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Resolution of myocardial phospholipase C into several forms with distinct properties

Martin G. LOW and William B. WEGLICKI
Cardiovascular Research Laboratory, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, U.S.A.

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1. Phospholipase C activity capable of hydrolysing phosphatidylinositol in bovine heart was resolved into four forms (I–IV) by ion-exchange chromatography. Some of these forms could only be detected if the assay was performed at acidic pH (I and IV) or in the presence of deoxycholate (II). 2. Gel-filtration chromatography indicated that the four forms had different molecular weights in the range 40000–120000. 3. I, II and III all had pH optima in the range 4.5–5.5. However, the major form (III) also had substantial activity at pH 7.0 and above. 4. The activities of I, II and III at pH 7.0 were stimulated by deoxycholate; this effect was most marked with I and II, which had very low activity at this pH. 5. All forms of the enzyme were inhibited by EGTA and required 2–5 mM-$CaCl_2$ for maximal activity. 6. When the fractions eluted from the ion-exchange and gel-filtration columns were assayed with polyphosphoinositides as substrates there was a close correspondence to the elution profile obtained with phosphatidylinositol as substrate; there was no evidence for the existence in heart of phospholipase C activities specific for individual phosphoinositides.

Phosphodiesterases (of the phospholipase C-type) that are relatively specific for the phosphoinositides are widely distributed in mammalian tissues (Shukla, 1982). These enzymes are thought to be involved in the stimulated metabolism of phosphoinositides and consequently much recent interest has centred on the mechanism by which their activity might be regulated *in vivo* (for references, see Hirasawa *et al.*, 1982*c*). Although phospholipase C activities against the phosphoinositides have been extensively studied since they were first demonstrated about 20 years ago, it is still not clear whether they are due to the occurrence of enzymes specific for the individual phosphoinositides or to one enzyme capable of hydrolysing all the phosphoinositides (Thompson & Dawson, 1964; Atherton & Hawthorne, 1968; Keough & Thompson, 1972; Tou *et al.*, 1973; Lapetina *et al.*, 1975, 1980; Abdel-Latif *et al.*, 1980; Shukla, 1982). Phospholipase C specific for phosphatidylinositol (compared with the major phospholipids) has recently been purified to various extents from liver, platelets, lung and brain (Takenawa & Nagai, 1981; Tai & Tai, 1981; Shen & Tai, 1981; Chau *et al.*, 1981; Hakata *et al.*, 1982), and other recent work has shown that the phospholipase C activity in these tissues can be resolved into more than one form by chromatofocusing or ion-exchange chromatography (Shen & Tai, 1981; Hirasawa *et al.*, 1982*a*,*b*; Chau & Tai, 1982). However, in these studies phosphatidylinositol was the only substrate used and the ability of these phospholipase C activities to hydrolyse the polyphosphoinositides was not reported. In the present paper we report the resolution of myocardial phospholipase C activity into four forms that have distinct physical properties but are able to hydrolyse all three phosphoinositides.

Materials and methods

Preparation of substrates

[^{3}H]Phosphatidylinositol was prepared by exchange of [^{3}H]inositol (New England Nuclear, Boston, MA, U.S.A.) into rat liver microsomes (Low & Zilversmit, 1980). Unlabelled phosphatidylinositol was prepared from soya-bean lipids by column chromatography on alumina (Luthra & Sheltawy, 1972). Phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were prepared from Folch fraction I of bovine brain (Sigma Chemical Co., St. Louis, MO, U.S.A.) by methanol precipitation and column chromatography on DEAE-cellulose as described by Hendrickson & Ballou (1964). The fractions were monitored by

chromatography on formaldehyde-treated papers (Wagner *et al.*, 1962). The concentration of phospholipids was determined by organic phosphorus assay; phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were assumed to have 1, 2 and 3 mol of phosphorus/mol respectively. Egg phosphatidylcholine and phosphatidylethanolamine were obtained from Supelco Inc. (Bellefonte, PA, U.S.A.). Bovine brain phosphatidylserine was obtained from Sigma Chemical Co.

Assay for phospholipase C activity

Hydrolysis of phosphatidylinositol was assayed in a mixture containing 50mM-Tris/maleate buffer, $CaCl_2$, [^{3}H]phosphatidylinositol (0.1mM; 25000–35000d.p.m.) and enzyme in a total volume of 0.4ml. The assay pH and $CaCl_2$ concentrations were varied considerably and are specified in the legends to Figures and in the text as appropriate. In some assays sodium deoxycholate at a concentration of 0.1% (w/v) was included. The assay mixture was incubated at 37°C for 15min and the reaction was terminated by the addition of 1.5ml of chloroform/methanol (1:2, v/v). The tubes were cooled on ice and 0.5ml each of chloroform and 1M-HCl were added. The tubes were vortex-mixed vigorously, centrifuged to separate the phases and a 1ml portion of the upper aqueous phase was mixed with 10ml of ACS (Amersham Corporation, Arlington Heights, IL, U.S.A.) for determination of radioactivity released into the aqueous phase.

Hydrolysis of polyphosphoinositides was assayed in a mixture containing 50mM-Tris/maleate buffer, pH6.6, 1mM-$CaCl_2$, phosphatidylinositol 4-phosphate or phosphatidylinositol 4,5-bisphosphate (0.25mM) and enzyme in a total volume of 0.2ml. Incubation and extraction were as described above except that 0.2ml of water was added before vortex-mixing. Portions of the upper phase were dried at 120°C and assayed for total phosphorus. Substrate hydrolysis was calculated from phosphorus released into the aqueous phase assuming that inositol bis- or tris-phosphates were the only water-soluble products. The release of phosphorus from phosphatidylinositol and other phospholipids was assayed by a similar procedure (0.4ml incubation volume). In experiments where the polyphosphoinositide phosphomonoesterase activity was determined the incubation volume was increased to 0.4ml (the addition of 0.2ml of water during the extraction was omitted) and equal portions were taken for both total and inorganic phosphorus assay. To prevent possible hydrolysis of the extracted inositol phosphates the inorganic phosphorus was determined (see below) without drying the upper-phase sample.

