Selective Release of Plasma-Membrane Enzymes from Rat Hepatocytes by a Phosphatidylinositol-Specific Phospholipase C

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When isolated hepatocytes are incubated with phosphatidylinositol-specific phospholipase C, three cell-surface enzymes show markedly different behaviour. Most of the alkaline phosphatase is released at very low values of phosphatidylinositol hydrolysis, whereas further phosphatidylinositol hydrolysis releases only a maximum of about one-third of the 5'-nucleotidase. Alkaline phosphodiesterase I is not released. If cells containing phosphatidyl[³H]inositol are similarly treated, then the released [³H]inositol is in the form of inositol phosphate: no evidence has been obtained for any covalent association between released [³H]inositol and alkaline phosphatase.

Treatment of plasma membranes, either in cells or as components of isolated subcellular fractions, with phosphatidylinositol-specific phospholipase C can lead to release of some membrane-bound enzymes in soluble form [alkaline phosphatase (Ikezawa *et al.*, 1976; Low & Finean, 1977a, 1978; Taguchi & Ikezawa, 1978; Ohyabu *et al.*, 1978), 5'-nucleotidase (Taguchi & Ikezawa, 1978; Low & Finean, 1978), acetylcholinesterase (Low & Finean, 1977b)]. These observations have suggested that phosphatidylinositol may be involved in the attachment of these enzymes at the external surface of the plasma membrane (for a review see Evans, 1979). We have now compared the effects of phosphatidylinositol-specific phospholipase C on the attachment at the cell surface of three different enzymes and on the hydrolysis of radioactively labelled phosphatidylinositol in hepatocytes from rats injected with [³H]inositol. The results demonstrate marked differences in the susceptibilities of different enzymes to phospholipase-induced release. They give no evidence for a covalent attachment of enzymes to phosphatidylinositol molecules at the plasma membrane.

Materials and Methods

Phosphatidylinositol-specific phospholipase C (*Staphylococcus aureus*) was purified and assayed as described previously (Low & Finean, 1977a). Hepatocytes were isolated after perfusion of rat liver *in situ* with a collagenase-containing Krebs–Ringer

buffer (Seglen, 1976). In experiments where phosphatidylinositol was labelled, rats were injected with 400 μ Ci of *myo*-[2-³H]inositol (The Radiochemical Centre, Amersham, Bucks., U.K.) and the hepatocytes were isolated 15–16 h after injection (Lewin *et al.*, 1976). Hepatocytes were washed twice with 0.25 M-sucrose, pH 7.2, by centrifugation at 400 g for 1 min. For the treatment of hepatocytes with phosphatidylinositol-specific phospholipase C, the incubation contained 0.25 ml of packed hepatocytes and the appropriate amount of enzyme in 1.75 ml of 0.32 M-sucrose, pH 7.2. A blank incubation without phospholipase was run in parallel. Incubations were at 37°C for 30 min with intermittent shaking. Cells were then sedimented at 150 000 g for 40 min at 4°C and the supernatants were assayed for various enzyme activities; pellets were immediately suspended in water at 4°C and their lipids were extracted (Shukla *et al.*, 1978). Lipid extracts were dried in scintillation vials and their radioactivity was determined. In some experiments, lipids were separated on formaldehyde-treated papers and their phosphatidylinositol content was estimated (Shukla *et al.*, 1979). The concentration of phosphatidylinositol was 1.51 $\pm$ 0.1 μ mol/ml of packed hepatocytes (mean $\pm$ S.E.M., nine experiments).

Enzyme assays were in triplicate and all values for the enzyme activities are expressed as means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. The alkaline phosphatase assay contained, in a total volume of 1.5 ml, 50 mM-ethanolamine/HCl buffer (pH 9.7), 10 mM-MgCl₂, 2.0 mM-*p*-nitrophenyl phosphate and enzyme. The reaction was at 37°C for 30 min and was stopped by the addition of 1.5 ml of 0.2 M-NaOH, followed by centrifugation.

