Multiple forms of phosphoinositide-specific phospholipase C of different relative molecular masses in animal tissues

Evidence for modification of the platelet enzyme by Ca^{2+} -dependent proteinase

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The M_r distribution of phosphoinositide-specific phospholipase C in the supernatants isolated from a variety of animal tissues was analysed by high-performance gel-filtration chromatography. In most tissues, at least four peaks of activity were resolved. However, different tissues showed quite marked differences in the distribution of activity between these peaks. In rat heart, lung and kidney, the predominant form had M_r approx. 90 000, whereas the predominant form in brain had M_r approx. 290 000. In liver, the M_r -90 000 form predominated, but this tissue also contained relatively large amounts of a form of M_r approx. 150 000. Phospholipase C in these tissues from other animal species gave similar distributions of activity between the peaks. In supernatants prepared from platelets sonicated in the presence of leupeptin (0.5 mM) or EGTA (20 mM), the M_r -290 000 form predominated. However, when leupeptin or EGTA (inhibitors of Ca^{2+} -dependent proteinase) was omitted from the sonication buffer, the M_r -290 000 form appeared to be replaced by a form of M_r 100 000. Similar changes in M_r were not demonstrated with the other tissues. These results may be relevant to the intracellular regulation of phospholipase C, since Ca^{2+} -dependent proteolysis has been reported to occur during platelet activation.

A major regulatory step of phosphoinositide metabolism in stimulus-response coupling is thought to be hydrolysis of phosphoinositides by a specific phospholipase C (for references see Shukla, 1982; Creba *et al.*, 1983; Berridge, 1983). Although this enzyme requires Ca^{2+} and its activity is known to be affected by the ionic and membrane-lipid environment of the substrate (Irvine *et al.*, 1979, 1984), the precise role of such factors in the intracellular regulation of phospholipase C activity by extracellular receptors remains uncertain. A further complication in studies of possible regulatory mechanisms of this enzyme is the great variation in molecular properties reported for phosphoinositide-specific phospholipases C isolated from different tissues. The reported M_r values of purified phospholipase C vary from approx. 70 000, for rat liver and sheep seminal vesicles, to 140 000 for bovine platelets (Takenawa & Nagai, 1981; Hofmann & Majerus, 1982; Hakata *et al.*, 1982). In several tissues multiple forms of phospholipase C have been reported (Shen & Tai, 1981; Hirasawa *et al.*, 1982*a,b*; Chau & Tai, 1982), which in some cases appear to have

significantly different M_r values (Atherton & Hawthorne, 1968; Quinn, 1973; Hofmann & Majerus, 1982; Low & Weglicki, 1983). It is not clear from the previous studies whether these variations in molecular properties might arise during isolation or are due to differences in analytical methods and tissue sources. In the present study, we have used high-performance gel-filtration chromatography to compare directly the M_r values of phospholipase C from a range of tissues from several different species. In addition, we have examined the possible role of proteolysis in generating these multiple- M_r forms. Of particular interest is evidence for such proteolytic modification in platelets, where Ca^{2+} -dependent proteolysis has been suggested to play a role in platelet activation.

Materials and methods

Preparation of supernatants

Preparation of supernatants was carried out at 0–4°C. Fresh tissues were homogenized in 0.15 M-NaCl/0.1 mM-dithiothreitol/1 mM-EDTA/10 mM-

Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH, pH 7.0 (9 ml/g wet wt.) with a Polytron homogenizer at setting 7 for 2×30 s. In some experiments pepstatin A (10 μ M), phenylmethanesulphonyl fluoride (0.1 mM) and leupeptin (0.5 mM) were included in the homogenization buffer. The homogenate was centrifuged at 10000 *g* for 20 min, and the supernatant was filtered through two layers of Miracloth (Calbiochem, San Diego, CA, U.S.A.). The filtrate was centrifuged at 150000 *g* for 40 min and the supernatant removed for chromatography as described below.

Animals used in this study were male Sprague-Dawley rats (approx. 200 g), New Zealand White rabbits (2.5–3.5 kg) and 11–16-day chicken embryos. Cardiac myocytes were also prepared from 11-day embryos by a modification of the method of DeHahn (1967). Hearts were collected on ice in phosphate-buffered saline (138 mM-NaCl/2.7 mM-KCl/1.5 mM-KH₂PO₄/8.1 mM-NaH₂PO₄), minced and then digested at 37°C in 0.05% (w/v) collagenase (Type II; Worthington, Freehold, NJ, U.S.A.) and 0.1% (w/v) trypsin. The myocytes were harvested at 5 min intervals for 30 min.