Analysis of lipid products of phosphoinositide hydrolysis

The incubation (total volume 0.4ml) and extraction were carried out as described above except that 0.5ml of 2M-KCl was substituted for 1M-HCl. The lower phase was dried under N_2 at 35°C, redissolved in 12μl of chloroform/methanol (4:1, v/v) and a 2μl portion was applied to a silicic acid-coated quartz rod (Chromarod-SII; Iatron Laboratories Inc., Tokyo). The rods were developed in the solvent hexane/diethyl ether/acetic acid (50:50:1, by vol.) for 30min, dried for 5min at 110°C and analysed for lipids by flame ionization in an Iatroscan TH-10 (Iatron Laboratories Inc.). The relative mobility of phospholipid, monoacylglycerol, 1,2- and 1,3-diacylglycerol and non-esterified fatty acid in this system was very similar to conventional flat-plate t.l.c. using the same solvent system.

Assay for phosphorus and protein

Total phosphorus was assayed by the method of Bartlett (1959). P_i was assayed by the method of Baginski *et al.* (1967), but was modified to permit the assay of P_i directly in the aqueous phase of incubation extracts without previous drying. A 0.8ml portion of the aqueous phase was mixed with 0.1ml of 12% (w/v) ascorbic acid/10mM-EDTA, 0.06ml of 5% (w/v) ammonium molybdate and 0.6ml of 2% (w/v) sodium citrate/2% (w/v) sodium arsenite. Absorbance at 840nm was determined after 20min. P_i standards carried through the extraction and assay showed that this modification had no significant effect on the colour yield of the assay. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Preparation of $(NH_4)_2SO_4$ fraction

The following steps in the enzyme purification were performed at 4°C. Fresh beef heart ventricle (200g) was homogenized in 1.8 litres of 0.25M-KCl / 0.1mM-dithiothreitol / 1mM-EDTA / 10mM-imidazole/HCl, pH7.0, with a Polytron homogenizer at setting 6 for 30s. The homogenate was centrifuged at 22000***g*** for 30min and the supernatant was filtered through two layers of Miracloth (Calbiochem, San Diego, CA, U.S.A.). Solid $(NH_4)_2SO_4$ was added to the filtered supernatant to give 35% saturation. The mixture was stirred for 15min and centrifuged at 5000***g*** for 60min. Additional $(NH_4)_2SO_4$ was added to the resulting supernatant to give 55% saturation and the mixture was re-centrifuged. The precipitate was dissolved in 160ml of 1mM-EDTA/10mM-acetic acid/NaOH buffer, pH4.8, centrifuged at 150000***g*** for 40min and the supernatant was dialysed overnight against 8 litres of the same buffer. When assayed at pH4.7 and 7.0 ([^{3}H]phosphatidylinositol as substrate;

1mM-$CaCl_2$ present) the specific activities of the 22000*g* supernatant were in the ranges 1.7–3.0 and 7.3–9.1nmol/min per mg of protein respectively. Under the same assay conditions the specific activities of the dialysed 35–55%-saturated-$(NH_4)_2SO_4$ precipitate were in the ranges 2.7–3.7 and 10–20nmol/min per mg of protein respectively.

Chromatography on CM Bio-Gel A

The non-diffusible material was centrifuged at 150000*g* for 40min and the supernatant (180ml; approx. 1g of protein) was applied to a column (1.5cm × 43cm) of CM Bio-Gel A (Bio-Rad Laboratories, Richmond, CA, U.S.A), equilibrated with 1mM-EDTA/10mM-acetic acid/NaOH buffer, pH4.8. The column was washed with approx. 70ml of the same buffer and then eluted at a flow rate of 40ml/h with 1100ml of a linear gradient of 0–0.5M-NaCl in the same buffer; 16ml fractions were collected. Fractions were assayed for phospholipase C activity as described above and the most active (with [^{3}H]phosphatidylinositol as substrate) fractions in each peak were pooled. Peaks are identified as I–IV in the order in which they elute from the column.

Chromatography on Sephacryl S-300

The pooled fractions were adjusted to pH7.0 with 1M-imidazole and concentrated to approx. 8ml in an Amicon ultrafiltration cell with a YM-10 membrane. The concentrate was applied to a column (2.5cm × 117cm; V_0 219ml) of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) equilibrated with 0.25M-KCl/0.1mM-dithiothreitol/1mM-EDTA/10mM-imidazole/HCl, pH7.0. Elution was continued with the same buffer at a flow rate of 12ml/h and 7ml fractions were collected. The fractions containing the highest phospholipase C activity (with [^{3}H]phosphatidylinositol as substrate) were pooled and used for characterization studies. The apparent molecular weights of the phospholipase C peaks were estimated by calibrating this column with proteins of known molecular weight: catalase, aldolase, bovine serum albumin. ovalbumin, chymotrypsinogen A, ribonuclease and cytochrome *c*.

Results

Chromatography on CM Bio-Gel A

Chromatography of the dialysed 35–55%-satd.-$(NH_4)_2SO_4$ fraction on CM Bio-Gel A revealed a single sharp peak of phospholipase C activity when the assay was conducted at pH7.0 in the presence of 1mM-$CaCl_2$ (peak III; Fig. 1*a*). However, if the fractions were assayed at pH4.7 in the presence of 1mM-$CaCl_2$ two additional peaks were observed (peaks I and IV; Fig. 1*b*). Although peaks I and III were widely separated there was always measurable activity in the fractions between, suggesting the presence of another form of the enzyme, which was relatively inactive in both sets of assay conditions. This was confirmed when the fractions were re-assayed in the presence of sodium deoxycholate. Under these conditions an additional peak (peak II, Fig. 1*b*) was observed but peaks I and IV were then barely detectable.

