Purification of a Phospholipase C from Rat Liver Cytosol That Acts on Phosphatidylinositol 4,5-Bisphosphate and Phosphatidylinositol 4-Phosphate*

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A soluble phospholipase C from rat liver was purified to homogeneity using phosphatidylinositol 4,5-bisphosphate (PIP2) as substrate. After ammonium sulfate fractionation, the purification involved chromatography on phosphocellulose, DEAE-Sepharose CL-6B, hydroxylapatite, Reactive Blue 2 dye-linked agarose, and Mono S cation exchanger. Under the conditions of the assay, the pure enzyme had a specific activity of 407 µmol/mg protein/min. It migrated as a single band with a molecular mass of 87 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The water-soluble product formed during the hydrolysis of PIP2 by the purified enzyme was inositol 1,4,5-trisphosphate. The enzyme shows one-half of maximum velocity at 2 mM Ca2+ with PIP2 as substrate. Between 0 and 100 mM Ca2+, the enzyme shows approximately the same activity with phosphatidylinositol 4-phosphate (PIP) as it does with PIP2, and very low activity with phosphatidylinositol. The enzyme is activated by low concentrations of basic proteins; for example, with PIP2 as substrate, 1 µg/ml histone activates the enzyme 3.6-fold. The enzyme shows an almost absolute requirement for monovalent salts which can be met by different alkali metal halides. A second, minor peak of PIP2-hydrolyzing phospholipase C activity was resolved during chromatography of the enzyme on hydroxylapatite.

The substrate specificity suggests that PIP and PIP2 are normal substrates of this enzyme. Under physiological conditions of activation, the enzyme may therefore generate inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate in amounts determined by the ratio of PIP and PIP2 present in the cellular membranes.

Hydrolysis of PIP2 is a key step in the regulation of cytosolic Ca2+ concentration, of protein kinase C activity, and probably of other processes (Nishizuka, 1984; Berridge, 1984; Williamson, 1986). Activation of phosphoinositide-specific phospholipase C by hormones may be mediated by a GTP-binding protein, in a manner similar to the modulation of adenylyl cyclase activity by hormones (Litoosh and Fain, 1986; Gilman, 1987). In the case of adenylyl cyclase, the regulatory proteins (G-proteins) and the enzyme itself are membrane-bound (Gilman, 1987). Membrane-associated phospholipase C activities have been reported (Downes and Michell, 1981; Cockcroft et al., 1984; Kamińska et al., 1986; Melin et al., 1986; Wang et al., 1986; Rock and Jackowski, 1987; Lee et al., 1987). However, the major portion of phosphatidylinositol-specific phospholipase C activity is found in the soluble fractions of tissue homogenates. It is not clear at present how the membrane-bound and soluble enzymes are related and whether both are involved in agonist-stimulated PIP synthesis.

A number of different, soluble enzymes with phospholipase C activity have been reported which can be classified according to their molecular weights. Many of these enzymes were purified or partially purified using PIP as substrate, but most if not all act on PIP and PIP2. The soluble activities can be resolved into multiple forms (Nakanishi et al., 1985; Hofmann and Majerus, 1982a; Low and Weglicki, 1983; Ryu et al., 1986; Banno et al., 1986), but the specificity of these different forms towards PI, PIP, and PIP2 has not been investigated in detail.

Rebecchi and Rosen (1987) reported phospholipase C isozymes in brain which use PIP2 as substrate. They purified one of these enzymes and showed it to have a molecular mass of 88 kDa. This isozyme hydrolyzes polyphosphoinositides in preference to PI. Banno and Nozawa (1987) showed that a partially purified phospholipase C from platelet membranes hydrolyzes PIP2 in preference to PI. This enzyme has a molecular mass of about 110 kDa.

We describe the purification to homogeneity of a polyphosphoinositide-prefering phospholipase C from rat liver cytosol. Two forms of the enzyme were separated by hydroxylapatite chromatography. The major peak, which eluted later and which accounted for a major fraction of the total cellular activity, was purified.

