Characterization of multiple forms of phosphoinositide-specific phospholipase C purified from human platelets

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The origin and physiological significance of the multiple M_r forms of phosphoinositide-specific phospholipase C in human platelets were investigated. The higher- M_r (400 000 and 270 000) forms of the phospholipase C were converted into the 100 000- M_r form without substantial loss of activity by incubation with a Ca^{2+} -dependent proteinase partially purified from human platelets. These three forms of the phospholipase C were purified approx. 200–500-fold from outdated human platelet supernatants. SDS/polyacrylamide-gel electrophoresis and gel-filtration analysis suggested that the higher- M_r forms of phospholipase C were complexes of 140 000- M_r subunits, whereas the lower- M_r form consisted of a single 95 000- M_r subunit. The substrate specificity of the purified phospholipase C was investigated by using ^{32}P -labelled polyphosphoinositide substrates purified from human platelets by a new method utilizing h.p.l.c. on an amino column. Activity against all three phosphoinositides was detected at micromolar concentrations of Ca^{2+} ; this hydrolysis was markedly stimulated by phosphatidylethanolamine and inhibited by phosphatidylcholine. Comparison of the different forms of purified phospholipase C revealed no major differences in Ca^{2+} -sensitivity or substrate specificity. Thus, although the suggestion that the high- M_r forms of human platelet phosphoinositide-specific phospholipase C were converted into a lower- M_r form by a Ca^{2+} -dependent proteinase has been substantiated, the physiological significance of this process remains to be determined.

INTRODUCTION

There is general agreement that the stimulated step in phosphoinositide metabolism is hydrolysis by phosphoinositide-specific phospholipase C, but, even though this enzyme has been studied in many tissues, the mechanisms by which it is regulated are unknown. Most recent evidence indicates that stimulated phosphoinositide metabolism in intact tissues and cells is not regulated by changes in intracellular Ca^{2+} concentrations and may involve hydrolysis by the phospholipase C of only one of the phosphoinositides (for reviews, see Berridge, 1984; Creba *et al.*, 1983). However, the isolated enzymes appear to be Ca^{2+} -dependent and exhibit little specificity towards the individual phosphoinositides (Low & Weglicki, 1983; Rittenhouse, 1983; Wilson *et al.*, 1984). This paradox appears to have been resolved now, since Irvine *et al.* (1984) have shown that, under conditions of Ca^{2+} concentration and substrate presentation which approximate to those found in the cell, phospholipase C in rat brain supernatants is considerably more active against phosphatidylinositol 4,5-bisphosphate than against phosphatidylinositol. It was also demonstrated that hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C was more sensitive to the lipid environment of the substrate than to changes in the Ca^{2+} concentration over the range likely to occur during cell stimulation. Consequently, they proposed that perturbation of the lipid bilayer was more likely as a controlling mechanism than changes in intracellular Ca^{2+} concentration (Irvine *et al.*, 1984).

An additional area of uncertainty concerns the M_r of the phospholipase C, since previous studies have shown

that many tissues contain multiple M_r forms of phospholipase C (Low *et al.*, 1984, and references therein). However, even though the existence of these multiple forms may account for the discrepancies in the reported M_r values for purified phospholipase C (Takenawa & Nagai, 1981; Hofmann & Majerus, 1982; Hakata *et al.*, 1982), their origin and physiological significance remain obscure. Furthermore, it is not clear whether the differences in molecular and enzymological properties of these multiple forms which have so far been identified *in vitro* (for references, see Low *et al.*, 1984; Nakanishi *et al.*, 1985) would result in differences in activity or response to regulatory factors in the intact cell.

In human platelets, unlike the other tissues tested, it appears that the principal low- M_r form of phospholipase C results from degradation of a higher- M_r form by a Ca^{2+} -dependent proteinase (Low *et al.*, 1984). In the present study, we have purified these forms of human platelet phospholipase C in order to study the proteolytic conversion in more detail. We have also compared their enzymological properties in order to determine if proteolytic conversion could have some role in intracellular regulation of phospholipase C activity.