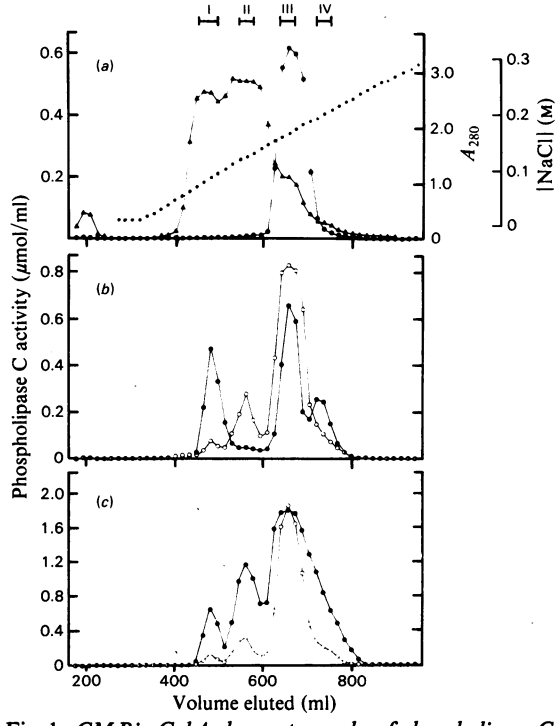

Fig. 1. *CM Bio-Gel A chromatography of phospholipase C*

Dialysed $(NH_4)_2SO_4$ fraction was applied to a column of CM Bio-Gel A, eluted as described in the Materials and methods section and the fractions were assayed for phospholipase C activity. The first 180ml eluted was collected as one fraction. The following substrates and assay conditions were used: (*a*) [^{3}H]phosphatidylinositol, 1mM-$CaCl_2$, pH7.0 (●); (*b*) [^{3}H]phosphatidylinositol, 1mM-$CaCl_2$, pH4.7 (●); [^{3}H]phosphatidylinositol, 5mM-$CaCl_2$, 0.1% (w/v) sodium deoxycholate, pH7.0 (○); (*c*) phosphatidylinositol 4-phosphate, 1mM-$CaCl_2$, pH6.6 (●); phosphatidylinositol 4,5-bisphosphate, 1mM-$CaCl_2$, pH6.6 (○). In (*a*) the A_{280} (▲) and NaCl concentration (····) are also shown. The values in these Figures were all obtained from one experiment; two other experiments gave similar results. Recovery of applied activity was 100%, 70% and 90% when assayed as described in the legends to Figs. 2(*a*), 3(*a*) and 4(*a*) respectively; specific activities of pooled fractions were 6.9, 2.3 and 60nmol/min per mg of protein for peaks I, II and III respectively. The fractions in each peak which were pooled are identified at the top of the Figure.

The fractions eluted from the CM Bio-Gel column were also assayed for phospholipase C activity using the polyphosphoinositides as substrates (Fig. 1*c*). Three peaks of activity were observed with both phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, which coincided with three of the peaks (peaks I, II and III; compare Figs. 1*a*–1*c*) already identified using phosphatidylinositol as a substrate. However, when the polyphosphoinositides were used as substrate there was no distinct peak of activity which corresponded to peak IV, even though these fractions contained significant activity against these substrates. Phospholipase C activity capable of hydrolysing the polyphosphoinositides was never reproducibly observed in fractions that had not previously been shown to contain phospholipase C activity against phosphatidylinositol.

Several unsuccessful attempts were made to resolve the different forms of phospholipase C in heart by the use of chromatofocusing. Activity was eluted over a wide pH range but no distinct peaks were observed. The situation was improved by using the modifications suggested by Hirasawa *et al.* (1982*a*,*b*) but the elution patterns obtained were not reproducible. An additional complication was the finding that phosphorus-containing compounds in the supernatant (or in the polybuffer used to elute the column) bound to the column and were eluted by 1 M-HCl. This prevented the use of these fractions for direct assay of polyphosphoinositide hydrolysis.

Chromatography on Sephacryl S-300

Fractions from the four peaks of phospholipase C activity obtained by CM Bio-Gel ion-exchange chromatography were applied to a column of Sephacryl S-300 (Figs. 2–4) and the eluent was assayed for phospholipase C activity with phosphatidylinositol as substrate (Figs. 2–4). In the case of I and III activity was restricted to a single peak (Figs. 2*a* and 4*a*), whereas II reproducibly exhibited a small additional peak with substantially lower molecular weight (Fig. 3*a*). The elution volumes for the major peaks differed slightly and although these differences were relatively small they were obtained in three separate experiments done with phospholipase C prepared from three different hearts (Table 1). Attempts were also made to analyse IV by chromatography on Sephacryl S-300. However, in this case when the pH of the pooled CM Bio-Gel fractions was adjusted to 7.0 protein precipitation and a substantial (approx. 75%) loss of phospholipase C activity was observed. Two peaks of activity were detected in the eluent from the Sephacryl S-300 column (Table 1) but because the levels of activity were very low it was not possible to characterize them further.