**Experimental Procedures**

**Materials**

Male rats of the Sprague-Dawley strain were purchased from Charles River Breeding Laboratory (Wilmington, MA); phosphocellulose P-11 from Whatman (Maidstone, Kent, United Kingdom); DEAE-Sepharose CL-6B and cation exchange resin Mono S from Pharmacia LKB Biotechnology Inc.; Reactive Blue 2 Agarose, crude phosphoinositide mixture, phosphorylase b of rabbit muscle, bovine serum albumin, ovalbumin, lysozyme (grade IV) from chicken egg white, carbonic anhydrase, soybean trypsin inhibitor, bovine hemoglobin, histone from calf thymus, and horse heart cytochrome c from Sigma; rabbit muscle lactate dehydrogenase from Boehringer (Mannheim, Federal Republic of Germany); hydroxylapatite from Bio-Rad;
Preparation of Phospholipid Substrate

Unlabeled PIP2 was purified from a crude phosphoinositide mixture by thin layer chromatography using the method of Dolles et al. (1981). Unlabeled PI and PC were purified from a Folch extract of rat liver by HPLC. [methyl-3H]Choline-labeled PC was prepared by methylation of phosphatidylethanolamine with [3H]methyl iodide (Patel et al., 1979), and purified as cited above for PC. Phospholipid concentration was determined after perchloric acid digestion (Kates, 1972) by measuring orthophosphate (Sanui, 1974).

Enzyme Assays

Phospholipase C activity was assayed with 17 µM [2-3H]inositol-labeled PIP2 (3-6 Ci/mmol), 2.4 mM sodium dodecylsulfate, 300 mM CaCl2, 100 µM EGTA, 180 mM NaCl, 100 µg/ml lysozyme from chicken egg white, and 50 mM Na-Hepes buffer, pH 7.1. The phospholipid, which was stored under nitrogen in a methanol/chloroform:water (20:10:1, by volume), was dried under nitrogen, and resuspended in the assay buffer by sonication in a Branson 12 sonicator for 1 min. Reaction was initiated by adding enzyme and was run in a final volume of 0.1 ml at 38 °C. It was stopped by adding 0.1 ml of 1.2 M HCl followed by 0.5 ml of chloroform:methanol (2:1). The mixture was vortexed vigorously for at least 20 s and centrifuged at 1,000 × g for 5 min. An aliquot of the resultant upper phase was removed and counted for radioactivity. Hydrolysis of PI, PIP, and PC was assayed under identical conditions using [2-3H]inositol-labeled PI and PIP and [methyl-3H]choline-labeled PC. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard, or by measuring the absorbance at 280 nm.

Analysis of Water-soluble Products

The reaction was stopped by adding 50 µl of bovine serum albumin (1 mg/ml) followed immediately by 50 µl of trichloroacetic acid. The mixture was placed on ice for 5 min and centrifuged at 12,000 × g for 10 min. An aliquot of the supernatant (150 µl) was removed and neutralized with 42 µl of 1 N NaOH. The mixture was diluted with 560 µl of distilled H2O and injected into a Dowex AG-MP1 HPLC column (4.6 × 250 mm) which had been washed with distilled H2O. After a 5-ml wash with distilled H2O, [3H]inositol phosphates were eluted with a linear gradient from 0 to 300 mM trichloroacetic acid over 45 ml. The flow rate was 1 ml/min. Fractions of 1 ml were collected and assayed for radioactivity. A mixture of AMP, ADP, and ATP (approximately 30 nmol each) was included as internal standard; their elution was detected by measuring the absorbance of the effluent at 260 nm.

Purification of Enzyme

All steps were carried out at 0-4 °C.

Step 1: Preparation of Cytosol—Male rats of the Sprague-Dawley strain (200-300 g) were killed by decapitation. The livers were excised, rinsed, and centrifuged at 1,000 × g for 10 min. An aliquot of the supernatant (150 µl) was removed and neutralized with 42 µl of 1 N NaOH. The mixture was diluted with 560 µl of distilled H2O and injected into a Dowex AG-MP1 HPLC column (4.6 × 250 mm) which had been washed with distilled H2O. After a 5-ml wash with distilled H2O, [3H]inositol phosphates were eluted with a linear gradient from 0 to 300 mM trichloroacetic acid over 45 ml. The flow rate was 1 ml/min. Fractions of 1 ml were collected and assayed for radioactivity. A mixture of AMP, ADP, and ATP (approximately 30 nmol each) was included as internal standard; their elution was detected by measuring the absorbance of the effluent at 260 nm.

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Electrophoresis and Molecular Weight Determination

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on slabs gels (10% polyacrylamide gel, 10 × 8 × 0.15 cm) according to Laemmli and Favre (1973). Protein was stained with 0.25% Coomassie Brilliant Blue R-250. The molecular weight standards used were: rabbit muscle phosphorylase a (94,000), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and horse heart cytochrome c (12,400).

