NON-LYTIC RELEASE OF ACETYLCHOLINESTERASE FROM ERYTHROCYTES BY A PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

Martin G. LOW* and J. Bryan FINEAN
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, England

Received 23 July 1977

1. Introduction

There is now substantial evidence that alkaline phosphatase and 5′-nucleotidase are specifically released from membranes by the action of phosphatidylinositol-specific phospholipase C [1–5]. Investigations on the mechanism of release of these enzymes [5] have revealed that the phospholipase C does not produce gross disruption of the membrane and that these plasma membrane ecto-enzymes are probably released from the membrane as individual molecules by a relatively specific process. As these enzymes are generally considered to have their active sites at the outer surface of the plasma membrane it would be expected that not only would release occur from the outer surface but also that the membrane phosphatidylinositol involved in their binding, would be located here as well. However, many of the previous experiments were done with tissue slices where accessibility of the phospholipase C to the cell surface might have been restricted and under conditions where substantial cell lysis, as judged by the release of lactate dehydrogenase, appeared to have occurred [6]. These technical limitations have precluded a definitive location of the essential molecular events of this process at the outer surface of the plasma membrane.

In the present paper we report on the release of acetylcholinesterase from mammalian erythrocytes by non-lytic exposure to the phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*. The results indicate that the phospholipase C acts on the outer surface of the erythrocyte and that breakdown of only a small proportion of the membrane phosphatidylinositol is required for release.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

*Present address: Division of Nutritional Sciences, Cornell University, Savage Hall, Ithaca, New York 14853, USA

2. Materials and methods

Most materials and methods were as described in previous papers [4,5] or as detailed in legends to tables. Acetylcholinesterase activity was measured by a modification of the method of Ellman et al. [8]. Incubation mixtures containing 2.3 ml 0.1 M phosphate buffer, pH 8.0, 0.5 ml enzyme or sucrose incubation medium (see table 1) and 0.1 ml 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) were preincubated at 20°C for 5 min. The reaction was started by the addition of 0.1 ml 50 mM acetylthiocholine iodide and the increase of absorbance at 412 nm followed using a spectrophotometer equipped with a chart recorder. Values in tables are means of duplicate assays and are expressed as percentages of the total activity assayed in a suspension of unincubated erythrocytes. Total erythrocyte acetylcholinesterase activity was routinely assayed using 0.1 ml of a suspension (2%, v/v) of erythrocytes in 0.1% w/v Triton X-100 (final concentration of Triton in assay 0.0033%, w/v). In some experiments intact or osmotically lysed erythrocytes were also assayed and similar values were obtained. Acetylcholinesterase was distinguished from pseudocholinesterase using the specific inhibitor BW 284 C51 (1,5-bis-(4-allyl dimethylammoniumphenyl)pentan-3-one dibromide) at a concentration of 30 μM.

Chromatography of released acetylcholinesterase was carried out by loading 1 ml of a supernatant from phospholipase C-treated erythrocytes onto a column (33 × 2.6 cm) of Sepharose 6B equilibrated in 0.1 M NaCl, 50 mM Tris/HCl, pH 8.0. The column was eluted (4°C) at a flow rate of 8 ml/h and 4 ml fractions collected. Acetylcholinesterase activities in 1.4 ml samples of these fractions were assayed by a modification of the above procedure in which the phosphate buffer was replaced by 1.4 ml 50 mM Tris/HCl, pH 9.0. The void volume of the column, assessed with blue dextran, was 60 ml.

3. Results

Treatment of pig erythrocytes with phosphatidylinositol-specific phospholipase C produced a large release of acetylcholinesterase into the supernatant. Although all the acetylcholinesterase was released by 1.25 units/ml (table 1), there was no increase in the release of haemoglobin from the cells even at phospholipase C concentrations 8-times greater. In four experiments with pig erythrocytes, haemoglobin release in both control and phospholipase C incubations was in the range 0.35–0.55%. The total acetylcholinesterase recovered did not appear to be affected by incubation with or without phospholipase C and analysis of the residual erythrocytes (after phospholipase C treatment and washing) showed that acetylcholinesterase had decreased to approximately 1% of its original value.