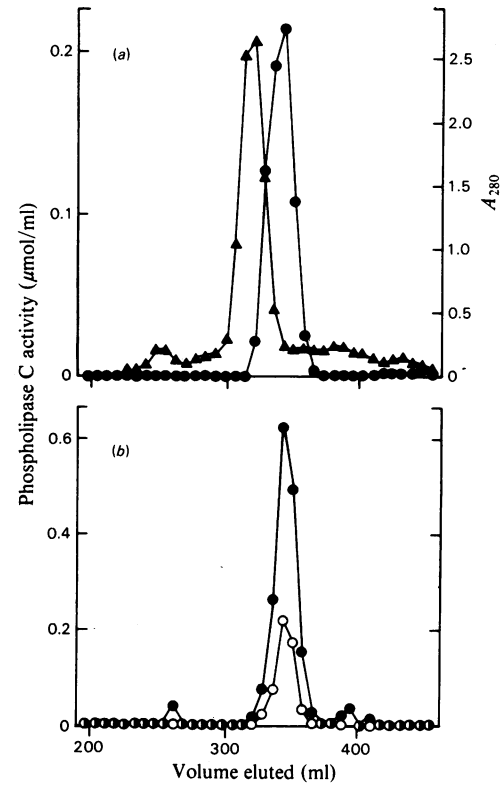

Fig. 2. *Sephacryl S-300 chromatography of phospholipase C (peak I)*

Pooled fractions (approx. 150 mg of protein) of the first peak eluted from the CM Bio-Gel A column were concentrated and applied to a column of Sephacryl S-300 (V_0 219 ml). The column was eluted and fractions assayed for phospholipase C activity as described in the Materials and methods section. The following substrate and assay conditions were used: (*a*) [^{3}H]phosphatidylinositol, 1 mM-$CaCl_2$, pH 4.7 (●); (*b*) phosphatidylinositol 4-phosphate. 1 mM-$CaCl_2$, pH 6.6 (●); phosphatidylinositol 4,5-bisphosphate, 1 mM-$CaCl_2$, pH 6.6 (○). In (*a*) the A_{280} (▲) is also shown. The values in these Figures were all obtained from one experiment; two other experiments gave similar results except that the small peaks eluted at 260 and 390 ml in (*b*) were not observed. Recovery of applied activity was approx. 40% when assayed as described in (*a*); specific activity of the pooled fractions was 6.2 nmol/min per mg of protein.

The fractions from the Sephacryl S-300 columns were also assayed for phospholipase C activity with polyphosphoinositides as substrates (Figs. 2*b*–4*b*). There was a close correspondence between the elution profiles obtained using the different substrates. Activity against the polyphosphoinositides was only detected in fractions that also contained activity against phosphatidylinositol; a similar result

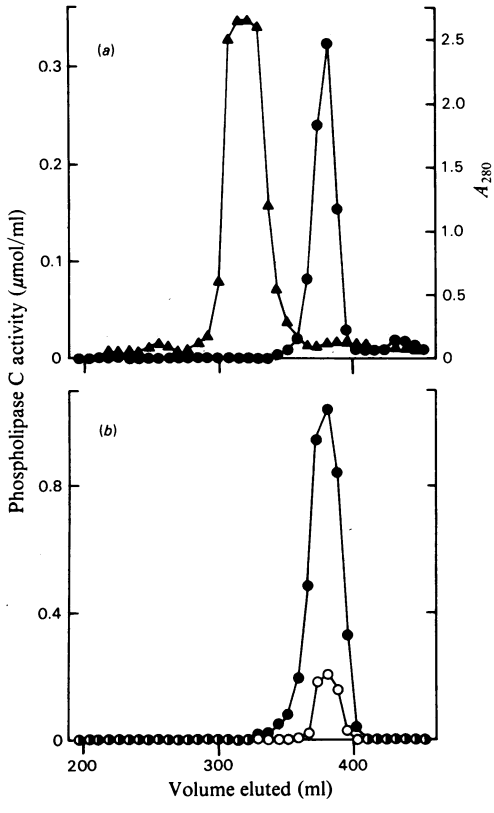

Fig. 3. *Sephacryl S-300 chromatography of phospholipase C (peak II)*

Pooled fractions (approx. 250mg of protein) of the second peak eluted from the CM Bio-Gel A column were concentrated and applied to a column of Sephacryl S-300, eluted and assayed for phospholipase C activity as described in the Materials and methods section. The substrates and assay conditions etc. were as described in the legend to Fig. 2 except that in (*a*) [^{3}H]phosphatidylinositol hydrolysis (●) was determined in the presence of 5mM-$CaCl_2$/0.1% (w/v) sodium deoxycholate, pH7.0. Values in these Figures were all obtained from one experiment; two other experiments gave similar results. Recovery of applied activity was approx. 40% when assayed as described in (*a*); specific activity of the pooled fractions was 152nmol/min per mg of protein.

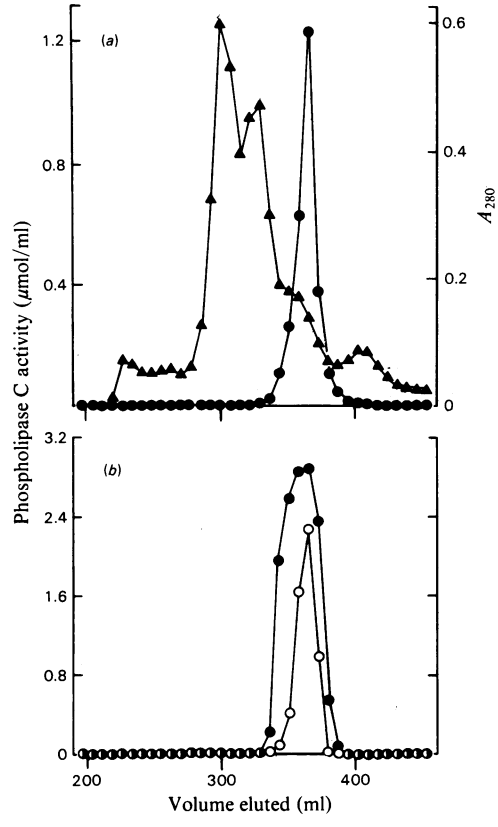

Fig. 4. *Sephacryl S-300 chromatography of phospholipase C (peak III)*

Pooled fractions (approx. 50mg of protein) of the third peak eluted from the CM Bio-Gel A column were concentrated and applied to a column of Sephacryl S-300, eluted and assayed for phospholipase C activity as described in the Materials and methods section. The substrates, assay conditions etc. were as described in the legend to Fig. 2 except that in (*a*) [^{3}H]phosphatidylinositol hydrolysis (●) was determined in the presence of 1mM-$CaCl_2$, pH7.0. Fractions were also assayed under the conditions described in the legend to Fig. 1(*b*) and similar elution profiles were obtained. Values in these Figures were all obtained from one experiment; two other experiments gave similar results. Recovery of applied activity was approx. 55% when assayed, as described, in (*a*); specific activity of the pooled fractions was 336nmol/min per mg of protein.

was obtained with IV (results not shown). The peak fractions from the Sephacryl S-300 columns were pooled and used for determination of the properties of the phospholipases as described below.

