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Release of Alkaline Phosphatase from Membranes by a Phosphatidylinositol-Specific Phospholipase C

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Purified phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* released a substantial proportion of the total alkaline phosphatase activity from a wide range of tissues from several mammalian species. Co-purification of the phospholipase C and alkaline phosphatase-releasing activities and the inhibition of both these activities by iso-osmotic salt solutions suggested that the releasing effect was unlikely to be due to a contaminant.

Slein & Logan reported that a factor present in *Bacillus cereus* culture filtrates, when injected into rabbits, produced a large increase in alkaline phosphatase activity in serum (Slein & Logan, 1962) and also released alkaline phosphatase from kidney and bone slices (Slein & Logan, 1963). They subsequently showed that, on column chromatography of the culture filtrates, the phosphatase-releasing activity was found in fractions that also contained a phospholipase C activity relatively specific for phosphatidylinositol (Slein & Logan, 1965). It was suggested that alkaline phosphatase might be bound to membrane phosphatidylinositol, presumably by specific interactions with the phosphatidylinositol molecule. Although many studies have indicated the existence of specific interactions between membrane lipids and proteins, the release of a protein from the membrane by disruption of such interactions is unprecedented and merits further detailed investigation.

In the original experiments only partially purified phospholipase C was available and, even though similar results have recently been obtained with a highly purified enzyme preparation (Ikezawa *et al.*, 1976), the possibility that the alkaline phosphatase release might still be caused by a contaminant was not eliminated. Furthermore, although the release of alkaline phosphatase by these phospholipase C preparations was well established, these studies did not indicate what proportion of the total alkaline phosphatase was involved.

We have now demonstrated that the phosphatidylinositol-specific phospholipase C from a different bacterium, *Staphylococcus aureus*, will release alkaline phosphatase from a wide range of tissues and that the released enzyme represents a substantial proportion of the total. We have also obtained further evidence that the release is a specific effect of the phospholipase C.

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Experimental

Phosphatidylinositol-specific phospholipase C (*S. aureus*) was prepared by a modification of the method described previously (Low & Finean, 1977). The eluate obtained from the Amberlite CG-50 column (2.6cm×40cm) was precipitated with $(NH_4)_2SO_4$ (0.65g/ml of eluate) and stored at −20°C as a suspension in saturated $(NH_4)_2SO_4$ (specific activity approx. 300units/mg of protein). $(NH_4)_2SO_4$ precipitates from several 4-litre batches of culture supernatant were pooled, dissolved in 50mM-Tris/HCl, pH7.4 (at 4°C), and applied to a Sephadex G-75 column (Low & Finean, 1977). Although this procedure gave a broader peak of phospholipase C activity than that obtained with concentrates prepared by ultrafiltration, electrophoretic analysis showed it to have a similar degree of purity (specific activity approx. 6.5×10^3 units/mg of protein). Phospholipase C activity was assayed as described previously (Low & Finean, 1976, 1977). A unit was defined as the amount of activity releasing 0.1 μmol of organic phosphorus into the upper phase in 2h at 37°C (Low & Finean, 1976). No alkaline phosphatase or proteolytic activity could be detected in the phospholipase C preparation. Purified *B. cereus* phospholipase C (Little *et al.*, 1975) was from Dr. C. Little, University of Tromsø, Tromsø, Norway, and an impure preparation of phospholipase C from *Clostridium perfringens* (AD 1051A) was obtained from Wellcome Research Laboratories, Beckenham, Kent, U.K.

Tissues (excepting lymphocytes) were sliced into small pieces with a scalpel, washed with 0.25M-sucrose, the excess of fluid drained off and the slices

weighed into centrifuge tubes. Homogenates in 0.25M-sucrose were also prepared from the same batch of tissue slices. Sliced tissue (0.5g), or the equivalent amount of homogenate, 0.25ml of phospholipase C (or, in control incubations, 0.25ml of 50mM-Tris/HCl, pH7.4 at 4°C) and 4.25ml of 0.25M-sucrose were incubated in centrifuge tubes for 90min at 37°C. The incubations were then cooled on ice and centrifuged at 100000*g* for 1h. The supernatants were removed and assayed for enzyme activities as described below. Lymphocytes from pig mesenteric lymph nodes (Allan & Michell, 1974) were washed and suspended in 0.25M-sucrose so that the final concentration in incubation mixtures was 25% (v/v). A microsomal fraction was prepared from rabbit kidneys. Homogenates prepared in 0.25M-sucrose (9ml/g of tissue) were centrifuged at 15000*g* for 20min and the resulting supernatant was then centrifuged at 100000*g* for 60min. The pellets were resuspended in 0.25M-sucrose (approx. 1ml/g of original tissue) and 1ml of this suspension was used for phospholipase C treatment (see above).