Platelets were prepared from fresh human blood by the method of Carroll *et al.* (1982). After elution from the Sepharose column, the platelets were pelleted by centrifugation at 1000 *g* for 10 min. Buffer (0.15 M-NaCl/0.1 mM-dithiothreitol/10 mM-Hepes/NaOH, pH 7.0) with either leupeptin (0.5 mM) or EGTA (20 mM) as indicated was added at a volume twice that of the pellet. The platelets were lysed by 15 s sonication with a Branson sonifier model 350 with a cup horn attachment. Membranes were pelleted by centrifuging for 45 min at 200000 *g*, and the supernatant was removed for chromatography as described below.

Gel-filtration chromatography on TSK-250

Gel-filtration chromatography was done at 4°C on a 7.5 mm $\times$ 75 mm Bio-Sil TSK-125 guard column connected to a 7.5 mm $\times$ 300 mm Bio-Sil TSK-250 column (both columns from Bio-Rad Laboratories, Richmond, CA, U.S.A.). The columns were equilibrated with 0.15 M-NaCl/0.1 mM-dithiothreitol/0.1 mM-EDTA/10 mM-Hepes/NaOH, pH 7.0. Portions of supernatant (0.2 ml) were applied to the guard column at a flow rate of 0.2 ml/min, and elution was continued with the same buffer at 0.2 ml/min. After the first 6 ml had eluted, 0.2 ml fractions were collected until a total of 14.5 ml had eluted. The whole of each 0.2 ml fraction was used for phospholipase C assay as described below. The column was routinely washed with 10 ml of buffer after each experiment at a flow rate of 1 ml/min; the column was then ready for application of another sample. This pro-

cedure permitted analysis of one sample every 90 min. The column was also washed with 20 ml of buffer before experiments at a flow rate of 1 ml/min if it had not been used for several hours.

The TSK-250 column was calibrated by applying duplicate samples of standard proteins (0.2 ml; 1 mg/ml) and eluting as described above. The elution volumes of the standards were determined by monitoring u.v. absorbance of the column eluate with a flow cell, and a calibration curve was constructed. The void volume of the column, determined with Blue Dextran, was 6.6 ml. The standard proteins used were catalase, aldolase, immunoglobulin G, alcohol dehydrogenase, transferrin, bovine serum albumin, ovalbumin, lactoglobulin, pepsin, carbonic anhydrase, chymotrypsinogen A, cytochrome *c*, ribonuclease A and myoglobin.

Assay for phospholipase C activity

Phospholipase C activity in the entire 0.2 ml fraction was assayed by the procedure described previously with [³H]phosphatidylinositol as substrate (Low & Weglicki, 1983). In the present studies all assays were carried out in the presence of 5 mM-CaCl₂, 0.1% (w/v) sodium deoxycholate and 25 mM-Tris/maleate buffer, pH 7.0 (total assay volume 0.4 ml), since these conditions were previously found to favour detection of the major forms of phospholipase C in bovine heart (Low & Weglicki, 1983). Incubation was routinely for 30 min at 37°C.

To facilitate comparison between different chromatographic experiments, the phospholipase C activity in each fraction was calculated as a percentage of the applied activity. These percentages were combined for groups of peak fractions as described in the Table legends. Since peak III was invariably poorly resolved from either peak II or IV, activity in only three fractions could be assigned unequivocally to this peak. Values given in the Tables therefore probably underestimate the activity actually present in this peak. In most of the tissues analysed, some of the peaks had relatively low phospholipase C activity, and therefore it was necessary to use the whole fraction for assay. However, this presented a problem in some tissues which had very high specific activity, and most of the eluted activity concentrated in one peak (e.g. rat lung). In these cases, the phospholipase C activity in the major peak was outside the linear range of the assay. In some experiments where less activity was applied, the same elution volume was obtained for the major peak. However, this was not done routinely, since this made it difficult to determine the elution volumes of the minor peaks accurately. Values for elution volumes represent the centres of the phospholipase C peaks deter-

mined graphically to the nearest 0.05ml. The elution volumes were used to estimate the M_r values by comparison with a calibration curve (see above). The values for peaks I and II are approximate, since they are eluted outside this calibration curve.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