Specificity of the phospholipase C

The specificity of the phospholipases C was studied by three methods. First, their ability to hydrolyse phospholipids other than phosphoinositides was determined. Incubation of phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine (0.25mM at pH5.2, in the presence of 1mM-$CaCl_2$ for 1h at 37°C) with the three phospholipases (0.05ml portions) gave no detectable release of water-soluble organic phosphorus (i.e. <0.5nmol/h). Under similar conditions phospha-

Table 1. *Molecular weights of phospholipase C*

The molecular weights of phospholipase C peaks eluting from the CM Bio-Gel column were estimated by gel-filtration chromatography on Sephacryl S-300 as described in the Materials and methods section. Phospholipase C activity was assayed using [^{3}H]phosphatidylinositol as substrate as described in the legends to Figs. 2–4. The molecular-weight values given were derived from four separate ion-exchange experiments each done with a different heart. Values in parentheses refer to the minor peaks detected in II and IV.

		Molecular weight			
Experiment no.	Peak no. ...	I	II	III	IV
1		–	–	72000	–
2		120000	40000 (9500)	64000	–
3		105000	43000 (10400)	58000	271000 (120000)
4		111000	42000 (11400)	76000	256000 (120000)

tidylinositol was hydrolysed at a rate of 7.5, 5.0 and 600 nmol/h by I, II and III respectively. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were also resistant to hydrolysis (i.e. <0.5 nmol/h) by peak III at pH 7.0, in the presence of 1 mM-$CaCl_2$.

Considerable difficulty was experienced in obtaining consistent values for the relative rates of hydrolysis of the different phosphoinositides by the pooled fractions from the Sephacryl S-300 columns. It was observed that the activity of the pooled fractions was often considerably lower than expected from assay of individual fractions. The reason for this is not known, although during the assay the incubation mixtures frequently became turbid. It is possible that proteins in the eluate from the Sephacryl S-300 columns are interacting with the polyphosphoinositides in the assay and inhibiting enzyme activity. Formation of strong complexes between phosphatidylinositol 4,5-bisphosphate and protein in the presence of bivalent cations has previously been observed (Hendrickson, 1969).

The lipid products of phosphatidylinositol hydrolysis by the three phospholipases were determined. In all cases the only product was diacylglycerol; significant increases in monoacylglycerol or non-esterified fatty acid were not observed. Similar results were obtained with the polyphosphoinositides as substrates. The products of phosphatidylinositol hydrolysis by peak III at pH 8.5 were also determined; non-esterified fatty acid was not released under these conditions either indicating that an alkaline-active phospholipase A or other deacylating enzyme was not responsible for the activity. Only 1,2-diacylglycerol was produced at pH 5.2, but at higher pH, isomerization (either during the incubation or extraction) appears to occur since approximately equal amounts of 1,2- and 1,3-diacylglycerol were detected at pH 8.5.

Finally, the amount of polyphosphoinositide phosphomonoesterase activity was determined using either phosphatidylinositol 4-phosphate or phosphatidylinositol 4,5-bisphosphate as substrates. After incubation for 1 h at 37°C (pH 6.6, in the presence of 1 mM-$CaCl_2$) the release of P_i could not be detected (i.e., less than 5% of the phospholipase C activity). This absence of phosphomonoesterase activity was obtained using pooled fractions from the gel-filtration or the ion-exchange columns or using the 35–55%-satd.-$(NH_4)_2SO_4$ precipitate applied to the ion-exchange column. This demonstrates that the peaks of activity eluted from the ion-exchange or gel-filtration columns were due to phospholipase C and not phosphomonoesterase activity.

Effect of pH on phospholipase C activity

The effect of pH on the hydrolysis of phosphatidylinositol by the 22000 *g* myocardial supernatant and the $(NH_4)_2SO_4$ fractions is shown in Fig. 5(*a*). Activity in the supernatant is optimum at pH 5.5–6.0, but substantial activity is also present even at pH 9.0; between pH 6.5 and 8.5, the activity is little affected by the assay pH. Phospholipase C activity in other tissues has also been reported over this broad pH range and in some cases two distinct peaks of activity have been observed (Allan & Michell, 1974; Hirasawa *et al.*, 1981, 1982*a*,*b*). The 35–55%-satd.-$(NH_4)_2SO_4$ precipitate has a pH curve that is very similar in shape to the original supernatant (Fig. 5). This fraction contains most of the phospholipase C activity, although in some experiments a substantial (up to 50%) loss of activity was observed. However, there was no indication of a specific loss of activity measurable in the range of pH 7.0 to 9.0 as was reported by Hirasawa *et al.* (1982*a*), for the phospholipase C from brain. Activity was lost on dialysis of the