MATERIALS AND METHODS

Purification of phospholipase C from outdated platelets

Outdated human platelets were obtained from the Oklahoma Blood Institute and used within 2 weeks of the expiration date. The elution volumes for phospholipase C peaks obtained by gel-filtration chromatography of supernatant from these platelets were similar to the values

obtained in a previous study using fresh platelets (Low *et al.*, 1984). All purification steps were done at 0–4 °C. The platelet suspensions were sedimented at 3500 *g* for 30 min. The pellets were washed once in one-half the original volume and once in one-fifth the original volume with 113 mM-NaCl/4.3 mM-K₂HPO₄/4.3 mM-Na₂HPO₄/24 mM-NaH₂PO₄/5.5 mM-glucose/5 mM-EGTA/NaOH, pH 7.0. The washed platelets were finally resuspended at a concentration of 20% (v/v) with 0.15 M-NaCl/10 mM-Hepes/0.1 mM-dithiothreitol/20 mM-EGTA/NaOH, pH 7.0, and sonicated with a Branson probe sonicator, model 350, at setting 5 for 2.5 min. The sonicated platelets were centrifuged at 33000 *g* for 3 h to remove the membranes, and the resulting supernatant was stored at –20 °C until required.

Thawed supernatant (200–250 ml) was centrifuged at 15000 *g* for 30 min to remove any precipitate that formed during freezing and thawing. The supernatant (sp. activity approx. 0.005 μ mol/min per mg) was diluted with an equal volume of water, adjusted to pH 8.0 with 2 M-Tris and applied to a column (4.5 cm $\times$ 30 cm) of DEAE-Bio-Gel A (equilibrated in 10 mM-Tris/1 mM-EDTA/0.1 mM-dithiothreitol, pH 8.0) at a flow rate of 100 ml/h. The column was eluted with approx. 200 ml of equilibrating buffer and then a linear 3-litre gradient which gave a final concentration of 0.5 M-NaCl in the equilibrating buffer; 18 ml fractions were collected. A broad peak of phospholipase C activity was eluted after approx. 1100 ml of the gradient; the most active fractions in this peak (generally about 200 ml total volume) were pooled, sufficient 100 mM-EDTA/NaOH, pH 7.0, was added to give a final concentration of 10 mM, and the solutions were concentrated in an Amicon ultrafiltration cell with a YM10 membrane to a volume of 40 ml. The concentrate was diluted with an equal volume of 10 mM-EDTA/0.1 mM-dithiothreitol/20 mM-Hepes/NaOH, pH 7.0, and re-concentrated to a volume of approx. 10 ml. The pH was adjusted to 7.8 with 1 M-imidazole and the concentrate centrifuged at 150000 *g* for 40 min. One-half of the supernatant was applied to a Mono Q HR5/5 column (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.), equilibrated with 0.15 M-NaCl/0.1 mM-EDTA/0.1 mM-dithiothreitol/20 mM-Hepes/NaOH, pH 7.6, at a flow rate of 1 ml/min. The column was eluted at the same flow rate with a linear 20 ml gradient (final concn. of 0.5 M-NaCl in the equilibration buffer). Fractions (0.5 ml) were collected in tubes containing 0.025 ml of 100 mM-EDTA/NaOH, pH 7.4. This same procedure was used for the other half of the Amicon concentrate. Phospholipase C activity was eluted at approx. 10 ml after the start of the gradient (approx. 0.3 M-NaCl), and peak fractions from both chromatographic separations (total volume 6–8 ml) were pooled.