M_r distribution of phospholipase C from different tissues and species

Supernatant fractions prepared from a variety of mammalian tissues were analysed by high-performance gel-filtration chromatography on columns of Bio-Sil TSK-250 (Figs. 1–5). The data from these and other experiments are summarized in Tables 1–3. To simplify presentation of the data, the peaks of phospholipase C activity in the different tissues which were eluted between specified volumes were assigned the same peak number on the assumption that they were related molecular species. In replicate experiments using supernatant fractions prepared from the same tissue obtained from different animals, the elution profiles obtained were highly reproducible, with respect to both the elution volumes of the peaks and the relative amounts of phospholipase C activity in each peak. In some cases the chromatographic analysis was repeated with the same sample of supernatant which had been stored at –20°C for several days; these experiments gave

Fig. 1. *Chromatography of phospholipase C from rat and chicken heart on TSK-250*

Supernatants from rat (■) or embryonic-chicken (●) heart ventricle were applied to a TSK-250 column, eluted and assayed for phospholipase C activity as described in the Materials and methods section. The values in the Figure were from typical experiments; data from these and similar experiments are summarized in Tables 1 and 2. The M_r scale along the top of the Figure was determined by calibration of the column with standard proteins as described in the Materials and methods section. Abbreviation: V_0 , void volume.

Table 1. *M_r distribution of phospholipase C from rat tissues*

Supernatants from various rat tissues were analysed by gel-filtration chromatography as described in the Materials and methods section. The amount of phospholipase C activity recovered in each peak is expressed as a percentage of applied activity ($\pm$ s.e.m.). Activity eluted in the ranges 7.75–8.95, 8.95–9.55 and 9.55–10.75 ml was used to calculate the recovery of phospholipase C in peaks II, III and IV respectively. Since the elution volumes for peaks II and III are similar for the different tissues and species, mean elution volumes ($\pm$ s.e.m.) of 8.20 ± 0.01 and 9.29 ± 0.01 ml, respectively, were calculated from all the experiments in this Table; mean elution volumes ($\pm$ s.e.m.) for peak IV calculated for each tissue are given in the Table. Values for peak I (see Table 3) are not given, since distinct peaks or shoulders were not observed in this region with these tissues. Except for rat kidney, less than 3% of the applied activity was eluted in the fractions preceding peak II. Distinct peaks of phospholipase C activity (2–15% of applied activity) were always observed in the fractions following peak IV (12–14 ml). However, the number and elution volumes of these peaks were not reproducible, and consequently these results are not included. Numbers in parentheses are the number of chromatographic experiments and the number of tissue samples, respectively. Specific activities for phospholipase C in the supernatants applied to the column were 3.7, 11.8, 1.3, 3.3 and 6.1 nmol/min per mg of protein for heart, brain, liver, kidney and lung respectively.

Tissue	Peak no. ...	Phospholipase C activity (% of applied)			Elution volume (ml) IV
		II	III	IV	
Heart (6, 5)		5.5 $\pm$ 0.4	7.4 $\pm$ 0.8	79.4 $\pm$ 4.2	10.13 $\pm$ 0.01
Brain (4, 3)		49.7 $\pm$ 5.3	12.0 $\pm$ 0.9	33.4 $\pm$ 1.4	10.09 $\pm$ 0.02
Liver (4, 3)		19.4 $\pm$ 2.4	19.7 $\pm$ 1.3	38.1 $\pm$ 2.3	10.06 $\pm$ 0.01
Kidney (3, 2)		3.8 $\pm$ 0.2	15.5 $\pm$ 1.4	61.8 $\pm$ 3.8	10.00 $\pm$ 0.03
Lung (3, 2)		13.6 $\pm$ 0.3	13.5 $\pm$ 0.8	58.9 $\pm$ 7.3	10.06 $\pm$ 0.02

similar results, except that a small increase in activity eluted in the void volume was sometimes observed. In all the experiments, phospholipase C activity was eluted with reasonable recovery (> 75% of applied activity).