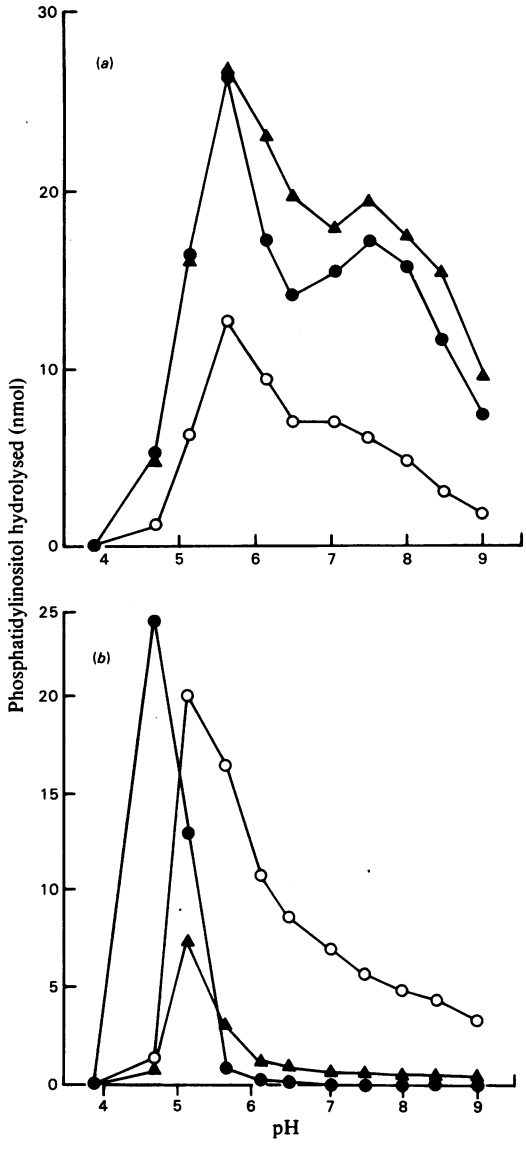

Fig. 5. *Effect of pH on phospholipase C activity*
Phospholipase C activity was determined at the pH values indicated in a series of 50mM-Tris/maleate buffers (1mM-$CaCl_2$) using [^{3}H]phosphatidylinositol as substrate. (*a*) 22000*g* supernatant (105μg of protein; ●), undialysed (△) and dialysed (○) 35–55%-satd.-$(NH_4)_2SO_4$ fractions were prepared as described in the Materials and methods section. In order to facilitate comparison of recoveries at different pH values samples of $(NH_4)_2SO_4$ fractions were diluted to the original volume of supernatant from which they were derived. (*b*) Peak I (27μg of protein; ●), peak II (7μg of protein; ▲) and peak III (1.8μg of protein; ○) were prepared by chromatography on Sephacryl S-300 as described in the Materials and methods section. Peaks I and II were assayed for 30min since their activities were relatively low compared with that of peak III. Values in these Figures are means of duplicate assays from single experiments. Similar results were obtained in a total of two to four experiments done with material obtained from different hearts.

35–55%-satd.-$(NH_4)_2SO_4$ precipitate at pH4.8, but even though this loss often seemed to be more pronounced when the assay was performed at pH7.0–9.0 compared with at pH5.0–6.0, substantial activity was always detectable at the higher pH values (Fig. 5*a*).

The efffect of pH on the hydrolysis of phosphatidylinositol by the three partially purified phospholipase C peaks is shown in Fig. 5(*b*). Peak I, which could only be detected by assay at acidic pH (Fig. 1), had a pH optimum of 4.5–5.0, with no activity at pH7.0. Peak III, by contrast, had a pH optimum in the range 5.0–5.5 but also exhibited substantial activity above neutrality. The broad asymmetric shape of this pH curve accounts for the detection of this form of the enzyme in the fractions from the CM Bio-Gel column when assayed at either pH7.0 or 4.7 (Fig. 1). Peak II (assayed in the absence of sodium deoxycholate) also displayed optimum activity at pH5.0–5.5 (Fig. 5*b*), with measurable activity at pH7.0. However, it is possible that this is due to contamination by peak III, which has the same pH optimum and is present in the eluate from the CM Bio-Gel column in much greater amounts.

Effect of Ca^{2+} on phospholipase C activity

The activities of all four phospholipases C were reduced (<0.5% of maximal activity) by inclusion of EGTA in the assay medium and were stimulated by the addition of $CaCl_2$; 2–5mM-Ca^{2+} gave maximum activity. Therefore they appear to be distinct from the Ca^{2+}-independent phospholipases reported in lysosomal fractions of liver and brain (Irvine *et al.*, 1978). Assay of peak III at pH7.0 in the presence of a series of EGTA-calcium buffers indicated that approx. 10% of maximal activity was present at 1μM free Ca^{2+}; under the same conditions peak I showed no detectable activity. Assay of peak II in the presence of 1μM free Ca^{2+} and deoxycholate at pH7.0 gave approx. 5% of maximal activity. It seems unlikely therefore that any of the major peaks isolated in the present study are related to the highly Ca^{2+}-sensitive forms of phospholipase C produced by proteolysis or autolysis of rat brain supernatant (Hirasawa *et al.*, 1982*c*). Furthermore, assay of the fractions eluted from the CM Bio-Gel column at pH7.0 in the presence of 1μM free Ca^{2+} did not reveal any new peaks that had maximal activity at this calcium concentration.

Effect of deoxycholate on phospholipase C activity

Peak II could only be distinguished in the fractions from the CM Bio-Gel column when the assay was conducted in the presence of sodium deoxycholate (see Fig. 1*b*). This apparent stimulatory effect of deoxycholate on the activity of peak II was therefore examined in more detail. At both 1mM- and 5mM-$CaCl_2$ (pH 7.0) deoxycholate produced a substantial increase in phosphatidylinositol hydrolysis. The maximum stimulation (about 20–30-fold) was produced by approx. 0.075% (w/v) deoxycholate; higher concentrations gave less stimulation. Deoxycholate also stimulated the activity of peaks I (>50-fold) and III (2–3-fold) when assayed at pH 7.0, in the presence of 1mM-$CaCl_2$; this effect was particularly striking with peak I since this form of the phospholipase C has no detectable activity at pH 7.0 in the absence of deoxycholate (Fig. 5*b*). These peaks were detectable in the CM Bio-Gel fractions in the absence of deoxycholate because they were assayed under conditions where they had substantial activity (i.e., pH 4.6 and 7.0; see Figs. 1 and 5*b*). Peak II by contrast has low activity at both these pH values if deoxycholate is not present (Figs. 1 and 5*b*).

