Biochimica et Biophysica Acta, 1029 (1990) 196–200
Elsevier

BBAMEM 70662

BBA Report

Biomembrane-modulated, lysosomal phospholipase A_2 contamination of chromaffin granule ghosts

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(Received 2 January 1990)
(Revised manuscript received 31 July 1990)

Key words: Phospholipase A_2; Liposome; Lysosome; Chromaffin granule; Adrenal medulla

It was reported that subcellular fractionation of bovine adrenal medulla results in the separation of distinct, non-calcium-dependent phospholipases A_2 — one associated with chromaffin granule ghosts, another with lysosomes. The basis of this distinction is pH optimum: in routine assays utilizing neat liposomal substrates, the chromaffin granule ghost-associated enzyme is alkaline-active whereas the lysosomal enzyme is acid-active (Husebye, E.S. and Flatmark, T. (1987) Biochim. Biophys. Acta 920, 120–130). We now report that biomembranes alter liposomal substrates and/or lysosomal phospholipase A_2 such that the enzyme now hydrolyzes them (at low cation concentration) with an alkaline pH optimum. In a lysosomal membrane fraction, phospholipase A_2 activity at pH 7.5 relative to activity at pH 5.0 increases as increasing amounts of lysosomal membranes are assayed. The pH optimum of chromaffin granule ghost-associated phospholipase A_2 toward liposomal substrates is likewise biomembrane-dependent and, when assayed carefully, is indistinguishable on the basis of optimal pH from the lysosomal enzyme. Although chromaffin granule ghost-associated phospholipase A_2 is most likely a lysosomal contaminant, its broad, biomembrane-modulated pH range may still allow it to participate in catecholamine secretion. More importantly, however, sensitivity of adrenal medullary lysosomal phospholipase A_2 to biomembranes broadens its potential physiologic pH range and may also play a role in the regulation of this potentially deleterious activity.

Secretion of catecholamines from adrenal medulla requires the fusion and subsequent lysis of chromaffin granule and plasma membranes. A role for phospholipase A_2 in this process is suggested by the fusogenic properties of its hydrolytic products [1,2], by an increase in unesterified arachidonic acid during catecholamine secretion [3], and by the enhancement of catecholamine release by prostaglandins [4]. A role for phospholipase A_2 in chromaffin granule physiology is also suggested by the large amount of 1-acyl-lysophospholipids on the inner aspect of chromaffin granule membranes [5]. Husebye and Flatmark [6] reported a non-calcium-requiring, alkaline-active phospholipase A_2 in chromaffin granule ghosts and hypothesized a role for it in secretion. However, the predominant phospholipase A_2 in bovine adrenal medulla is a non-calcium requiring, mostly soluble, lysosomal enzyme with an acidic pH optimum when hydrolyzing liposomal phospholipids [7], but with a pH optimum varying from pH 4.5 to pH 7.5 (with decreasing concentrations of cations) when hydrolyzing biomembranous substrates [8,9]. Husebye and Flatmark [6] demonstrated neither co-enrichment of phospholipase A_2 with chromaffin granule marker biomolecules nor absence of lysosomal contamination — their only criterion distinguishing chromaffin granule from lysosomal phospholipase A_2 being pH optimum [6]. This report demonstrates that biomembranes shift the pH optimum of lysosomal phospholipase A_2 for neat liposomal substrates from acid to alkaline. The conclusion of distinct lysosomal and chromaffin granule ghost-associated phospholipases A_2 [6] is based on an erroneous premise of distinct pH optima which, in all probability, resulted from a chromaffin granule membrane-induced shift in pH optimum of contaminating lysosomal phospholipase A_2.