The elution profiles for phospholipase C activity were generally similar for the same tissue obtained from different species. However, comparison of the elution profiles obtained with different tissues revealed considerable differences. Thus in rat heart ventricle (Fig. 1; Table 1) approx. 75% of the eluted activity was contained in one major peak (peak IV), with the remainder being distributed between the minor peaks (II and III). A similar distribution of activity was obtained with supernatants prepared from the right and left atria of rat heart (one experiment each with atria pooled from 10 rats; results not shown) or rabbit heart ventricle (Table 2). Embryonic-chicken heart (Fig. 1, Table 2) and embryonic-chicken heart myocytes (Table 2) also had peak IV as the major form, but peak II was not detectable. The elution volume of peak IV appeared slightly different in the different species (approx. 10.4 ml and 9.8 ml for rabbit and chicken respectively). The lower elution volume for peak IV from chicken obscured the peak-III region, and consequently the elution volume of this peak (if present) could not be determined.

In rat liver, peak IV was also the largest; however, it only contained approx. 30–40% of the applied activity (Fig. 2, Table 1). Peaks II and III, which were generally very small in heart, together contained approx. 40% of the applied activity. A similar distribution of activity between the peaks

was obtained with rabbit liver; however (as with rabbit heart), the elution volume of peak IV was significantly greater (Table 2).

Peak III was also relatively large in kidney, but, in this tissue, peak II was not routinely detectable (Fig. 3, Table 1). Furthermore, kidney, unlike all

Fig. 2. Chromatography of phospholipase C from rat and rabbit liver on TSK-250

Supernatants from rat (■) or rabbit (●) liver were applied to a TSK-250 column, eluted and assayed for phospholipase C activity as described in the Materials and methods section. For further details see the legend for Fig. 1.

Table 2. M_r distribution of phospholipase C from rabbit and embryonic-chicken tissues

Supernatants were analysed by gel-filtration chromatography as described in the Materials and methods section. The amount of phospholipase C activity recovered in each peak is expressed as a percentage of the applied activity. The mean elution volumes ($\pm$ S.E.M.) of peaks II (8.22 ± 0.02 ml) and III (9.28 ± 0.01 ml) were very similar to those observed for rat tissues (see legend to Table 1), and the activity recovered in these peaks was therefore calculated as described in the legend to Table 1. Elution volumes for peak IV differed significantly between species, and mean values determined for each tissue are included in the Table. Recovery of phospholipase C in peak IV was calculated from activity eluted in the ranges 9.95–11.15 and 9.35–10.55 ml for rabbit and chicken tissues respectively. Values for the recovery of activity in peak III from embryonic chicken could not be determined, since peak IV overlaps this position. Values for phospholipase C activity eluted before peak II and after peak IV are not included for the reasons given in the legends to Table 1. Numbers in parentheses are the number of chromatographic experiments and numbers of tissue samples, respectively. Specific activities for phospholipase C in the supernatants applied to the column were 2.1, 8.0, 0.7, 8.9 and 15.1 nmol/min per mg of protein for rabbit heart, brain and liver and embryonic-chicken heart and myocytes respectively.

Tissue	Peak no. . . .	Phospholipase C activity (% of applied)			Elution volume (ml) IV
		II	III	IV	
Rabbit heart (3, 2)		7.3 ± 0.7	5.4 ± 0.3	61.8 ± 7.8	10.47 ± 0.02
Rabbit brain (2, 2)		59.5	6.6	20.1	10.38
Rabbit liver (2, 1)		13.2	18.3	59.9	10.43
Embryonic-chicken heart (3, 3)		2.0 ± 0.1	—	90.1 ± 1.8	9.78 ± 0.02
Embryonic-chicken heart myocytes (3, 3)		0.6 ± 0.3	—	75.8 ± 1.4	9.85 ± 0.03

Fig. 3. Chromatography of phospholipase C from rat lung and kidney on TSK-250

Supernatants from rat lung (●) or kidney (■) were applied to a TSK-250 column eluted and assayed for phospholipase C activity as described in the Materials and methods section. For further details see the legend for Fig. 1.

Fig. 4. Chromatography of phospholipase C from rat and rabbit brain on TSK-250

Supernatants from rat (■) or rabbit (●) brain were applied to a TSK-250 column eluted and assayed with phospholipase C activity as described in the Materials and methods section. For further details see the legend for Fig. 1.

the other tissues examined, contained a substantial amount of activity (10–15% of applied activity) which was excluded from the column and eluted in the void volume; this result was obtained with both fresh and frozen supernatants. Rat lung (Fig. 3, Table 1) and rat skeletal muscle (results not shown) gave elution profiles of phospholipase C activity similar to those obtained with rat heart.