Discussion

Previous work with the soluble fraction of brain and kidney has demonstrated the occurrence of a phospholipase C activity capable of hydrolysing phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate but not phosphatidylinositol (Thompson & Dawson, 1964; Tou *et al.*, 1973; Lapetina *et al.*, 1975). However, similar studies by others with the soluble fraction from intestinal mucosa, brain or iris smooth muscle revealed that all three phosphoinositides were hydrolysed to some extent (Atherton & Hawthorne, 1968; Keough & Thompson, 1972; Abdel-Latif *et al.*, 1980). Therefore, it is not clear whether hydrolysis of the phosphoinositides is catalysed by one enzyme with broad specificity for these three phospholipids or by several phospholipases that are relatively specific for individual phosphoinositides (Lapetina *et al.*, 1980; Shukla, 1982). This last possibility seems reasonable since several studies have shown that phospholipase C activity capable of hydrolysing phosphatidylinositol from brain, liver, kidney and platelets can be resolved into more than one form by ion-exchange chromatography or by chromatofocusing (Quinn, 1973; Hirasawa *et al.*, 1982*a*,*b*; Chau & Tai, 1982). Similarly, our preliminary results showed that phospholipase C from myocardial tissue (Low & Weglicki, 1982) could also be resolved into more than one form by ion-exchange chromatography (M. G. Low & W. B. Weglicki, unpublished work). However, in none of these studies was the specificity of the various forms of the phospholipase C for the different phosphoinositides compared.

In the present study ion-exchange chromatography of the beef heart supernatant revealed the presence of four distinct forms of phospholipase C activity using phosphatidylinositol as substrate under a variety of assay conditions. Three of these forms (I, II and III) coincided with the peaks of activity obtained when phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were used as substrates. Furthermore, gel-filtration chromatography of each of the three peaks from ion-exchange chromatography revealed similar elution patterns for phospholipase C activity with each of the three different substrates. However, a distinct peak of activity with the polyphosphoinositide substrates was not observed using peak IV from the ion-exchange column. The activity against polyphosphoinositides observed in these fractions might be due to contamination of a phosphatidylinositol-specific enzyme by the much greater and less specific peak III activity. However, gel-filtration chromatography of peak IV revealed two peaks of activity against all three substrates neither of which corresponded (in molecular weight) to peak III. These results demonstrate that the major forms of phospholipase C (detected using phosphatidylinositol as substrate) in heart and possibly in other tissues also, are capable of hydrolysing polyphosphoinositides as well as phosphatidylinositol. This result is not unexpected since there are no reports of phospholipase C activity (detected using phosphatidylinositol as substrate) being unable (when tested) to hydrolyse the polyphosphoinositides. However, there are several reported instances of phospholipase C activity specific for polyphosphoinositides. In our study many of the column fractions were assayed for activity against polyphosphoinositides even though they did not contain detectable activity against phosphatidylinositol. Phospholipase C activity against the polyphosphoinositides was found only in fractions that also contained significant activity against phosphatidylinositol. These results clearly indicate that phospholipases C specific for individual phosphoinositides do not form a large portion of the total cardiac phospholipase C activity.

At present there seem to be several possible explanations for our inability to detect polyphosphoinositide-specific phospholipase C activity in heart when such activities have been detected in other tissues. One explanation might be that the assay conditions we have used (e.g., low pH, deoxycholate etc.) are optimal for detection of phosphatidylinositol hydrolysis by the minor forms of the enzyme. For example, I and II have barely detectable activity against phosphatidylinositol at

pH 7 in the absence of deoxycholate (and therefore might not have been detected in previous studies) even though they are able to hydrolyse polyphosphoinositides under the same conditions. Another possibility is that the conditions of preparation inactivated the phospholipase C specific for polyphosphoinositides. This seems quite reasonable since substantial activity against phosphatidylinositol is lost during $(NH_4)_2SO_4$ precipitation (and during chromatography) as reported here (see Fig. 5*a*) and previously (Hirasawa *et al.*, 1982*a*). However, in this respect it is important to note that a similar $(NH_4)_2SO_4$ precipitation technique was used for enzyme preparation in the original reports of phospholipase C specific for polyphosphoinositides (Thompson & Dawson, 1964; Tou *et al.*, 1973; Lapetina *et al.*, 1975). A final possibility is that there are genuine differences in the specificities of phospholipase C contained in different tissues or cell types; if such differences exist and some tissues (e.g., brain and kidney) contain specific phospholipases C, then the application of the techniques described here and previously (Hirasawa *et al.*, 1982*a*,*b*) should be able to identify them.

In these studies relatively large amounts of myocardial tissue were required in order to detect the various forms of phospholipase C after column chromatography and therefore it was not feasible to centrifuge the supernatant at high speed to remove small membrane vesicles. Consequently the heterogeneity of the phospholipase C activity may have resulted in part from solubilization of a membrane-bound phospholipase C activity during the $(NH_4)_2SO_4$ precipitation. Membrane-bound phospholipase C activity has been demonstrated in erythrocytes and iris smooth muscle (Allan & Michell, 1978; Akhtar & Abdel-Latif, 1978; Abdel-Latif *et al.*, 1980; Downes & Michell, 1982). Phospholipase C activity has also been solubilized from the particulate fraction of brain by high-salt extraction (Keough & Thompson, 1972), but this may be due largely to release of trapped soluble activity (Irvine & Dawson, 1978). However, even though solubilization of membrane-bound phospholipase C activity might account for the present results it cannot readily explain the heterogeneity observed in other tissues where high-speed supernatants were used (Hirasawa *et al.*, 1982*a*,*b*; Chau & Tai, 1982).

The pH optima for activity in peaks I and III, although consistently different, were very close (pH 4.5–5.0 and 5.0–5.5 respectively). A more striking difference was the fact that peak I had no activity in the pH range 7.0–9.0, whereas peak III had substantial activity in this region. The reason for this broad pH dependency curve is not clear. However, Hirasawa *et al.* (1982*a*,*b*) reported that some of the fractions (pI 4.4–4.8) of phospholipase C activity obtained by chromatofocusing of brain, liver and kidney supernatants had similar activity at both pH 5.5 and 8.0, whereas others had activity only at the acidic pH. At present we cannot exclude the possibility that those chromatofocusing fractions and (in the present work) peak III contained additional forms of the phospholipase C that were not resolved by ion-exchange chromatography or chromatofocusing.