Gel filtration on a Ultrogel AAc-34 column was performed according to the method of Andrews (1965) with the additional molecules: thyroid weight standard of rabbit muscle lactate dehydrogenase (140,000).

RESULTS

Purification of Enzyme—The enzyme activity eluted as a single peak from the phosphocellulose column (Fig. 1); however, on DEAE-Sepharose CL-6B chromatography it resolved into a major and a minor peak (Fig. 2). Both active fractions were combined and applied to the hydroxylapatite column. The resolution of enzyme activity into two peaks was also observed on the hydroxylapatite column (Fig. 3). The major
FIG. 1. Elution of phospholipase C from phosphocellulose column. The dialyzed ammonium sulfate fraction (105 ml) was applied to a phosphocellulose column (2.6 × 34 cm). The column was washed with 180 ml of Buffer PC and eluted with a linear gradient (1500 ml) going from 0 to 1 M KCl in Buffer PC over 1500 ml, as described under "Purification of Enzyme." O, activity; solid line without symbols, A_{280} (protein); broken line without symbols, concentration of KCl.

FIG. 2. Elution of phospholipase C from DEAE-Sepharose CL-6B column. The desalted enzyme fraction from the phosphocellulose column was applied to a DEAE-Sepharose CL-6B column (2.6 × 17 cm). The column was washed with 90 ml of Buffer DEAE and eluted with a linear gradient going from 0 to 6 M KCl in Buffer DEAE over 440 ml, as described under "Purification of Enzyme." O, activity; solid line without symbols, A_{280} (protein); broken line without symbols, concentration of KCl.

FIG. 3. Elution of phospholipase C from hydroxylapatite column. The desalted enzyme fraction from the DEAE-Sepharose CL-6B column was applied to a hydroxylapatite column (1.4 × 13.5 cm). The column was washed with 21 ml of Buffer HPT and eluted with linear gradient from 10 to 200 mM potassium phosphate buffer, pH 7.0, containing 0.2 mM PMSF and 0.5 mM DTT over 100 ml, as described under "Purification of Enzyme." O, activity; solid line without symbols, A_{280} (protein); broken line without symbols, concentration of potassium phosphate.

FIG. 4. Elution of phospholipase C from Reactive Blue 2-agarose column. The desalted enzyme fraction from hydroxylapatite was applied to a Reactive Blue 2-agarose column (0.5 × 10 cm). The column was washed with 2 ml of Buffer RB and eluted with linear gradient from 0 to 1.5 M KCl in Buffer RB over 40 ml, as described under "Purification of Enzyme." O, activity; solid line without symbols, A_{280} (protein); broken line without symbols, concentration of KCl.

FIG. 5. Elution of phospholipase C from first cation exchange HPLC column. The desalted enzyme fraction from the Reactive Blue 2-agarose column was applied to a Mono S cation exchange HPLC column (0.5 × 5 cm). The column was washed with 4 ml of Buffer PC and eluted with linear gradient from 0 to 0.7 M KCl in Buffer PC over 14 ml, as described under "Purification of Enzyme." O, activity; solid line without symbols, A_{280} (protein); broken line without symbols, concentration of KCl.

FIG. 6. Elution of phospholipase C from second cation exchange column. The enzyme fraction from the first cation exchange HPLC was reapplied to the same column and eluted with linear gradient from 0 to 0.5 M KCl in Buffer PC over 20 ml, as described under "Purification of Enzyme." O, activity; solid line without symbols, A_{280} (protein); broken line without symbols, concentration of KCl.

As a single peak from both columns. Gel electrophoresis of individual fractions after the first cation exchange chromatography (Fig. 5) showed that an 87-kDa protein correlated well with enzyme activity (Fig. 7A). The first cation exchange chromatography resulted in near electrophoretic homogeneity. Minor amounts of proteins did not correlate with enzyme activity. Minor amounts of proteins did not correlate with enzyme activity.
Phospholipase C from Rat Liver Cytosol

Fig. 7. Polyacrylamide gel electrophoresis of cytosolic phospholipase C. A, 15 μl each of fractions 33–39 from the first cation exchange HPLC; and B, 50 μl each of the pooled fractions of the activity peak from the second cation exchange HPLC (lane 1) and the molecular weight markers (lane 2) were subjected to SDS-polyacrylamide gel electrophoresis using a 10% gel as described under “Experimental Procedures.” The molecular mass markers were (in kilodaltons): phosphorylase from rabbit muscle (97.4); bovine serum albumin (66); ovalbumin (48); carbonic anhydrase from bovine erythrocytes (31); and soybean trypsin inhibitor (20.5).