Solid (NH₄)₂SO₄ was added to give a concentration of 1 M, the pH was re-adjusted to 7.6 with 1 M-HCl and the mixture centrifuged at 15000 *g* for 20 min to remove the precipitate. One-half of the supernatant (approx. 4 ml) was applied to a 7.5 mm $\times$ 75 mm Bio-Gel Phenyl TSK 5-PW column (Bio-Rad Laboratories, Richmond, CA, U.S.A.) equilibrated with 1 M-(NH₄)₂SO₄/0.1 mM-EDTA/0.1 mM-dithiothreitol/20 mM-Hepes/NaOH, pH 7.6, at a flow rate of 0.5 ml/min, followed by 5 ml of the same buffer, 15 ml of a decreasing linear gradient [final buffer as above, but without the 1 M-(NH₄)₂SO₄] and finally a further 10 ml of the buffer without

(NH₄)₂SO₄; 1 ml fractions were collected. This same procedure was used for the other half of the pooled fractions from the Mono Q column. Two peaks of activity, designated phenyl fractions A and B, were eluted, at 13–14 ml and 17–18 ml respectively after the start of the gradient. Peak fractions were pooled and concentrated to approx. 0.4 ml in a Centricon 10 apparatus (Amicon, Danvers, MA, U.S.A.). The concentrate was re-diluted to 2 ml with 0.15 M-NaCl/0.1 mM-EDTA/0.1 mM-dithiothreitol/10 mM-Hepes/NaOH, pH 7.0, and re-concentrated twice. The final concentrate (approx. 0.4 ml) was applied to a 7.5 mm $\times$ 300 mm Bio-Sil TSK-250 column (with a 7.5 mm $\times$ 75 mm Bio-Sil TSK-125 guard column) at a flow rate of 0.2 ml/min essentially as described by Low *et al.* (1984). Peaks I, II and IV were pooled on the basis of the elution volumes previously reported (Low *et al.*, 1984), i.e. 7.2–8.0, 8.0–8.8 and 9.6–10.4 ml respectively.

The procedure described above gave approx. 200–600-fold purification of peak-II enzyme (i.e. the major form in fresh human platelets), with a specific activity of approx. 1.2–3.4 μ mol/min per mg. Although not completely homogeneous, this compares favourably with previous attempts to purify the phospholipase C from human and bovine platelets (Hakata *et al.*, 1982; Chau & Tai, 1982; Nakamura *et al.*, 1985). When they were present in the original supernatant, the specific activities of the other forms of platelet phospholipase C were similar to those of peak II. This is consistent with the observation that peak II is converted into peak IV with relatively little loss in activity (Low *et al.*, 1984).

Specific activities and recoveries for individual steps in the purification procedure were monitored, but varied considerably between preparations, probably as a result of the variation in the distribution of the different *M_r* forms. In general, purifications of approx. 3–5-fold with approx. 50% recovery were obtained for each step.

Purification of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate

Platelets prepared from 60 ml of fresh human blood were labelled with [³²P]P_i (Carroll *et al.*, 1982). The lipids were extracted with chloroform/methanol/HCl (500:1000:13, by vol.) for 30 min at 25 °C. The dried extract was redissolved in 0.5 ml of solvent A (chloroform/methanol/water, 20:9:1, by vol.) and applied at a flow rate of 1 ml/min to a 4.5 mm $\times$ 250 mm Amino-NP column (5 μ m spherical silica with n-propylamine bonded phase; IBM Instruments, Danbury, CT, U.S.A.) equilibrated in solvent A. The column was eluted with 5 ml of solvent A, a 25 ml linear gradient from 100% solvent A to 100% solvent B (0.6 M-ammonium acetate dissolved in solvent A), and 20 ml of solvent B; 1 ml fractions were collected and radioactivity was determined by liquid-scintillation counting (a typical elution profile is shown in Fig. 1a). Approx. 2–6 μ Ci of [³²P]phosphatidylinositol 4-phosphate and 1–3 μ Ci of [³²P]phosphatidylinositol 4,5-bisphosphate were obtained by this procedure.

A methanol precipitate (approx. 150 μ mol of lipid P) prepared from 1 g of bovine brain, Folch fraction I (type I brain extract from Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in 5 ml of solvent A and was applied at a flow rate of 2.5 ml/min to a 10 mm $\times$ 250 mm amino column (IBM Instruments) with a 4.5 mm $\times$ 50 mm guard column (same packing material) equilibrated with solvent A. The column was eluted at a

Fig. 1. Purification of polyphosphoinositide substrates by h.p.l.c.