Table 1
Effect of phosphatidylinositol-specific phospholipase C on release of acetylcholinesterase from erythrocytes

Phospholipase C (units/ml)	Acetylcholinesterase released (%)		
	human	pig[a]	ox
0	0.02	0.28	0.33
0.25	2.5	35	108
0.50	–	55	113
1.25	–	102	113
2.5	4.8	104	111
5.0	–	112	–
10.0	4.8	108	114

[a]Mean of 4 experiments

Erythrocytes were washed in isotonic saline and then washed and suspended (22%, v/v) in 0.29 M sucrose, 10 mM glucose, 10 mM NaCl, 1 mM Hepes/NaOH, pH 7.4. Of this suspension, 3.6 ml were incubated with 0.4 ml phospholipase C, diluted to the appropriate concentration with 50 mM Tris/HCl, pH 7.4, for 90 min at 37°C. The incubation mixtures were centrifuged at 2000 × *g* for 5 min and 2 ml samples of the supernatants were re-centrifuged at 150 000 × *g* for 40 min. Duplicate samples of the final supernatants were assayed for acetylcholinesterase activity as described in Materials and methods

Inclusion of 0.15 M NaCl or KCl (previously shown to inhibit the phospholipase C [4,9]) in the incubation medium reduced the release of acetylcholinesterase (from pig erythrocytes) by phospholipase C (0.25 units/ml) from approx. 30% to less than 2%. This decrease was not due to inactivation of the acetylcholinesterase as more than 90% of the initial activity was recovered in the sedimented cells.

In three experiments with pig erythrocytes the amounts of phosphatidylinositol hydrolysis which occurred during phospholipase C treatment were measured (table 2). Incubation with 10 units/ml for 90 min at 37°C, which released essentially all the acetylcholinesterase, only decreased the phosphatidylinositol content by approx. 5%. However, as table 2 shows, a decrease as small as this is not statistically significant. This result is in agreement with our previous work where no detectable phosphatidylinositol hydrolysis occurred on treatment of pig, ox or sheep erythrocytes with the phospholipase C [7]. Such low levels of hydrolysis might be more convincingly demonstrated by analysing for diglyceride produced, perhaps by gas–liquid chromatography.

Similar experiments carried out with human erythrocytes also showed a large increase in the release of acetylcholinesterase (tables 1 and 2). However the activity released only amounted to approx. 5% (range from 4 experiments, 4.8–7.0%) of the total. The amount released was not increased by raising the phospholipase C concentration (table 1). The relatively slight release of acetylcholinesterase from these cells was not due to inactivation of the enzyme by the phospholipase C as more than 90% of the original activity was recovered from both control and phospholipase C incubations. The amount of acetylcholinesterase released from human erythrocytes was not increased:

(i) By varying the age of the blood used (0–9 days old).

Table 2
Effect of phosphatidylinositol-specific phospholipase C on erythrocytes

	Release of component (%)		PI/PL (%)
	Haemoglobin	Acetylcholinesterase	
Pig			
Control	0.41	0.28	2.59 ± 0.052
Phospholipase C-treated	0.35	108	2.45 ± 0.065
Human			
Control	0.59	0.13	–
Phospholipase C-treated	0.70	5.4	–

Experimental conditions were essentially as described in legend to table 1 (phospholipase C, 10 units/ml). For pig erythrocytes the total volume of the incubation mixture was increased and several 2.5 ml samples were extracted and analysed for phosphatidylinositol content as described previously [7,9]. Samples of the low-speed supernatant were also assessed for haemolysis by comparing their absorbance at 412 nm with that of appropriate dilutions, in water, of the erythrocyte suspensions. Values for release are the means of 4 experiments. PI/PL is the amount of phosphatidylinositol (PI) expressed as a percentage of the other phospholipids (PL) and values are means ± S.E.M. of data from 3 separate experiments (14 determinations). The control and phospholipase C-treated values are not significantly different ($P < 0.1$)

(ii) By varying the period of incubation (30–240 min) in the presence of excess phospholipase C.
(iii) By washing sedimented, phospholipase C-treated erythrocytes with isotonic saline.
(iv) By omitting the high speed centrifugation.

