellet	0.9	1.3	1.41
105,000-*g* pellet	3.1	1.3	0.42
105,000-*g* supernatant	58.8	245.8	4.18

To measure the subcellular distribution of phospholipase C activity, aliquots (~80 μg protein) of different subcellular fractions of amnion tissue were incubated under standard assay conditions (Methods). The small amount of enzymatic activity found in the 10,000-*g* pellet and in the 105,000-*g* pellet exhibited a pH dependence and a Ca^{2+} dependence similar to those of the large amount of enzymatic activity present in the 105,000-*g* supernatant fraction. Recoveries of protein and enzymatic activity throughout the subcellular fractionation procedure were 86 and 93%, respectively (average of the results of two experiments).

of diacylglycerol was less than that of inositol-1-phosphate plus inositol-1,2-cyclic phosphate. Therefore, we assayed diacylglycerol lipase activity in each of these tissues using 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol as the substrate. The rate of formation of radiolabeled free fatty acid was determined. The specific activities of diacylglycerol lipase (nmol × mg^{-1} protein × h^{-1}) observed in the 750-*g* supernatant fractions of amnion, chorion laeve, and uterine decidua were, respectively, 9.6±1.9 (mean±SE, n=4), 12.1 ±4.4 (n=4) and 38.4±4.5 (n=4). We also observed that the subcellular distribution of diacylglycerol lipase activity in these tissues was similar to that of NADPH cyctochrome *c* reductase activity, a finding which suggests a microsomal location of diacylglycerol lipase (Okazaki et al., manuscript in preparation). Although the substrate used to measure diacylglycerol lipase activity was radiolabeled in the fatty acid in the *sn*-2 position, the assay procedure does not allow a distinction between a diacylglycerol lipase with *sn*-2 positional specificity and a diacylglycerol lipase acting in conjunction with a monoacylglycerol lipase.

DISCUSSION

In the present study, phosphatidylinositol-specific phospholipase C activity was demonstrated in subcellular fractions prepared from human amnion, chorion laeve, and uterine decidua. This enzymatic activity was characterized partially in the 105,000-*g* supernatant fraction of amnion tissue. The specific activity of the enzyme in amnion was much greater than that in either chorion laeve or uterine decidua, and also was greater than the specific activity of phospholipase C of human platelets, which has been reported by others (14, 15, 33). Most of the phosphatidylinositol-specific phospholipase C enzymes studied in other mammalian tissues are either cytosolic (18, 21, 33–35) or membrane bound (20), and require Ca^{2+} for activity. A second group of phosphatidylinositol-specific phospholipase C enzymes of lysosomal origin has a more acidic pH optimum and is not inhibited by EDTA (21, 25, 26). From the results of the present study, it is evident that the phospholipase C activity of human fetal membranes and uterine decidua belongs to the former group.

Some of the phosphatidylinositol-specific phospholipase C enzymes are activated by the detergent deoxycholate (20, 36, 37). It has been postulated that deoxycholate might mimic some endogenous anionic amphiphile which regulates the activity of the enzyme in vivo. Irvine et al. (37) presented evidence that supported the view that this amphiphilic regulator may be a free fatty acid, since the hydrolysis of membrane-bound phosphatidylinositol in rat liver microsomes by the soluble phospholipase C from rat brain was stimulated markedly by oleic and arachidonic acids. Although stimulation of the same enzyme in fetal membranes by free arachidonic acid is an attractive hypothesis, we were unable to demonstrate a substantial increase in activity of phospholipase C in amnion when arachidonic acid was added to the assay mixture. A slight increase (30–40%) in enzyme activity was observed when the concentration of arachidonic acid was in the range of 20 to 40 μM. We observed that deoxycholate, at all concentrations tested, inhibited phospholipase C activity; of the other detergents tested, only cetylpyridinium chloride, a cationic detergent, stimulated phospholipase C activity.

Phosphatidylinositol of human fetal membranes, as in other tissues, is known to be rich in arachidonic acid (7). This enrichment with arachidonic acid, together with the presence in fetal membranes of phosphatidylinositol-specific phospholipase C, may lead to the formation of a diacylglycerol enriched in arachidonic acid, from which arachidonic acid may be released during parturition. Such a process would involve a diacylglycerol lipase. In the present study, using 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol as substrate, we demonstrated the presence of diacylglycerol lipase activity in fetal membranes and uterine decidua. In addition, even though the present assay conditions were optimized for measurement of phospholipase C activity, monoacylglycerol formation was detected when the 105,000-*g* supernatant fraction of amnion tissue was incubated with phosphatidylinositol. This finding also suggests the presence of a diacylglycerol lipase in amnion tissue and contrasts with the failure to observe an accumulation of monoacylglycerol as a result of diacylglycerol lipase activity in platelets (13).

When phosphatidylinositol-specific phospholipase C activity was measured under the conditions described above, we found no difference in the specific activity of this enzyme in fetal membranes (or uterine decidua) obtained after labor compared with that in the corresponding tissues obtained before labor but at the equivalent time in gestation. Although no effect of labor on in vitro phospholipase C activity could be detected, this finding does not preclude the possibility that phospholipase C activity is regulated in vivo during labor. Although a regulatory function for diacylglycerol lipase cannot be discounted (13, 16), it is possible that, in vivo, phosphatidylinositol-specific phospholipase C is regulated by Ca^{2+}, fatty acids, or neurohormones. There is precedent for suggesting that phosphatidylinositol-specific phospholipase C is a regulatory enzyme. In many tissues, a response to extracellular stimuli is observed that involves specifically an enhanced turnover of phosphatidylinositol. It has been proposed that in such responses, phosphatidylinositol-specific phospholipase C activity is greatly increased (38). When considering phosphati-

dylinositol as the source of arachidonic acid for prostaglandin biosynthesis, it is recognized that the concentration of this glycerophospholipid in human fetal membranes is low compared with that of phosphatidylcholine and phosphatidylethanolamine. However, in many tissues the turnover of phosphatidylinositol is great compared with that of other glycerophospholipids. Moreover, the optimal specific activity of the phosphatidylethanolamine-specific phospholipase A_2 in human fetal membranes under optimal in vitro conditions (9) is very low compared with that of phosphatidylinositol-specific phospholipase C activity in these tissues. For these reasons, phospholipase C of fetal membranes, as already suggested for that of human platelets (13), may play a key role in the regulation of the selective release of arachidonic acid for conversion to prostaglandins in the initiation and/or maintenance of human parturition.

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