Polyphosphoinositides were purified by h.p.l.c. as described in the Materials and methods section. (a) Elution profile of a lipid extract of $[^{32}\text{P}]\text{P}_i$ -labelled human platelets. Peaks eluted at the volumes specified were identified by comparison with the elution volumes of standard lipids: phosphatidylcholine (5 ml), phosphatidic acid and phosphatidylinositol (21–22 ml), phosphatidylinositol 4-phosphate (33 ml) and phosphatidylinositol 4,5-bisphosphate (39 ml). The peak eluted at 29 ml was reproducibly present, but was not identified. (b) Elution profile of acid-washed methanol-precipitated Folch Fraction I from bovine brain. Phosphatidylinositol 4-phosphate and 4,5-bisphosphate were eluted at 140–150 ml and 170–180 ml respectively. The peak eluted at 85–95 ml was not analysed further, but was probably a mixture of phosphatidylserine and phosphatidylinositol.

flow rate of 2.5 ml/min with 25 ml of solvent A, a 125 ml gradient of 100% solvent A to 100% solvent B, and 100 ml of 100% solvent B; 5 ml fractions were collected. The fractions were assayed for organic P; a typical elution profile is shown in Fig. 1(b). This procedure produced approx. 10–15 μmol each of phosphatidylinositol 4-phosphate and 4,5-bisphosphate. Purity of the polyphosphoinositides was checked by t.l.c. on oxalate/EDTA-impregnated plates (Wilson *et al.*, 1984).

Phospholipase C assays

Phospholipase C activity in column fractions was routinely assayed with $[^3\text{H}]\text{phosphatidylinositol}$ as substrate by the procedure described previously (Low & Weglicki, 1983). Incubations were done at 37 °C for 10–30 min and contained 5 mM- CaCl_2 , 0.1% (w/v) sodium deoxycholate and 25 mM-Tris/maleate buffer, pH 7.0.

The Ca^{2+} -sensitivity and substrate specificity of the phospholipase C was assayed by a modified version of the above procedure. All of these assays were incubated at 37 °C for 15 min and contained 80 mM-KCl/1 mM- MgCl_2 /50 mM-Tris/maleate, pH 7.0, and substrate (see below). All incubations also contained 18.75 mM-NaCl contributed by the TSK-250 column buffer (a total of 0.05 ml added to a final assay volume of 0.4 ml) in which the phospholipase C was finally purified. In many assays, the Ca^{2+} concentration was varied, by using Ca^{2+} -EGTA buffers. All Ca^{2+} -EGTA buffers contained 2 mM-EGTA (final concn.) and the appropriate amount of CaCl_2 to give the desired Ca^{2+} concentration (Raaflaub, 1956); for Ca^{2+} concentrations of 0.1 mM and above the appropriate amount of CaCl_2 was added in excess of the EGTA (2 mM final concn.). Other variations to this basic assay are indicated in the text or Figure legends. The pure substrates were used (0.1 mM final concn. in assay) unless specified otherwise. These were prepared by mixing (in chloroform) $[^3\text{H}]\text{phosphatidylinositol}$ (Low & Weglicki, 1983) or individual $[^{32}\text{P}]\text{polyphosphoinositides}$ (prepared from human platelets; see above) with unlabelled phosphatidylinositol (soya-bean; Serdary, London,

Ontario, Canada) or unlabelled polyphosphoinositides (prepared from bovine brain; see above) respectively. In some experiments, other lipids were added and mixed in chloroform with the polyphosphoinositide substrate (0.05 mM). Phosphatidylcholine (pig brain; Serdary) and phosphatidylethanolamine (pig liver; Serdary) were added to give a final concentration of 0.5 mM. In experiments designed to measure activity in the presence of competing substrates, a mixture containing phosphatidylinositol (0.5 mM final concn.), phosphatidylinositol 4-phosphate (0.05 mM), phosphatidylinositol 4,5-bisphosphate (0.05 mM), phosphatidylethanolamine (2.5 mM) and the appropriate radiolabelled phosphoinositide was used.