Abbreviations: Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Fresh bovine adrenal glands were kept on ice until the medullae were removed by dissection. Chromaffin granule ghosts were prepared by established procedures [6]. Purified lysosomes [9] were frozen and thawed, dialyzed against 2.5 mM Hepes (pH 7.5), sonicated, and separated into soluble and membrane fractions by sedimentation at $110000 \times g$ for 90 min, with the membrane fraction washed once and resedimented. Lysosomal phospholipase A_2 of p*I* 8.1 was purified as previously described [9]. Phospholipase A_2 was assayed essentially as described [9] except that fractionation of 1-palmitoyl-2-[1-^{14}C]linoleoylphosphatidylcholine hydrolyzates into phosphatidylcholine, lysophosphatidylcholine and fatty acid was by two successive one dimensional chromatographic runs in solvent systems consisting of chloroform/methanol/ammonium hydroxide (29%)/water (90 : 54 : 5.5 : 5.5, v/v) followed by chloroform/methanol/acetic acid/water (90 : 40 : 12 : 2, v/v). Assay conditions are given in figure and table legends. Each experiment is representative of at least two repetitions, and each data point is the average of duplicate determinations. Protein was determined by the Coomassie brilliant blue method using bovine serum albumin as standard [10]. Phospholipid phosphorus was measured by an established procedure [11] after hydrolysis of lipid extracts with 70% perchloric acid at 170–180 °C for 1 h in thick-walled tubes topped with marbles. Subcellular markers were assayed as previously described [12].

When assayed at low cation concentrations, lysosomal phospholipase A_2 from bovine adrenal medulla hydrolyzes the phospholipids in biomembranes [9] and in liposomes (Fig. 1A, solid line, and [9]) optimally at alkaline and acidic pH values, respectively. And biomembranes, in a dose-dependent manner, shift the optimal pH for hydrolysis of liposomal phospholipid from acidic to alkaline (Fig. 1A, broken lines). Thus, biomembranes alter liposomal phospholipids or lysosomal phospholipase A_2 such that the enzyme now hydrolyzes them with the same pH profile that it hydrolyzes biomembranes themselves.

In contrast to lysosomal phospholipase A_2, chromaffin granule ghosts, during routine assay, hydrolyze both biomembranous and liposomal substrates optimally at alkaline pH (Fig. 1B, solid lines and [6]). However, because of the relatively low specific activity of ghost-associated phospholipase A_2 (Table I), 490 nmol of ghost phospholipid are added to an assay system containing only 15 nmol of liposomal phospholipid. To determine if this relatively large amount of biomembrane has shifted the pH optimum for hydrolysis of liposomal phospholipid by the ghost-associated phospholipase A_2, a reduced amount of ghosts (62 nmol phospholipid) was assayed over a much longer incubation time. Strikingly, the ghost-associated phospholipase A_2 is now, like the lysosomal enzyme, optimally active at acid pH (Fig. 1B, dashed line); and, as with the lysosomal enzyme, its pH optimum becomes alkaline with increased amounts of biomembrane (Fig. 1B, dot-dash line).