Alkaline phosphatase (EC 3.1.3.1) was assayed in an incubation mixture containing 2mM-*p*-nitrophenyl phosphate, 5mM-$MgCl_2$ and 50mM-Na_2CO_3/$NaHCO_3$ buffer, pH10.1, in a final volume of 1ml. Incubation was for 10–30min at 37°C. Values of alkaline phosphatase activity quoted in Tables 1 and 2 are means of duplicate incubations (reproducible within ±5%) and are expressed as percentages of the activity determined at the same time (day of preparation) in samples of non-incubated homogenates and of lymphocyte or microsomal suspensions.

Lipid phosphorus and phosphatidylinositol contents of microsomal fractions were determined as described previously (Low & Finean, 1976, 1977).

Results

Phospholipase C-induced release of alkaline phosphatase

Incubation of various tissue preparations with the phosphatidylinositol-specific phospholipase C from *S. aureus* caused the release, in all cases, of a substantial proportion of the alkaline phosphatase activity that could be assayed in the homogenate (Table 1). In the case of rabbit kidney or liver the release from homogenates was significantly greater than that from slices and approached 100% (Table 1).

Alkaline phosphatase (homogenate and released enzyme) from each type of tissue was sensitive to inhibition by EDTA (10mM). In most cases this inhibition was greater than 95%, although intestinal enzyme was inhibited by only about 70%. However, there was less than 5% inhibition of either the released or the homogenate activities in the presence of 25mM-NaF.

Results shown in Table 2 indicate that a substantial proportion of the alkaline phosphatase was also released into the supernatant on treatment of a rabbit kidney microsomal fraction with phospholipase C. In this system it was possible also to determine the amount of activity remaining in the membrane pellet, and this showed a substantial decrease. The value of pellet activity/supernatant activity decreased from 40 to 0.5 on treatment with phospholipase C. Three experiments in which different concentrations of phospholipase C were used showed that even at maximum alkaline phosphatase release (70–95% of original activity) some 30–45% of the original activity remained in the pellet. The total recovery of activity following phospholipase C treatment was 10–30% greater than that of the control.

The pellets from phospholipase C-treated microsomal fractions were analysed for phospholipid content. Only 5% of the total phospholipid had been hydrolysed (Table 2), and this decrease could be accounted for by extensive hydrolysis of microsomal phosphatidylinositol (approx. 7.5% of the microsomal phospholipid was phosphatidylinositol).

In further experiments the effect of varying phospholipase C concentration both on the release of alkaline phosphatase and on hydrolysis of phosphatidylinositol was investigated. 85% of maximum release occurred with a phospholipase C concen-

Table 1. *Effect of phosphatidylinositol-specific phospholipase C on the release of alkaline phosphatase from various tissues*

Tissue slices and homogenates were incubated with phospholipase C (kidney and liver, 20 units/ml; lymphocytes, 10 units/ml; pancreas, smooth muscle and intestine, 5 units/ml), centrifuged and the supernatants assayed for released enzyme activities. The released alkaline phosphatase activity is expressed as a percentage of the activity assayed in the homogenate (non-incubated). Values for kidney are means (±S.D.) of results from three experiments.

		Alkaline phosphatase released (% of homogenate)	
Animal	Tissue	Control	Phospholipase C-treated
Rabbit	Kidney		
	Slices	1.1±0.6	58±3.9
	Homogenate	9.9±7.7	96±4.5
Rabbit	Liver		
	Slices	3.4	61
	Homogenate	21.6	117
	Homogenate	12.0	97
Pig	Lymphocytes	1.5	70
Guinea pig	Pancreas	3.8	138
Guinea pig	Ileum smooth muscle	2.0	38
Guinea pig	Intestine	4.3	28

Table 2. *Effect of phosphatidylinositol-specific phospholipase C (S. aureus) on the release of alkaline phosphatase from rabbit kidney microsomal fractions*

Rabbit kidney microsomal fractions were incubated with *S. aureus* phospholipase C (5 units/ml), *B. cereus* phospholipase C (1 μg/ml) or *C. perfringens* phospholipase C (50 μg/ml), centrifuged and the supernatants and pellets were assayed for alkaline phosphatase activity, total phosphorus and phosphatidylinositol. The values are expressed as percentages of the alkaline phosphatase assayed in the original microsomal suspension (non-incubated) or as percentages of total lipid phosphorus or phosphatidylinositol present in the control pellet. Values are means (±S.D.) of results from three experiments.