Phosphomonoesterase activity was determined by using a sample of the upper phase from the chloroform/methanol extraction by extracting the $[^{32}\text{P}]\text{P}_i$ from the mixture as a complex with molybdate (Downes *et al.*, 1982). No significant phosphomonoesterase activity (i.e. less than 1% of the phospholipase C activity) was detected in the human platelet phospholipases C purified as described above.

Miscellaneous assays

Protein was assayed by a modification of the Coomassie Blue procedure of Bradford (1976), with bovine serum albumin as a standard. Ca^{2+} -dependent proteinase was assayed by using ^{125}I -labelled casein essentially as described by Kishimoto *et al.* (1983). Organic P was determined by the method of Bartlett (1959). M_r values of purified phospholipases C were determined by high-performance gel-filtration chromatography on TSK-250 (Low *et al.*, 1984) or by SDS/polyacrylamide-gel electrophoresis (Studier, 1973).

RESULTS

Relationship between high- and low- M_r forms of platelet phospholipase C

Previous studies (Low *et al.*, 1984) had indicated that one of the high- M_r forms of human platelet phospholipase

Fig. 2. Effect of Ca^{2+} -dependent proteinase on M_r of human platelet phospholipase C

Supernatants from outdated human platelets were chromatographed on a Mono Q anion-exchange-chromatography column (see the Materials and methods section) to resolve the phospholipase C and Ca^{2+} -dependent proteinase. The high- M_r forms of phospholipase C were further purified by gel-filtration chromatography on TSK-250. The phospholipase C was incubated (1 mM- Ca^{2+} , pH 7.0) at 4 °C for 20 h in the presence ($\square$) or absence ($\blacksquare$) of the platelet proteinase. The incubation mixtures were then applied to the TSK-250 gel-filtration column, eluted and assayed for phospholipase C activity as described in the Materials and methods section. The proteinase used in these experiments did not contain detectable phospholipase C activity.

C appeared to be converted into a lower- M_r form, which was still active, by a Ca^{2+} -dependent proteinase. We therefore attempted to isolate the proteinase responsible. Supernatants from outdated platelets were chromatographed on Mono Q to separate the proteinase and the phospholipase C. The fractions containing phospholipase C were further purified by gel-filtration chromatography to give a preparation which contained only the higher- M_r forms. Incubation of this phospholipase C with the partially purified proteinase (which did not itself contain detectable phospholipase C activity) resulted in its conversion into a form with an M_r value similar to that of peak IV (Fig. 2). This observation supported the suggestion that a Ca^{2+} -dependent proteinase was responsible for the changes in M_r observed previously (Low *et al.*, 1984). However, although a similar result was obtained in several independent experiments, difficulty was experienced in obtaining proteinase preparations which reproducibly gave this effect; this precluded further purification and characterization of the proteinase(s) responsible. This variability could be due to a number of factors, e.g. auto-digestion of the proteinase to a less Ca^{2+} -sensitive form (Zimmerman & Schlaepfer, 1984) or inhibition by an endogenous factor such as 'calpastatin' (Murachi *et al.*, 1980; Murachi, 1983).

M_r and subunit structure of purified phospholipase C

The M_r values of the purified platelet phospholipases C were determined by rechromatography on the TSK-250 gel-filtration column. These experiments showed that the purified phospholipases C have similar M_r values (approx. 400 000, 270 000, 140 000 and 100 000 for peaks I–IV respectively) to those observed for peaks of phospholipase C activity in crude cytosol from fresh platelets (Low *et al.*, 1984). They also showed that peaks I and II contained relatively little peak IV, and vice versa (Fig. 3). This indicated that neither peak I nor peak II was likely to be a simple aggregate of peak IV. By contrast,

Fig. 3. Gel-filtration chromatography of purified human platelet phospholipase C

Phospholipase C purified from outdated human platelets was applied to a TSK-250 gel-filtration column eluted and assayed as described in the Materials and methods section. (a) $\blacksquare$, Peak II; $\square$, peak IV. (b) $\blacksquare$, peak I; $\square$, peak IIIB, i.e. activity eluted at 9.2–9.6 ml during preparative gel-filtration chromatography of phenyl-column fraction B.