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The absorbance of the supernatant at 400nm was recorded. Hepatocytes showed an activity of 29 ± 4 (6) μ mol of substrate hydrolysed/h per ml of hepatocytes. The assay for 5'-nucleotidase was that of Michell & Hawthorne (1965), except that phosphate was estimated by the method of Baginski *et al.* (1967). The specific activity was 274 ± 35 (5) μ mol of P_i liberated/h per ml of packed hepatocytes. The alkaline phosphodiesterase I assay contained 33mM-glycine/NaOH buffer (pH 9.6), 10mM-MgCl₂, 1mM-*p*-nitrophenyl-5'-phosphothymidine and enzyme in a final assay volume of 1.5 ml. Incubations were at 37°C for 15 min and subsequent treatments and measurements of enzyme activities were similar to those described for alkaline phosphatase. The specific activity was 1138 ± 51 (5) μ mol of substrate hydrolysed/h per ml of packed hepatocytes. Lactate dehydrogenase was assayed as described by Stolzenbach (1966). In all cases total enzyme activities were determined by using hepatocytes pretreated with 0.25% (v/v) Triton X-100 and incubated in the presence of 0.008% (v/v) Triton X-100.

When the water-soluble products were to be examined, the supernatants obtained from control or phospholipase-treated samples were loaded on Sephadex G-25 (coarse) columns (38cm $\times$ 1.5cm). Samples were fractionated by eluting with 50mM-Tris/HCl buffer (pH 7.1), containing 0.1M-NaCl. Sixty fractions, each of 2 ml, were collected and A_{280} , radioactivity and activity of alkaline phosphatase were determined in each fraction.

Results and Discussion

Treatment of hepatocytes for 30 min with 0.1 unit of phosphatidylinositol-specific phospholipase C/ml (the units of enzyme activity are as defined by Low & Finean, 1977a) released about 75% of the total alkaline phosphatase (Fig. 1), but the same treatment liberated very little 5'-nucleotidase (75–80% of the activity of the whole cells, both for alkaline phosphatase and 5'-nucleotidase, was non-latent and therefore presumably located at the cell surface). Under these conditions only about 1% of the total phosphatidylinositol of the cells was hydrolysed, and the lack of liberation of lactate dehydrogenase indicated that very few cells were lysed. When the concentration of phospholipase was increased, little more alkaline phosphatase was released, but increased release of 5'-nucleotidase occurred, reaching a maximum of about 30% release (i.e. about 40% of the non-latent activity) at 10 units of phospholipase/ml of incubation (Fig. 1). At this stage, the extent of phosphatidylinositol hydrolysis indicated by the loss of radioactivity was in close agreement with that derived from direct chemical determinations of cell phosphatidylinositol content

Fig. 1. Selective release of enzymes from the surface of rat hepatocytes by phosphatidylinositol-specific phospholipase C

Isolation of hepatocytes and treatment with phospholipase were as described in the Materials and Methods section. The results presented are from one of three experiments that each gave similar results. Symbols: Δ , alkaline phosphatase; $\square$, 5'-nucleotidase; ∇ , alkaline phosphodiesterase I; $\blacktriangle$, lactate dehydrogenase; $\blacksquare$, phosphatidylinositol hydrolysis.

($7.7 \pm 1.0\%$; mean $\pm$ s.e.m. for eight experiments), and there was still no lysis. At 10–20 units of phospholipase/ml of incubation there was a further increase in phosphatidylinositol hydrolysis and some cell lysis, but no additional release of membrane enzymes (Fig. 1). Homogenization of the hepatocytes so as to expose all membranes to the phospholipase did not alter the extent of membrane enzyme release at any phospholipase concentration (see also Higgins & Evans, 1978); nor did increasing the period of incubation with 10 units of phospholipase/ml to 2 h. Under none of the conditions tested was there any release in soluble form of alkaline phosphodiesterase I, a third externally located plasma-membrane enzyme (Fig. 1).