TABLE I

Purification of cytosolic phospholipase C from rat liver

The purification started with 208 g of liver from 21 male rats. Phospholipase C was assayed as described under “Enzyme Assays” at protein concentrations for which activity was linear with respect to time of incubation. One unit is the amount of enzyme that hydrolyzes 1 μmol of PIP2/min under the conditions described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Specific activity (units mg⁻¹)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cytosol</td>
<td>12,900</td>
<td>272</td>
<td>0.021</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium sulfate (0-50%) and pH 7.4 dialysis</td>
<td>3,710</td>
<td>208</td>
<td>0.056</td>
<td>76</td>
</tr>
<tr>
<td>3. pH 6.0 dialysis</td>
<td>2,470</td>
<td>190</td>
<td>0.077</td>
<td>70</td>
</tr>
<tr>
<td>4. Phosphocellulose</td>
<td>117</td>
<td>160</td>
<td>1.37</td>
<td>59</td>
</tr>
<tr>
<td>5. DEAE-Sepharose CL-6B</td>
<td>22.7</td>
<td>87.9</td>
<td>3.87</td>
<td>32</td>
</tr>
<tr>
<td>6. Hydroxylapatite</td>
<td>3.75</td>
<td>51.4</td>
<td>13.7</td>
<td>19</td>
</tr>
<tr>
<td>7. Reactive blue 2-agarose</td>
<td>0.923</td>
<td>39.2</td>
<td>42.5</td>
<td>14</td>
</tr>
<tr>
<td>8. First cation exchange HPLC</td>
<td>0.066</td>
<td>23.9</td>
<td>362</td>
<td>8.8</td>
</tr>
<tr>
<td>9. Second cation exchange HPLC</td>
<td>0.030</td>
<td>12.2</td>
<td>407</td>
<td>4.5</td>
</tr>
</tbody>
</table>

activity (Fig. 7A). The second cation exchange chromatography (Fig. 6) removed these impurities (Fig. 7B). Table I summarizes the procedure that resulted in a 19,400-fold purification of the enzyme. Note that the assay used for the purification contained 17 μM PIP2, which is well below the K_{app} for PIP2. The specific activity of the enzyme under conditions of V_{max} is discussed below.

Purified enzyme was applied to a calibrated Ultrogel AcA-34 column. It emerged from the column at the position where a globular protein with a molecular weight of approximately 73,000 is eluted (Fig. 8). It is possible that phospholipase C interacted weakly with this type of gel filtration column to give a slightly lower molecular weight than was observed by SDS gel electrophoresis. This result suggests that the enzyme is composed of a single polypeptide. The optimal pH for the cleavage of PIP2 by the purified enzyme was about 7.1 (Fig. 9). Ion exchange chromatography of the water-soluble products formed from [2-3H]inositol-labeled PIP2 showed a single radioactive product which ran identically with inositol 1,4,5-trisphosphate.

Stability—The purified enzyme, 0.01 mg/ml, can be stored in 50 mM potassium phosphate, pH 6.0, 1 mM DTT, 0.2 mM PMSF, 1 mM EDTA, and 0.2 M KCl at 4 °C for at least 1 month with no appreciable loss of activity. Freezing at −20, −80, or −190 °C and thawing once causes 70−80% loss of activity. Glycerol (10 or 50%) did not stabilize the enzyme.

Effect of Other Proteins—We observed that recovery after the hydroxylapatite step was often less than 30%; this low recovery was improved by adding the breakthrough fraction of the phosphocellulose or DEAE columns which had no PIP2-phospholipase C activity. Such activation was also observed with other proteins. Typical effects are shown in Fig. 10. Histone (2 μg/ml), lysozyme (25 μg/ml), cytochrome c (50 μg/ml), and the breakthrough fraction of the phosphocellulose column (25 μg/ml) caused a 4-fold activation of activity. Hemoglobin and bovine serum albumin were less effective.
Phospholipase C from Rat Liver Cytosol