peaks I and II appeared to be reversibly converted into one another, since rechromatography of each peak usually revealed the presence of considerable activity that was eluted in a position corresponding to the other peak (results not shown). The factors which govern the apparently reversible conversion of peaks I and II have not been studied in detail. However, it is relevant to note that both forms are present in fresh platelet cytosol (Low *et al.*, 1984), and under conditions of ionic strength approximating to those found in the intact cell. Activity eluted between peaks II and IV during the final purification step on TSK-250 was also rechromatographed in an attempt to locate peak III. Activity eluted in this region, although prominent in many mammalian tissues, is only observed in human platelet cytosol that has undergone Ca^{2+} -dependent proteolysis and even then is present as a poorly resolved shoulder on peak IV (see Low *et al.*, 1984). However, as shown in Fig. 3(b), peak III does appear to be a distinct entity; it is also present in both the A and B fractions from the phenyl column. The properties of this peak-III form of phospholipase C were not studied further, since it was always contaminated with peak II or IV (Fig. 3b). In the purification scheme utilized here, activity was eluted from the hydrophobic phenyl TSK 5-PW column in two distinct fractions (fractions A and B, see the Materials and methods section). However, gel-filtration chromatography of these two fractions revealed that they contained a mixture of the different M_r forms. Thus fraction A contained mostly peaks I and II, with very little of peak IV. Fraction B, on the other hand, usually contained a mixture of approximately equal amounts of all three peaks. Similar M_r values were obtained for these peaks derived from either the A or the B phenyl-column fractions.

The purified platelet phospholipases C were also analysed by SDS/polyacrylamide-gel electrophoresis in order to determine the subunit structure. Although it was not completely homogeneous, it was possible to identify the phospholipase C polypeptide in several preparations by the following criteria: (i) the relative amounts of the polypeptide in fractions eluted from the TSK-250 column corresponded closely to the relative phospholipase C activities in these fractions; (ii) it was the major polypeptide in the peak fraction(s); (iii) other polypeptides were not consistently present in the different preparations

analysed. The M_r values obtained from these analyses were approx. 140000 for peaks I and II and approx. 95000 for peak IV. It is relevant to note that electrophoretic analysis of peak IV did not reveal the presence of any bands in the M_r -140000 region. Conversely, peaks I and II did not contain any bands in the M_r -95000 region of the gel. The data from this electrophoretic analysis were consistent with the conclusion from the gel-filtration experiments (see above), i.e. that peak I is interconvertible with peak II, but peaks I or II are not themselves derived from peak IV by aggregation. Instead, since peak IV has a lower subunit M_r than either peak I or II, these data suggest that peak IV is derived from peak II by a proteolytic cleavage.

Substrate specificity and Ca^{2+} -sensitivity of purified platelet phospholipase C

As reported previously (Chau & Tai, 1982; Rittenhouse, 1983; Lenstra *et al.*, 1984; Nakamura *et al.*, 1985), the activity of phospholipase C from the human platelet was Ca^{2+} -dependent; however, the Ca^{2+} concentration required for maximal activity depended on the substrate. Phosphatidylinositol 4,5-bisphosphate showed maximal activity in the 0.01–0.1 mM range, whereas phosphatidylinositol 4-phosphate exhibited a marked stimulation of activity at Ca^{2+} concentrations in excess of 0.1 mM (Fig. 4a). Phosphatidylinositol also gave maximal activity in the millimolar range (results not shown), but significant activity was also detected at 1 μM - Ca^{2+} (about 30% of that given by phosphatidylinositol 4,5-bisphosphate). Although activity at 1 mM- Ca^{2+} against phosphatidylinositol 4-phosphate was substantially greater than that against phosphatidylinositol 4,5-bisphosphate, the activities at physiological Ca^{2+} concentrations were closer (Fig. 4a). These experiments were done in the presence of 80 mM-KCl and 1 mM- MgCl_2 in an attempt to duplicate the intracellular ionic environment (Irvine *et al.*, 1984), and under these conditions phosphatidylinositol 4-phosphate is the preferred substrate (Fig. 4a). However, if assays were done without these additions, the activity against phosphatidylinositol 4,5-bisphosphate was the same as, or slightly greater than, that exhibited against phosphatidylinositol 4-phosphate (results not shown).