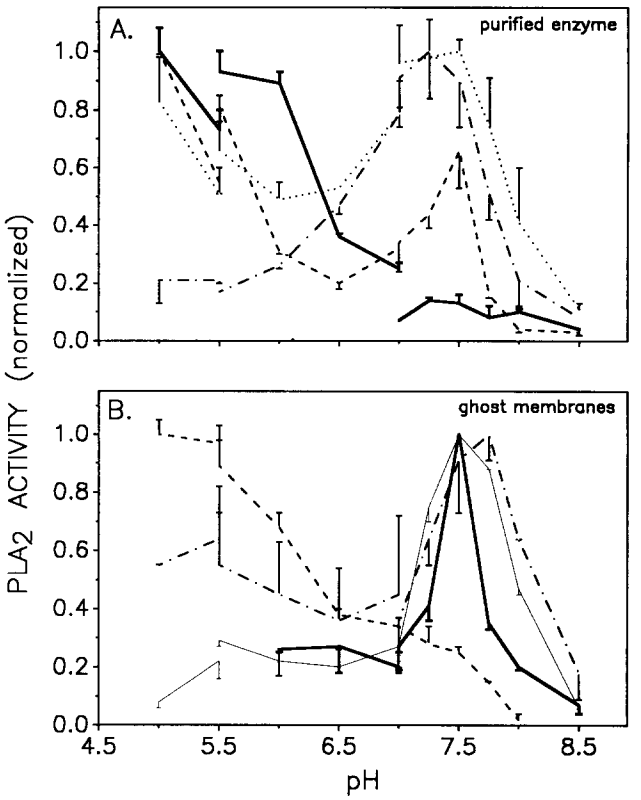

Fig. 1. Modulation by biomembranes of the hydrolysis of liposomal phosphatidylcholine by adrenal medullary, non-calcium-dependent phospholipase A_2. (A) Hydrolysis by 0.55 μg 1900-fold purified, lysosomal phospholipase A_2 of p*I* 8.1 [9] under the following conditions: 7.5 mM buffer (pH 5.0–5.5, acetate; pH 5.5–7.0, Bistris; pH 7.0–8.5, glycylglycine), 0.2 mM EDTA, 0.00025% Triton X-100, 15 nmol (30 μM) 1-palmitoyl-2-[1-^{14}C]linoleoylphosphatidylcholine, incubated at 37 °C for 5 h in the absence (solid line) or presence of non-radiolabelled, autoclaved *E. coli* membranes (7.5 nmol *E. coli* phospholipid, dashed line; 25 nmol *E. coli* phospholipid, dotted line; and 150 nmol *E. coli* phospholipid, dotted-dashed line). (B) Hydrolysis by chromaffin granule ghosts at 7.5 mM buffer, 0.2 mM EDTA, 0.00025% Triton X-100 and: (1) 15 nmol radiolabelled phosphatidylcholine, 490 nmol ghost phospholipid, 312 μg protein incubated at 37 °C for 5 h (heavy solid line); (2) 15 nmol radiolabelled phosphatidylcholine, 62 nmol ghost phospholipid, 27.2 μg protein incubated at 37 °C for 18 h (dashed line); (3) 15 nmol radiolabelled phosphatidylcholine, 62 nmol ghost phospholipid, 27.2 μg protein and 150 nmol non-radiolabelled *E. coli* membrane phospholipid incubated at 37 °C for 18 h (dotted-dashed line); (4) 15 nmol radiolabelled *E. coli* membranes, no liposomal phosphatidylcholine, 10 nmol ghost phospholipid, 4.54 μg protein incubated at 37 °C for 5 h (light solid line). For ease in comparing the various pH profiles, values are normalized separately for each plot (normalized activity = individual activity/highest activity).

Husebye and Flatmark [6] concluded that lysosome- and chromaffin granule-associated phospholipases A_2 are distinct because liposomal phosphatidylcholine is hydrolyzed by purified lysosomes optimally at acid pH and by purified chromaffin granule ghosts at alkaline

TABLE I

Phospholipase A_2 activity and phospholipid in subcellular fractions

Phospholipase A_2 was assayed under standard conditions [9]: 25 mM Bistris (pH 6.0), 25 mM NaCl, 0.5 mM EDTA, 0.00025% Triton X-100 (w/v), 15 nmole (30 μM) *E. coli* phospholipid, and appropriate amounts of cellular fractions incubated at 37 °C for 1 h, 5 h, and 15 min for the lysosomal, chromaffin granule ghost, and purified enzyme fractions, respectively. When assayed under conditions similar to those of Fig. 1, i.e., 7.5 mM glycylglycine (pH 7.5), 0.2 mM EDTA, 0.00025% Triton X-100 (w/v), 15 nmol (30 μM) *E. coli* phospholipid, the relative specific activities among the five fractions do not change. The last column represents the amount of phospholipid contributed by the fraction being assayed for phospholipase A_2 under standard conditions when enough of the fraction is added to hydrolyze at one nmol/mg protein per min.

Fraction	Endogenous phospholipid (nmol/mg protein)	Phospholipase A_2 activity (nmol/mg protein per min)	Endogenous phospholipid / Phospholipase A_2 (nmol/nmol per min)
Lysosomes	580	9.7	60
Lysosol	99	4.9	20
Lysosomal membranes	2820	21.7	130
Chromaffin granule ghosts	1930	0.60	3200
Purified enzyme	–	1830	–