FIG. 8. Gel filtration of cytosolic phospholipase C on Ultrogel AcA-34. A solution containing the purified enzyme and a mixture of molecular markers was applied to a column of AcA-34 (0.9 x 44 cm) equilibrated with 50 mM potassium phosphate buffer, pH 6.0, containing 300 mM potassium chloride, 1 mM DTT, 1 mM EDTA, and 0.4 mM PMSF. The column was eluted with the same buffer at a flow rate of 3.5 ml/h, and fractions of 0.4 ml were collected and assayed for enzyme activity. The molecular weight markers were (in kilodaltons): 1, lactate dehydrogenase from rabbit muscle (140); 2, bovine serum albumin (67); 3, ovalbumin (45); 4, myoglobin from horse heart (18); and 5, cytochrome c from horse heart (13).

Histone produced 3.6- and 4.8-fold activation at concentrations of 1 and 10 μg/ml, respectively, indicating its superiority as an activator, when compared to the other proteins that were tested. The activation observable after the phosphocellulose step was slight, it increased during subsequent steps of the purification. No activation of the enzyme was observed with active fractions obtained before phosphocellulose chromatography.

The K_{app} for PIP_{2} was estimated to be 31 and 83 μM from Lineweaver-Burk plots in the absence and presence of lysozyme in the assay mixture, respectively (Fig. 11a). The activity of the pure enzyme assayed with 17 μM PIP_{2} in the presence of lysozyme was 407 μmol/mg/min. Using a K_{app} of 83 μM, it can be calculated that the specific activity of the enzyme at V_{max} was 2390 μmol/mg/min.

The double-reciprocal plot for PIP was slightly concave upward (Fig. 11b). The PIP concentration at 1/2 V_{max} was 31 μM. V_{max} with PI as substrate was only 50 μmol/mg/min, as compared to 2390 μmol/mg/min with PIP_{2}. The K_{app} for PI was 143 μM.

Effect of Monovalent Salts—Both cations and anions affect the activity of the enzyme. Sodium ion is most effective among the alkali metal cations and chloride is the most effective among the halide anions (Table II). Ammonium and cesium...
that the incubation time was 2 min.

The standard assay was used except that the concentration of deoxycholate was varied as indicated. The activities in the presence of fluoride are somewhat lower and ions appear to fall outside the typically observed sequence. In the presence of iodide are substantially lower than in the oxycholate (Fig. 12).

The standard assay conditions were used with 0.5 ng of purified enzyme, except that the Ca²⁺ concentration was varied as shown and the substrate was either: e, PIP₂; O, PIP; or △, PI at a concentration of 50 μM. Calcium-EGTA buffers were used to control the free Ca²⁺ concentration (Raafraulx, 1956). The total EGTA concentration was 0.1 mM. Note the different scale for PI.

Preparation has been repeated five times with essentially the same results shown in Table I. The conditions used to assay the enzyme during the purification were optimum except for the PIP₂ concentration, which, at 17 μM, was kept below the Kₘ (Fig. 11A), in order to economize on substrate. The soluble brain phospholipase C, which was purified by Rebecchi and Rosen (1987) to near purity, has a molecular mass of 88 kDa. Previously, Takenawa and Nagai (1981) isolated a phospholipase C from rat liver cytosol using PI as substrate. The molecular mass of this enzyme was 68 kDa. Two peaks of phospholipase C activity from the soluble fraction of rat liver were separated by hydroxylapatite column chromatography (Fig. 3). Multiple forms of phospholipase C have been observed in the soluble fraction from several other tissues. At least two immunologically distinct isozymes exist in seminal vesicles (Hoffmann and Majerus, 1982b), brain ( Ryu et al., 1987b), and uterus (Bennett and Crooke, 1987).

A survey of molecular weights of soluble phospholipase C isozymes, determined by SDS-polyacrylamide gel electrophoresis and gel filtration, is given in Table III. The enzymes appear to fall into three groups: 62–70 kDa, 85–88 kDa, and 143–154 kDa. The relationship between the different forms of phospholipase C remains to be determined. A membrane-bound form of phospholipase C has been shown to be hormonally regulated via a G-protein (Litosch, 1987; Taylor and Exton, 1987). It is not clear whether or not the soluble forms of the enzyme are also regulated by hormones. The hormone-stimulated hydrolysis of PIP₂ generates inositol 1,4,5-trisphosphate, which acts as a second messenger for the release of Ca²⁺ ions into the cytosol. The Ca²⁺ so released may then activate the soluble forms of phospholipase C, thus amplifying the original signal. The possibility cannot be ruled out that the soluble forms of phospholipase C are artifacts of preparing homogenates, and that in the intact cell they are to a greater or lesser extent also membrane-bound. If the soluble forms of phospholipase C are membrane bound in situ, this still leaves open the function of these forms of the enzyme. Presumably they are regulated differently from the membrane-anchored enzyme(s), either via different G proteins, or by mechanisms not involving G proteins.