	Alkaline phosphatase (% of original activity)		Phospholipid content of pellets (% of control)	
	Pellet	Supernatant	Total lipid phosphorus	Phosphatidylinositol
Control	94±7.7	2.9± 0.9	100	100
Phospholipase C-treated (*S. aureus*)	32±4.8	81.0±12.9	97±1.7	17±7.4
Phospholipase C-treated (*B. cereus*)	112±2.8	4.0± 1.8	26±2.4	84±5.7
Phospholipase C-treated (*C. perfringens*)	109±4.0	3.4± 1.9	34±0.5	80±2.6

tration as low as 0.02 unit/ml (approx. 3 ng/ml). However, even at this concentration, significant (approx. 15%) phosphatidylinositol hydrolysis was detected.

Inclusion of 0.15 M-KCl or 0.15 M-NH_4Cl in the incubation medium, previously shown to inhibit phosphatidylinositol hydrolysis (Low & Finean, 1976, and unpublished work), decreased by approx. 75% the amount of alkaline phosphatase released from kidney microsomal fractions. This decrease could not be accounted for by inhibition of the alkaline phosphatase, as approx. 90% of the original activity was recovered in the pellet.

The possibility that a contaminant in the phospholipase C preparation was responsible for the alkaline phosphatase release was investigated by assaying the ratio of phospholipase C and releasing activity (with kidney microsomal fractions) at different stages of the purification. This ratio was similar (i.e. within 50%) for the culture supernatant, the $(NH_4)_2SO_4$ precipitate applied to the Sephadex G-75 column and for the pooled phospholipase C-containing fractions eluted from the column. When this material was further purified on DEAE-Sephadex A-50 (in 50 mM-Tris/HCl, pH 9.0) the peaks of eluted phospholipase C and alkaline phosphatase-releasing activity coincided.

The relatively non-specific phospholipase C from *B. cereus* or *C. perfringens* produced only small increases in the release of alkaline phosphatase activity from microsomal fractions despite a substantial decrease in the phospholipid content of the microsomal fractions. This included a 10–20% decrease in phosphatidylinositol content (Table 2).

Discussion

The release of alkaline phosphatase induced by the phosphatidylinositol-specific phospholipase C from *S. aureus* appears to be similar to that reported previously (Slein & Logan, 1965; Ikezawa *et al.*, 1976) for the enzyme from *B. cereus* and, in addition, appears to represent a substantial proportion of the total activity. We have, however, obtained significantly greater release from homogenates than from tissue slices, whereas Ikezawa *et al.* (1976) reported a relatively poor release from homogenates. The difference might be accounted for by the higher spontaneous release of alkaline phosphatase from control incubations of homogenates which would make the effect of phospholipase C appear less dramatic.

The possibility that the observed release could be brought about by a contaminating substance appears unlikely for several reasons. First, it is unlikely that the same contaminant would be present in phosphatidylinositol-specific phospholipase C preparations derived from both *B. cereus* and *S. aureus*, two taxonomically unrelated bacteria. Secondly, we have also demonstrated that the phospholipase C activity and the alkaline phosphatase-releasing activity co-purify on gel filtration and ion-exchange chromatography which supports the idea that the two activities are due to the same protein. Finally, neither we nor previous workers (Ikezawa *et al.*, 1976) have been able to demonstrate proteolytic activity in the purified preparations. Indeed alkaline phosphatase activity is not readily released from membranes by treatment with detergents, proteinases, lipase or neuraminidase (Wachsmuth & Hiwada, 1974; Louvard *et al.*, 1973; Nordstrom, 1972; Thomas & Klinne, 1972). The activity is, however, readily released by the phosphatidylinositol-specific phospholipase C preparation, and this release is markedly decreased by NH_4Cl, or KCl, which inhibit phosphatidylinositol hydrolysis by this enzyme.

It was reported previously that the non-specific phospholipase C from *B. cereus* did not release alka-

line phosphatase (Ikezawa *et al.*, 1976), and the data in the present paper support this conclusion. However, we have also demonstrated that the non-specific phospholipases C from *B. cereus* and *C. perfringens* did hydrolyse significant amounts of phosphatidylinositol in these experiments. Perhaps the hydrolysis of the major phospholipid components of the membrane in some way modifies the release induced by (subsequent) phosphatidylinositol hydrolysis.

Extension of the observations to a wider range of tissues from several mammalian species has emphasized that the releasing effect of phosphatidylinositol-specific phospholipase C is not restricted to tissues rich in membrane-bound alkaline phosphatase (e.g. intestinal mucosa, bone and kidney), where this enzyme might be expected to have some important physiological role. It probably applies to this particular molecule in whatever tissue it might occur and may reflect fundamental factors in its structural incorporation into plasma membranes.

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