pH. But the relatively large amounts of chromaffin granule membranes in the assays of Husebye and Flatmark (approx. 300 nmol phospholipid [6]) very likely have altered the pH optimum of ghost-associated phospholipase A_2 resulting in only an apparent difference in pH optimum between it and lysosomal phospholipase A_2. Due to their substantially higher phospholipase A_2 activity (Table I), lysosomes contribute much less biomembrane during in vitro assay than do chromaffin granule ghosts, and are therefore much less likely to optimally hydrolyze liposomal phospholipids at alkaline pH under routine assay conditions. Indeed, phospholipase A_2 activity in purified lysosomes is predominantly acid-active toward liposomal substrate (Table II) when assayed in the presence of the low amounts of lysosomal membranes typical during in vitro assay of relatively highly-active lysosome preparations (Table I). But when much greater amounts of lysosomal membranes are present during assay, substantial phospholipase A_2 activity is detected at pH 7.5. For example, with 5.3 nmol lysosomal phospholipid there is 45-times as much activity at pH 5.0 as at pH 7.5, whereas at 53 nmol lysosomal phospholipid there is only 2.2-times as much (Table II). Moreover, when lysosomes are fractionated into soluble and membrane fractions, phospholipase A_2 activity at pH 7.5 is barely detectable over a ten-fold concentration range of lysosol, whereas in the membrane fraction activity at pH 7.5 is substantial, and increases relative to activity at pH 5.0 with increasing membrane phospholipid (Table II). If phospholipase A_2 associated with purified chromaffin granule ghosts were due to contamination by small amounts of lysosomal membranes, then the amount of phospholipase A_2 per unit of membrane would be relatively low requiring addition of greater amounts of membranes during routine assay of ghost preparations. This would result in increased activity of phospholipase A_2 at pH 7.5 relative to pH 5.0, and thereby lead to the erroneous conclusion that the chromaffin granule-associated activity is different from that in purified lysosomes.

The shapes of our pH curves in the alkaline region differ from those of Husebye and Flatmark [6] in that

TABLE II

Hydrolysis of phosphatidylcholine by lysosome fractions

Lysosomal fractions were prepared as described in the text. Lysosomes (580 nmol phospholipid per mg protein), lysosol (99 nmol phospholipid per mg protein), and lysosomal membranes (2800 nmol phospholipid per mg protein) were incubated for the times indicated at 37 °C with 15 nmol (30 μM) 1-palmitoyl-2-[1-^{14}C]linoleoylphosphatidylcholine, 7.5 mM acetate (pH 5.0), or 7.5 mM glycylglycine (pH 7.5) and 0.2 mM EDTA.

Fraction	Lysosomal phospholipid (nmol)	Incubation time (min)	pH 5.0		pH 7.5	
			lyso-PC (%)	fatty acid (%)	lyso-PC (%)	fatty acid (%)
Lysosomes	5.3	360	7.8	18	0.1	0.4
	53	72	15	19	0.5	8.6
Lysosol	0.48	260	10	3.8	0.2	0.5
	4.8	72	15	9.2	0.1	0.0
Membranes	27	360	2.2	23	0.0	5.9
	270	144	3.3	28	0.1	24

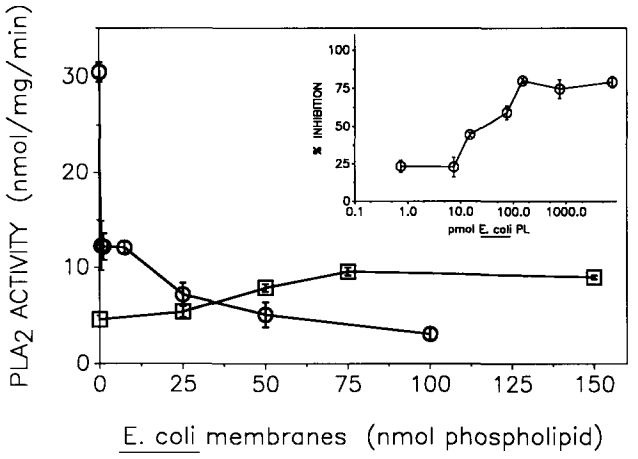

Fig. 2. Effects of *E. coli* membranes on the hydrolysis of liposomal phosphatidylcholine by 1900-fold purified lysosomal phospholipase A_2 of p*I* 8.1 [9]. Hydrolysis of 15 nmol (30 μM) 1-palmitoyl-2-[1-^{14}C]linoleoylphosphatidylcholine by 0.103 μg enzyme, 0.2 mM EDTA, 0.00025% Triton X-100, incubated at 37°C for 5 h with variable amounts of non-radiolabelled *E. coli* membranes and 7.5 mM acetate (pH 5.0) (circles), or 7.5 mM glycylglycine, (pH 7.5) (squares). Inset: lower amounts of *E. coli* membrane phospholipids at pH 5.0. The somewhat different degrees of inhibition between the main figure and inset are due to variability among experiments.