Rat brain, by contrast, gave an elution profile quite different from that obtained with the other rat tissues (Fig. 4, Table 1); peak II was now the largest and usually contained more activity than peaks III and IV combined. A similar distribution of activity between the peaks was obtained with rabbit (Table 2); however, the elution volume of peak IV was higher than the value obtained from rat brain.

Supernatants obtained by sonicating freshly prepared human platelets in the presence of EGTA or leupeptin exhibited an elution profile (Fig. 5, Table 3) similar to that obtained with brain; most of the activity was contained in peak II. Peak IV was present, but peak III, which is generally observed in a variety of rat and rabbit tissues, was often not visible as a distinct peak or shoulder, even though activity was detected in this region.

Since the elution volume for peak IV in platelets is slightly lower than is usually observed in these other tissues, it is likely that peak III is always present, but is sometimes obscured by peak IV. Significant activity (approx. 5% of that applied) was also frequently detected in the region designated as peak I (Table 3). However, even when present it was a poorly resolved shoulder on peak II (Fig. 5). This peak was not seen in other tissues. Bovine platelets (two experiments, each with a different platelet preparation) gave similar results with respect to the elution volumes of the peaks and to the distribution of activity between peaks (results not shown).

In all the tissues examined in the present study, significant amounts of activity were detected in fractions eluted after peak IV (Figs. 1–5). However, the elution volumes of these peaks were extremely variable and, in many cases, two peaks could be distinguished. Activity recovered in this region was generally about 2–5% of applied activity; however, tissues such as liver and heart often had more than 10% of applied activity eluted in this region. Small amounts of low- M_r phospholipase C activity were also observed in our previous studies with bovine heart (Low & Weglicki, 1983).

Table 3. *Effect of proteinase inhibitors on M_r of human platelet phospholipase C*

Fresh human platelets were sonicated in the presence or absence of leupeptin (0.5 mM) or EGTA (20 mM), and the supernatants were analysed by gel-filtration chromatography as described in the Materials and methods section. Activity eluted in the ranges 7.15–7.75, 7.75–8.95, 8.95–9.55 and 9.55–10.75 ml was used to calculate recovery of phospholipase C in peaks I, II, III and IV respectively. Since the elution volumes of peaks II and IV were unaffected by the sonication conditions, the mean elution volumes ($\pm$ S.E.M.) of 8.29 ± 0.01 ml and 9.91 ± 0.02 ml, respectively, were calculated from all the experiments in the Table. The elution volumes for peak I (approx. 7.6 ml) and III (approx. 9.3 ml) could not be determined accurately, since they only appeared as shoulders. Values for phospholipase C activity eluted before peak I and after peak IV are not included for the reasons given in the legend to Table 1; however, the amount of activity eluted in these fractions was not reproducibly changed by the presence of leupeptin or EGTA. Numbers in parentheses are the number of chromatographic experiments and the number of platelet preparations, respectively. The specific activity of phospholipase C in supernatants prepared in the presence of leupeptin was 3.5 nmol/min per mg of protein. The relative phospholipase C activities of the supernatants applied to the column were 1.0 (no additions), 1.5 (leupeptin) and 0.9 (EGTA).

Additions to sonication buffer	Peak no.	Phospholipase C activity (% of applied)			
		I	II	III	IV
No additions (4, 4)		1.1 ± 0.1	7.3 ± 0.9	20.5 ± 1.5	69.4 ± 1.7
Leupeptin (5, 4)		6.3 ± 1.8	56.8 ± 6.1	8.0 ± 0.2	12.3 ± 2.0
EGTA (2, 2)		10.9	85.6	13.3	28.2

Fig. 5. *Chromatography of phospholipase C from human platelets on TSK-250*

Supernatants prepared from fresh human platelets sonicated in the absence (●) or presence (■) of 0.5 mM-leupeptin were applied to a TSK-250 column, eluted and assayed for phospholipase C activity as described in the Materials and methods section. The values in this Figure were from a typical experiment; data from this and similar experiments are summarized in Table 3.

The apparent M_r values of the phospholipase C peaks were estimated by comparing their elution volumes with those of protein standards of known M_r . The approximate M_r values for peaks I, II and III are 420 000, 290 000 and 150 000 respectively.