The effect of the addition of other membrane phospholipids to the substrate on phospholipase C

Fig. 4. Effect of Ca^{2+} on polyphosphoinositide hydrolysis by human platelet phospholipase C

Phosphatidylinositol 4-phosphate (□) and phosphatidylinositol 4,5-bisphosphate (■) were incubated with purified human platelet phospholipase C at the Ca^{2+} concentrations indicated and hydrolysis determined as described in the Materials and methods section. Purified phospholipase C (phenyl-column fraction B) was from: (a) peak II (0.7 μg); (b) peak IV (1.0 μg).

Fig. 5. Effect of phosphatidylethanolamine and phosphatidylcholine on hydrolysis of phosphatidylinositol 4,5-bisphosphate by human platelet phospholipase C

Phosphatidylinositol 4,5-bisphosphate, mixed with phosphatidylethanolamine (□) or phosphatidylcholine (○) or no additions (■) at a molar ratio 10:1, was incubated with purified human platelet phospholipase C at the Ca^{2+} concentrations indicated, and hydrolysis was determined as described in the Materials and methods section. Values are given as phospholipase C activities relative to those exhibited at $1 \mu\text{M-Ca}^{2+}$, no additions, which were 0.11 and $0.04 \mu\text{mol/min per mg}$ of protein for (a) and (b) respectively. Purified phospholipase C (phenyl-column fraction B) was from: (a) peak II; (b) peak IV.

activity was also examined. The hydrolysis of phosphatidylinositol 4,5-bisphosphate, when the Ca^{2+} concentration was in the range $0.1\text{--}10 \mu\text{M}$, was markedly stimulated by the addition of a 10-fold molar excess of phosphatidylethanolamine to the substrate (Fig. 5a) and approached the activity attained when phosphatidylinositol is used as substrate under optimal conditions (i.e. 5 mM-Ca^{2+} , 0.1% deoxycholate). Conversely, inhibition (approx. 50% at $1 \mu\text{M-Ca}^{2+}$) of activity was observed when phosphatidylcholine was used instead of phosphatidylethanolamine (Fig. 5a). Similar stimulatory and inhibitory effects of phosphatidylethanolamine and phosphatidylcholine were observed when phosphatidylinositol 4-phosphate was used as a substrate at these Ca^{2+} concentrations (results not shown). By contrast, in all these substrate mixtures, the increases in activity occurring when the Ca^{2+} concentration was raised from 0.1 to $1 \mu\text{M}$ (i.e. 2–4-fold) were much smaller (Fig. 5a).

An attempt was also made to assess the relative rates of hydrolysis of the individual phosphoinositides in the presence of the competing substrates. To duplicate the relative intracellular concentrations more closely, phosphatidylinositol and phosphatidylethanolamine were present at 10- and 50-fold greater concentrations respectively (see the Materials and methods section) than the polyphosphoinositides. Under these conditions (in contrast with the pure substrate), phosphatidylinositol 4,5-bisphosphate was hydrolysed at approx. 50% faster rate than the 4-phosphate; phosphatidylinositol was hydrolysed at about 50% the rate of the 4-phosphate.

We also compared the properties of the various forms of purified phospholipase C with respect to the properties described above; some of the data for one of the low- M_r forms are shown in Figs. 4(b) and 5(b). All the forms exhibited qualitatively similar Ca^{2+} -sensitivities, as judged by the concentration required for maximal activity and response to Ca^{2+} concentrations in the physiological range. The relative substrate specificities at $1 \mu\text{M-Ca}^{2+}$ for the three phosphoinositides were also similar. Minor quantitative differences in some of the properties were sometimes observed, but these differences were not reproducible between different preparations of the purified phospholipase C forms. The stimulation of

activity by phosphatidylethanolamine and inhibition by phosphatidylcholine were also exhibited by the other forms of the phospholipase C. However, the stimulatory effect of phosphatidylethanolamine on phosphatidylinositol 4,5-bisphosphate hydrolysis appeared to be substantially higher for peak II than it did for peak IV (Fig. 5). For example, at $1 \mu\text{M-Ca}^{2+}$ peaks II and IV were stimulated 40-fold and 9-fold respectively. This result was obtained with these forms of phospholipase C from two different preparations.