These observations give further support to the idea that certain plasma-membrane enzymes are linked specifically to phosphatidylinositol at the external surface of the plasma membrane. In addition, the clear difference in susceptibility to release between alkaline phosphatase and 5'-nucleotidase indicates that these two enzymes are associated with two populations of phosphatidylinositol molecules that, at least so far as access to phosphatidylinositol-specific phospholipase C is concerned, are in distinct environments. Similarly, the recognition that, particularly for 5'-nucleotidase, there is a substantial population of enzyme molecules unaffected by the action of this phos-

pholipase may mean that these molecules are attached to the membrane either through phosphatidylinositol that is inaccessible to the enzyme or in some chemically different manner.

To investigate the nature of the involvement of phosphatidylinositol in attachment of alkaline phosphatase and 5'-nucleotidase to membranes, the phosphatidylinositol of hepatocytes was labelled to high specific radioactivity with [^3H]inositol and the nature of the soluble products released by the phosphatidylinositol-specific phospholipase C was investigated. In particular, we wished to determine whether any of the released [^3H]inositol might be in covalent association with the liberated membrane enzymes. Fractionation on Sephadex G-25 columns of supernatants both from control cells and from phospholipase-treated cells revealed two peaks of absorption at 280nm (Fig. 2). One peak was macromolecular and contained, in the supernatant from phospholipase-treated cells, all of the released alkaline phosphatase, whereas the other peak was of low molecular weight. In each sample, there was a small peak of ^3H -labelled material that coincided with the macromolecular peak. More than half of this labelled material, which contained radioactivity equivalent in quantity to about 1% of the total cellular phosphatidylinositol, could be separated from protein either by dialysis or by precipitation of proteins with trichloroacetic acid, and therefore appeared not to be covalently linked to protein (results not shown). There was no significant difference between the amounts of [^3H]inositol associated with the high-molecular-weight peaks in the control and phospholipase-treated samples.

The bulk of the released ^3H -labelled material was of low molecular weight, and the radioactivity of this peak increased substantially in the phospholipase-treated samples (Fig. 2). The extra radioactivity in these samples appeared likely to be inositol phosphate released by the phospholipase, whereas the radioactivity in the untreated control samples seemed likely to be mainly [^3H]inositol that had leaked out of the cells. This was confirmed by experiments (results not shown) in which these compounds were separated on Dowex-1 columns by the method of Ellis *et al.* (1963).

As a result of these experiments, it is clear that the interaction of alkaline phosphatase with the hepatocyte plasma membrane is, like the attachment of acetylcholinesterase to ox or pig erythrocytes (Low & Finean, 1977b), particularly sensitive to attack on membrane phosphatidylinositol by phospholipase C. In view of the specificity of the phospholipase-induced release, this might provide a most effective initial step in future routes to the purification of these membrane-bound enzymes. Our inability to detect [^3H]inositol in association with the enzymes released by the phospholipase suggests to us that the

Fig. 2. Fractionation on Sephadex G-25 columns of the material released by phosphatidylinositol-specific phospholipase C

The supernatants from phospholipase-treated hepatocytes were fractionated as described in the Materials and Methods section. The results are from a typical experiment, one of four that gave similar results. Symbols: $\circ$, A_{280} ; $\bullet$, ^3H radioactivity; Δ , alkaline phosphatase activity.

association between enzymes and phosphatidylinositol at the membrane surface may not be covalent. We estimate that we would have detected such an interaction unless it involved substantially less than 0.1% of the total phosphatidylinositol of the cell. Although the distribution of phosphatidylinositol in the plasma membrane of hepatocytes is not known, this 0.1% value would probably represent only a few percent of the externally located phosphatidylinositol of the plasma membrane.

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