The soluble enzyme purified by us shows similar activities with PIP₂ and PIP₃ over a Ca²⁺ concentration range of 0.1–10 μM. A comparable study for the membrane-anchored enzyme is not available. Thus, the soluble enzyme at least is capable of generating both inositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate, and the amount of diacylglycerol gener-

**TABLE II**

<table>
<thead>
<tr>
<th>Salt added</th>
<th>PIP₂ hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.002</td>
</tr>
<tr>
<td>NaF</td>
<td>0.211</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.392</td>
</tr>
<tr>
<td>NaBr</td>
<td>0.351</td>
</tr>
<tr>
<td>NaI</td>
<td>0.007</td>
</tr>
<tr>
<td>LiCl</td>
<td>0.358</td>
</tr>
<tr>
<td>KCl</td>
<td>0.307</td>
</tr>
<tr>
<td>RbCl</td>
<td>0.142</td>
</tr>
<tr>
<td>CsCl</td>
<td>0.332</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.451</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0.248</td>
</tr>
</tbody>
</table>

**FIG. 13.** Effect of Ca²⁺ concentration on the hydrolysis of three phosphoinositides by phospholipase C. The standard assay was used except that the Ca²⁺ concentration was varied as shown and the substrate was either: □, PIP₂; ◯, PIP; or △, PI at a concentration of 50 μM. Calcium-EGTA buffers were used to control the free Ca²⁺ concentration (Raafraulx, 1956). The total EGTA concentration was 0.1 mM. Note the different scale for PI.

**FIG. 12.** Effect of sodium deoxycholate on phospholipase C. The standard assay was used except that the concentration of deoxycholate was varied as indicated.
activated is equivalent to the sum of inositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate generated. The actual amounts of PIP and PIP₂ hydrolyzed probably depend on the relative amounts of these substances present in the cell membranes as well as on the relative affinity of the enzyme for these substrates. Fig. 11 shows that the apparent affinities for PIP and PIP₂ are of the same order of magnitude when the detergent-micelle assay is employed. A better measure of relative affinities may be obtained using detergent-free liposomes. This is under investigation.

Comparison of published kinetic data for the different isozymes is problematical, since standardized assay conditions were not used by different workers. The activity of phospholipase C is affected by calcium ions, phospholipids, detergents, ionic strength, pH, type of buffer, and protein concentration (Hofmann and Majerus, 1982b; Wilson et al., 1984; Rebecchi and Rosen, 1987). Moreover, these variables may exert different effects, depending on whether the substrate used is PI, PIP, or PIP₂, and whether the substrate is presented in the form of detergent micelles or as liposomes. The assay employed in the work described here has a sharp optimum for deoxycholate at 0.1%. The enzyme is activated by various alkali metal salts; in the chloride series the activation is smallest with Rb⁺ ions, but similar activation is observed with sodium bromide. The extent to which this activation represents ionic strength and specific ion effects remains to be determined.

The enzyme isolated by us is activated by basic proteins (Fig. 10). A relatively low concentration of histone (2 µg/ml) produces a 4-fold activation. Bovine serum albumin is much less effective. Activators of the enzyme also occur in the breakthrough eluate from the phosphocellulose column (step 4). The high sensitivity of the enzyme to basic proteins is indicative of a potential mechanism for regulating its activity. We are currently conducting a search for the most effective activators of this type. Additional work is required to ascertain whether activation of PIP₂ hydrolysis by basic proteins is due to an effect on the substrate, the enzyme, or both. Preliminary experiments show that the activation also occurs when the substrate is presented in the form of detergent-free liposomes, instead of detergent-containing micelles, which were used to produce the data shown in Fig. 10.

In conclusion, phospholipase C activity is regulated in a complex manner. It seems likely that the different forms of the enzyme which have been reported (Table III) will exhibit differences in the regulation of their activity. The physiological importance of the different types of activation and inhibition of the enzyme remains to be determined.

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