ours have a relatively narrow pH optimum whereas those of Husebye and Flatmark continue increasing with increasing pH. The somewhat different pH buffering agents utilized in our two laboratories do not account for these differences since our results do not change when we use the buffers of Husebye and Flatmark (not shown). The most likely explanation is the use of a calcium-EDTA containing assay system by Husebye and Flatmark in contrast to our EDTA containing assay system. Assay in the absence of free calcium is necessary to inhibit the calcium-dependent, alkaline-active, membrane-associated phospholipase A_2 in adrenal medulla [13,14], which is about equally enriched in both fractions when adrenal medulla is fractionated into plasma-microsomal and lysosomal-mitochondrial-chromaffin granule membrane fractions (not shown).

The data in Fig. 1 have been normalized within each curve to facilitate comparisons among several curves which differ widely in enzyme activities. To demonstrate both qualitatively and quantitatively the effects of biomembranes on hydrolysis of liposomal phosphatidylcholine by lysosomal phospholipase A_2, modulation of enzyme activity as a function of *Escherichia coli* membrane phospholipid concentration is shown in Fig. 2. It is apparent that biomembranes shift the pH optimum by reducing activity at acid pH (circles) while enhancing activity at alkaline pH (squares). At pH 5.0, 50% inhibition occurs with only 0.01 nmol *E. coli* phospholipid, which is less than 0.07% of liposomal substrate phospholipid. Thus, inhibition is clearly not due to substrate competition or dilution, but most likely results from a qualitative change in the liposomal substrate or a direct effect on the enzyme. The increased activity at pH 7.5, which is much less dramatic than the decreased activity at acid pH, may result from conversion of a less favorable liposomal substrate to a more favorable membrane-like substrate [9] or, again, may result from a direct effect on the enzyme. Since the added (*E. coli*) membranes are also being hydrolyzed at pH 7.5 [8,9], it is somewhat surprising that a 6-fold increase in biomembrane concentration does not appear to compete with liposomal phosphatidylcholine, although it is possible that these results reflect the net effect of both activation and competition. It appears that different membranes modulate activity to different extents since, for example, at 62 nmol ghost phospholipid, activity is still predominantly acid-active, i.e., the shift to an alkaline pH optimum has not yet begun (Fig. 1B, dashed line); whereas, at only 7.5 nmol *E. coli* membrane phospholipid the shift is already evident since activity is about equal at pH's 5.5 and 7.5 (Fig. 1A, dashed line). Different susceptibility to modulation by various biomembranes may be a mechanism for 'targeting' lysosomal phospholipase A_2.

Thus, lysosomal phospholipase A_2 in bovine adrenal medulla is extremely sensitive to biomembranes which change its observed properties through substrate and/or enzyme alteration(s). The observation that a 1900-fold purified enzyme and crude cellular fractions respond identically to biomembranes (Fig. 1) suggests the presence of a single enzyme; but until purification to homogeneity is achieved, multiple enzymes cannot be ruled out. Since various organelle biomembranes may have distinct effects and are present to widely varying degrees in isolated subcellular fractions, it is important to consider such biomembrane-induced alteration(s) in intracellular localization studies. Contrary to the report of Husebye and Flatmark [6], our results demonstrate that chromaffin granule ghosts do not contain a non-calcium-dependent phospholipase A_2 with a pH optimum toward liposomal substrate distinct from the lysosomal enzyme. More importantly, however, our report shows that lysosomal phospholipase A_2 can act at non-lysosomal, physiological pH values, which raises the possibility that this enzyme may function outside lysosomes. As a secreted soluble, membrane-associated, or perhaps even receptor-bound enzyme [15,16], the lysosomal phospholipase A_2 may have a direct role in catecholamine secretion. Finally, since modulation of lysosomal phospholipase A_2 by biomembranes varies with membrane source, properties such as membrane composition, surface charge, or pressure may influence in vivo targeting and/or regulation of this enzyme in bovine adrenal medulla.

This work was supported by Grant DK-34558 from the National Institutes of Health, U.S.A. Excellent technical assistance was provided by Ella Segal.

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