The M_r values for peaks II and III appeared to be very similar for all the tissues and species studied, whereas the M_r for peak IV varied between animal species, i.e. approx. 110 000 (embryonic-chick heart), 100 000 (platelets), 90 000 (rat heart, brain, liver etc.) and 75 000 (rabbit heart, brain and liver). As a check on the validity of these values, the M_r values of the three partially purified phospholipases C from bovine heart (Low & Weglicki, 1983) were determined by chromatography on TSK-250 under the same conditions used for the present study. The values obtained, 125 000, 80 000 and 48 000, are in reasonably good agreement with the ranges of 105 000–120 000, 58 000–76 000 and 40 000–43 000 obtained previously with Sephacryl S-300 (Low & Weglicki, 1983).

Effect of proteinase inhibitors on chromatographic behaviour of phospholipase C

In some experiments, outdated platelets from the blood bank were used to prepare the supernatant. These supernatants usually contained a larger proportion of their phospholipase C activity in peak IV than did supernatants prepared from fresh platelets, and in one batch of platelets peak IV was actually larger than peak II. However, the relative amounts of activity in peaks II and IV varied quite markedly from batch to batch (results not shown). Since the Ca^{2+} -dependent proteinase is known to become more active in outdated platelets (Robey *et al.*, 1979), it seemed possible that peak II might be a substrate for this proteinase. This was confirmed in experiments where the same preparation of platelets was used, but the composition of the sonication buffer was varied (Fig. 5, Table 3). When no additions were made, most of the phospholipase C activity was in peak IV, with only

about 5–10% in peak II. These supernatants also contained substantial activity in peak III; however, this was not well resolved and appeared as a shoulder on peak IV. If leupeptin was included in the sonication buffer, there was a 5–10-fold decrease in the amount of activity in peak IV, with a corresponding 5–10-fold increase in the activity in peaks I and II; peak III was decreased approx. 2-fold (Fig. 5, Table 3). A similar result was obtained when EGTA was used instead of leupeptin (Table 3). Addition of leupeptin or EGTA to the supernatant before application to the column had no apparent effect on the distribution of phospholipase C activity between the peaks (results not shown).

Since rat brain also contained relatively large amounts of peak II, the possibility that this could be converted into peak IV by a mechanism similar to that observed in platelets was investigated. However, when the supernatant was prepared from brain homogenized in the absence of EDTA, there did not appear to be any marked changes in the relative sizes of peaks II and IV. Furthermore, addition of Ca^{2+} (1 mM) to the supernatant followed by incubation at 0°C for 2 h (in the presence or absence of 0.5 mM-leupeptin) did not affect distribution of activity between the peaks.

The observation of relatively large amounts of activity in peak IV in tissues such as heart, lung, liver and kidney suggested that very rapid proteolysis might be occurring after homogenization, in spite of the presence of EDTA in the homogenization buffer. However, inclusion of additional proteinase inhibitors such as pepstatin A, leupeptin and phenylmethanesulphonyl fluoride in the homogenization buffer had no effect on the elution profile obtained for any of these tissues.

Discussion

Several tissues have been shown by others (Shen & Tai, 1981; Hirasawa *et al.*, 1982a,b; Chau & Tai, 1982) to contain multiple forms of phosphoinositide-specific phospholipase C. Our previous work has demonstrated that in heart these multiple forms may differ substantially in M_r (Low & Weglicki, 1983). The results in the present paper indicate that the multiple forms observed in the other tissues also differ in M_r . The M_r values of two of these forms seem to be very similar in the different tissues and species studied (i.e. approx. 290 000 and 150 000), whereas that of a third form varies in the range 75 000–110 000, depending on species. The different tissues show great variation in the relative amounts of these multiple forms. Thus brain and platelets contain mainly the M_r -290 000 form, whereas in kidney, lung and heart the M_r -75 000–110 000 form predominates,

with relatively little of the M_r -290 000 form. In liver, the M_r -75 000–110 000 form is also the major peak; however, the M_r -290 000 and -150 000 forms are also present in substantial amounts. The relative amounts of the different forms appear to be a characteristic of the particular tissue, since broadly similar results were obtained with the same tissue from the different animal species tested.