An unexpected result of these studies was the small but reproducible difference in the apparent molecular weight of peaks I, II and III obtained by gel-filtration chromatography. There appear to be no recent reports of such differences since the molecular weights of the multiple forms of brain, kidney, liver and platelet phospholipase C resolved by chromatofocusing and ion-exchange chromatography were not determined (Shen & Tai, 1981; Hirasawa *et al.*, 1982*a*,*b*; Chau & Tai, 1982). The molecular weights of purified liver and platelet phospholipase C have recently been determined (by gel-filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) and were found to be approx. 70000 and 140000 respectively (Takenawa & Nagai, 1981; Hakata *et al.*, 1982). However, these authors reported the existence of only one form of the enzyme even though others have shown that these tissues contain multiple forms of the enzyme (Hirasawa *et al.*, 1982*a*,*b*; Chau & Tai, 1982). There seem to be several possible explanations for these differences in the apparent molecular weight. First, the different forms of the enzyme may be a result of aggregation of phospholipase C either with itself or with other protein molecules, during preparation. The work of Quinn (1973) with phospholipase C from brain suggests that it binds to tubulin to produce several forms that can be distinguished by ion-exchange chromatography and density gradient centrifugation. We cannot exclude the possibility of aggregation in our studies since insufficient material was available to purify all three forms to homogeneity and compare their molecular weights as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. However, it seems unlikely that the differences in molecular weight reported for the purified phospholipases C from liver and platelets (Takenawa & Nagai, 1981; Hakata *et al.*, 1982) could have been due to aggregation since molecular-weight determinations by gel-filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were in close agreement. A further possibility is that the lower-molecular-weight forms of the phospholipase C were generated by proteolysis of a higher-molecular-weight precursor (e.g., peak I) during the dialysis at low pH before application to the CM Bio-Gel column. However, the pH-dependency curve of the original myocardial supernatant seems to be more similar to that

obtained with peak III than with peak I. This suggests that peak III was the major form of phospholipase C in the 22000*g* supernatant and the material applied to the CM Bio-Gel column as well as in the fractions eluted from the column. A final possibility is that some or all of these forms of the enzyme are structurally unrelated and formed from quite distinct polypeptide chains. The predominant form may be different in different tissues or cell types, which could explain the differences in molecular weight reported previously for the purified phospholipases C from liver and platelets (Takenawa & Nagai, 1981; Hakata *et al.*, 1982) and (in the present work) from a heterogeneous tissue such as heart. Presently we are unable to distinguish between these possibilities or to determine the physiological significance of the existence of distinct forms of the phospholipase C.

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References

Abdel-Latif, A. A., Luke, B. & Smith, J. P. (1980) *Biochim. Biophys. Acta* **614**, 425–434

Akhtar, R. A. & Abdel-Latif, A. A. (1978) *Biochim. Biophys. Acta* **527**, 159–170

Allan, D. & Michell, R. H. (1974) *Biochem. J.* **142**, 591–597

Allan, D. & Michell, R. H. (1978) *Biochim. Biophys. Acta* **508**, 277–286

Atherton, R. S. & Hawthorne, J. N. (1968) *Eur. J. Biochem.* **4**, 68–75

Baginski, E. S., Zak, B. & Foa, P. P. (1967) *Clin. Chem.* **13**, 326–332

Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468

Chau, L. Y. & Tai, H. H. (1982) *Biochim. Biophys. Acta* **713**, 344–351

Chau, L. Y., Tai, H. H. & Elko, E. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 1629

Downes, C. P. & Michell, R. H. (1982) *Biochem. J.* **202**, 53–58

Hakata, H., Kambayashi, J. & Kosaki, G. (1982) *J. Biochem. (Tokyo)* **92**, 929–935

Hendrickson, H. S. (1969) *Ann. N.Y. Acad. Sci.* **165**, 668–676

Hendrickson, H. S. & Ballou, C. E. (1964) *J. Biol. Chem.* **239**, 1369–1373

Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1982*a*) *Biochem. J.* **205**, 437–442

Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1982*b*) *Biochem. Biophys. Res. Commun.* **107**, 533–537

Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1982*c*) *Biochem. J.* **206**, 675–678

Irvine, R. F. & Dawson, R. M. C. (1978) *J. Neurochem.* **31**, 1427–1434

Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1978) *Biochem. J.* **176**, 475–484

Keough, R. M. W. & Thompson, W. (1972) *Biochim. Biophys. Acta* **270**, 324–336

Lapetina, E. G., Seguin, E. B. & Agranoff, B. W. (1975) *Biochim. Biophys. Acta* **398**, 118–124

Lapetina, E. G., Billah, M. M. & Cuatrecasas, P. (1980) in *The Regulation of Coagulation* (Mann, K. G. & Taylor, F. B., eds.), pp. 491–497, Elsevier/North-Holland, Amsterdam

Low, M. G. & Weglicki, W. B. (1982) *Circulation* **66**, II–110

Low, M. G. & Zilversmit, D. B. (1980) *Biochim. Biophys. Acta* **596**, 223–234

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Luthra, M. G. & Sheltawy, A. (1972) *Biochem. J.* **126**, 251–253

Quinn, P. J. (1973) *Biochem. J.* **133**, 273–281

Shen, R. F. & Tai, H. H. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 1629

Shukla, S. D. (1982) *Life Sci.* **30**, 1323–1335

Tai, C. L. & Tai, H. H. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 347

Takenawa, T. & Nagai, Y. (1981) *J. Biol. Chem.* **256**, 6769–6775

Thompson, W. & Dawson, R. M. C. (1964) *Biochem. J.* **91**, 237–243

Tou, J. S., Hurst, M. W., Baricos, W. H. & Huggins, C. G. (1973) *Arch. Biochem. Biophys.* **154**, 593–600

Wagner, H., Lissau, A., Holzl, J. & Horhammer, L. (1962) *J. Lipid Res.* **3**, 177–180