The acetylcholinesterase released from human, pig and ox erythrocytes was inhibited by the specific acetylcholinesterase inhibitor BW 284 C51 at a concentration of 30 μM and is therefore unlikely to be due to residual serum pseudocholinesterase.

In contrast, ox erythrocytes appeared to be exceptionally sensitive as all the acetylcholinesterase was released (table 1) at a phospholipase C concentration of 0.25 units/ml (haemoglobin release 0.1–0.2%).

Incubation of human or pig erythrocytes with the non-specific phospholipase C from *Bacillus cereus* (1 μg/ml) for 90 min at 37°C did not release any acetylcholinesterase. It should be noted however, that this enzyme does release acetylcholinesterase from human erythrocyte ghosts [10].

Chromatography of the released acetylcholinesterase on a column of Sepharose 6B gave a single peak with an apparent mol. wt in the region 100 000–200 000 and no activity could be detected in the void volume. V_e/V_0 values were 2.00 for human and 1.95 for pig (mean of three experiments) erythrocyte acetylcholinesterase and were similar to those reported for alkaline phosphatase and 5′-nucleotidase released from membranes by a similar procedure [5]. Approximately 90% of the applied activity was recovered in the eluate.

4. Discussion

The results presented in this paper clearly demonstrate that acetylcholinesterase can be quantitatively released from pig or ox erythrocytes without significant cell lysis. It therefore appears likely that this ecto-enzyme is released from the outer surface of the membrane by the attack of phosphatidylinositol-specific phospholipase C on phosphatidylinositol at the same surface. Although similar data for the phospholipase C-induced release of alkaline phosphatase and 5′-nucleotidase are not available at present we feel that further investigations will reveal that these enzymes too are released by processes occurring at the outer surface of the plasma membrane.

Phosphatidylinositol in intact erythrocytes appears to be largely inaccessible to the action of phospho-

lipase C, possibly as a result of its location at the inner surface of the membrane [7]. The release of all the acetylcholinesterase from intact ox and pig erythrocytes by the phospholipase C therefore suggested that only a small proportion of the total membrane phosphatidylinositol was involved in the release process.

It has been well established, both in the present and in previous papers [1–5], that several plasma membrane ecto-enzymes are specifically released from membranes by phosphatidylinositol-specific phospholipase C. The exact molecular mechanism of this process is not clear but the available evidence supports the suggestion that the effect of the phospholipase C is to disrupt relatively specific interactions between individual enzyme molecules and phosphatidylinositol. The data are consistent with the identification of phosphatidylinositol as the binding site for these enzymes and perhaps for other membrane proteins. The phosphatidylinositol-specific phospholipase C might provide a useful means for completely removing specific proteins from cell surfaces and perhaps a starting point for their purification.

Acknowledgements

This work was supported by a grant from the Medical Research Council.

References

[1] Slein, M. W. and Logan, G. G. (1965) J. Bacteriol. 90, 69–81.
[2] Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T. and Ohyabu, T. (1976) Biochim. Biophys. Acta 450, 154–164.
[3] Low, M. G. and Finean, J. B. (1977) Biochem. Soc. Trans. in press.
[4] Low, M. G. and Finean, J. B. (1977) Biochem. J. in press.
[5] Low, M. G. and Finean, J. B. (1977) submitted.
[6] Low, M. G. and Finean, J. B. (1977) unpublished.
[7] Low, M. G. and Finean, J. B. (1977) Biochem. J. 162, 235–240.
[8] Ellman, G. L., Courtney, D., Andres, V. and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–98.
[9] Low, M. G. and Finean, J. B. (1976) Biochem. J. 154, 203–208.
[10] Zwaal, R. F. A., Roelofsen, B. and Colley, C. M. (1973) Biochim. Biophys. Acta 300, 159–182.