DISCUSSION

Our previous studies had indicated that, although many tissues contained multiple M_r forms of phospholipase C, brain and platelets were distinct from the other tissues in that they contained mainly the high- M_r forms. However, that study (Low *et al.*, 1984) only used gel filtration for determination of M_r , and the possibility that the different forms were simply aggregates either with other proteins or with other phospholipase C molecules could not be precluded. Phospholipase C had not been purified previously from human platelets or brain, and estimates of the M_r for phospholipase C in human platelet cytosol (by gel filtration; Lenstra *et al.*, 1984) were much lower than ours (Low *et al.*, 1984). In the present study, therefore, the subunit M_r values were determined by SDS/polyacrylamide-gel electrophoresis. Although the purified phospholipase C preparations were not completely homogeneous, the results obtained allow some general conclusions to be drawn about the origin of the multiple forms. In human platelets, peaks I and II appear to be complexes containing two or more subunits each of M_r 140000, whereas peak IV is a monomer of M_r 950000. Activity corresponding to peak III, which in human platelets appeared only as a shoulder on peak IV (Low *et al.*, 1984), was identified during our purification scheme, but because of its low yield could not be purified further, and consequently no information on its subunit structure could be obtained. The differences in subunit M_r obtained for the purified phospholipase C are consistent with the observation reported here and

previously (Low *et al.*, 1984) that peaks I and II are converted into peak IV by Ca^{2+} -dependent proteinase.

The value of approx. 140000 obtained for the subunit M_r of the major phospholipase C species in human platelets is in good agreement with that obtained (i.e. 143000) by Hakata *et al.* (1982), with phospholipase C purified from bovine platelets. It is therefore rather surprising that they also obtained an M_r of 140000 by gel filtration, since our previous studies (Low *et al.*, 1984) show that bovine platelet cytosol, in common with human platelets, contained mainly the peak-II form of phospholipase C. This difference may be due to the relatively low ionic strength used for gel-filtration analysis of the purified bovine platelet phospholipase C or to irreversible dissociation by the high concentration of ethylene glycol used to elute the phospholipase C from the octyl-Sepharose column during purification (Hakata *et al.*, 1982).

The present studies support the suggestion (Low *et al.*, 1984) that the low- M_r form in human platelet supernatants arises as a result of proteolytic degradation of the high- M_r form. Ca^{2+} -dependent proteinases have been identified in many tissues (Murachi *et al.*, 1980; Murachi, 1983), and they have been suggested to have a role in intracellular regulatory processes. This raised an important question: could proteolysis of the phospholipase C affect its sensitivity to changes in the intracellular environment and thereby have a role in the regulation of phosphoinositide metabolism? Marked changes in phospholipase C activity were produced by alterations in the Ca^{2+} concentration and lipid composition of the substrate that were similar to results obtained by other investigators (Irvine *et al.*, 1984; Wilson *et al.*, 1984). However, our studies also revealed that only relatively minor differences in sensitivity to these changes existed between the various forms of the phospholipase C. This result is rather puzzling, since peak IV appears to be produced from peak II by removal of a 45000- M_r fragment, and it seems unlikely that such a large portion of the enzyme molecule would be completely redundant. It is conceivable that differences in other regulatory properties not studied here (e.g. interaction with guanine-nucleotide-binding proteins) do exist, but assessment of such possibilities will have to await further investigation.

This work was supported by grants from the National Institutes of Health (HL-34445 and HL-28985) and by a Grant-in-Aid from the American Heart Association with funds

contributed in part by the American Heart Association, Oklahoma Affiliate, Inc. We are grateful to Ms. Julie Butler for excellent technical assistance and artwork. The advice and encouragement of Dr. William B. Weglicki is also greatly appreciated.

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Received 13 August 1985/21 January 1986; accepted 3 March 1986