The observation that multiple M_r forms of phosphoinositide-specific phospholipase C exist in several animal tissues is supported by the work of other investigators. Thus M_r values have been reported for purified phospholipase C, of approx. 70 000 for rat liver and sheep seminal vesicles (Takenawa & Nagai, 1981; Hofmann & Majerus, 1982) and 140 000 for bovine platelets (Hakata *et al.*, 1982). It is relevant to note that in the present study the predominant form in platelets had a substantially higher M_r than did the predominant form in liver. However, the M_r values that we obtained for these forms were not very close to the reported values. This discrepancy is particularly marked with the platelet phospholipase C, where we find the M_r of the largest peak (i.e. peak II) to be approximately double that reported by Hakata *et al.* (1982) for the purified enzyme. It is possible that they purified a form equivalent to peak III or that peak II is a dimer, but these possibilities are difficult to assess, since their report makes no mention of the existence of multiple M_r forms or their distribution during the purification procedure. Additional, indirect, evidence that platelets and liver contain quite distinct forms of the phospholipase C comes from the work of Hofmann & Majerus (1982). They showed that antibodies directed against a purified phospholipase C from seminal vesicles inhibited most of the phospholipase C activity in liver, but not in platelets. Furthermore, phospholipase C in brain, which we find to have a similar M_r distribution to platelet phospholipase C, was also resistant to inhibition by this antibody.

In the present study, the conditions for assay of phospholipase C activity were deliberately chosen in an attempt to detect all the different forms of the enzyme in the tissues analysed. However, by analogy with our previous work on the multiple M_r forms of bovine heart phospholipase C (Low & Weglicki, 1983), it is probable that they will differ quite markedly in their specificity for the physical and chemical nature of the substrate. Since such factors have been proposed as intracellular regulators of phospholipase C activity (Irvine *et al.*, 1979, 1984), an analysis of the 'substrate specificity' of the different M_r forms identified in the present study may give an insight into their functions in the cell.

The origin of the multiple M_r forms of the phospholipase C in the different tissues is not clear. However, our observations with human platelet phospholipase C suggested a possible mechanism. If the platelet supernatant was prepared in the absence of leupeptin or a bivalent-cation chelator, the M_r -420000 and -290000 forms of phospholipase C were replaced by forms with lower M_r (150000 and 100000). Thus it seems likely that the phospholipase C or some macromolecular complex containing phospholipase C is a substrate for the Ca^{2+} -dependent proteinase which has been described in platelets and other tissues (Philips & Jakabova, 1977; Murachi *et al.*, 1980). However, other explanations, such as release of phospholipase C from an association with the membrane or cytoskeleton, cannot be eliminated at present. This appears to be the first report of the action of an endogenous proteinase on phospholipase C in platelets. The occurrence of endogenous or exogenous proteolytic modification of rat brain phospholipase C has been observed (Hirasawa *et al.*, 1982c). These modifications were reported to have increased the Ca^{2+} -sensitivity of the phospholipase, but this effect has since been shown to be due to the presence of inhibitory proteins (Irvine & Dawson, 1983).

The similarity in M_r value of the degraded form of platelet phospholipase C to that of one of the phospholipase C peaks (peak IV) found in rat tissues suggested a common mechanism of origin. However, in several experiments with these other tissues, we have been unable to obtain similar evidence for the involvement of proteolysis. Although we cannot preclude the occurrence of rapid proteolysis of the phospholipase C during preparation of the supernatants, our results indicate that the various forms of phospholipase C that we have demonstrated exist in the intact tissue. Whether the lower M_r forms in these tissues arise as a consequence of proteolytic processes *in vivo* remains an interesting question for further studies.

It has been reported that modification of protein kinase C by a Ca^{2+} -dependent proteinase abolishes the dependence of the kinase on Ca^{2+} , phospholipid and diacylglycerol (Inoue *et al.*, 1977; Kishimoto *et al.*, 1983). This observation may be of relevance to the present results with platelet phospholipase C, since the physiological regulator of protein kinase C, diacylglycerol, is produced in cells by phosphoinositide-specific phospholipase C. An intriguing possibility is that such proteolytic processing may occur during stimulus-response coupling in platelets. Since degradation of cytoskeletal proteins by Ca^{2+} -dependent proteinase has been suggested to occur during platelet activation (Fox *et al.*, 1983), proteolytic modification

of protein kinase C and phospholipase C may also occur and be of significance in regulating their activities or substrate specificities.

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