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DECREASED IODINATION OF THE RED CELL SURFACE FOLLOWING PHOSPHOLIPASE C TREATMENT

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Summary

Human red blood cells were treated with phospholipase C from *Clostridium welchii*. Lipase concentrations which produced <1% hemolysis and 10—15% hydrolysis of the membrane phospholipids reduced markedly (>80%) the accessibility of membrane proteins to the external surface as measured by lactoperoxidase-catalyzed iodination.

A number of studies suggest that in biological membranes two lipid regions exist; that is, a region in which the bulk lipid acts independently of protein [1—6], and one in which the lipid is closely associated with protein [7—11]. Intrinsic membrane proteins including those which span the erythrocyte membrane interact presumably with the membrane phospholipids. It is possible that this interaction can be perturbed by treating the red cells with lipolytic enzymes. Whether such a perturbation could be detected as a difference in protein accessibility at the surface was tested in this study using phospholipase C from *Clostridium welchii* to cleave the hydrophilic portion of the membrane phospholipids of intact cells. It has been reported that the phospholipase C from *Cl. welchii* [6] but not from *Bacillus cereus* [5,12] will hydrolyze the phospholipids of intact human erythrocytes. That this effect could be due to the ability of the *Cl. welchii* enzyme preparation to hydrolyze sphingomyelin is suggested by the observation that pure phospholipase C from *B. cereus* together with sphingomyelinase will also hydrolyze the phospholipids of intact cells [5].

Treatment of intact erythrocytes with phospholipase C from *Cl. welchii* (Sigma, Type 1, 7.6 units/mg) was carried out at 37°C for 10 min in 10 mM Tris-buffered saline, pH 7.4, containing 1 mM $CaCl_2$ at 5% cell density. Phos-

Abbreviations: EGTA, ethanedioxybis(ethylamine)tetraacetic acid; SDS, sodium dodecyl sulfate

pholipase C solutions (0.5 mg/ml) were freshly prepared and in some cases were heated for 15 min at 95°C to destroy proteolytic activity [13]. The reaction was terminated by addition of 2 mM Tris/EGTA and the erythrocytes were centrifuged. The supernatants were collected for determination of haemolysis. This was accomplished by measuring the absorbance at 540 nM of the supernatants and comparing this to the supernatant of erythrocytes lysed in distilled water (100% haemolysis). The red cells were washed twice with 20 vol. Tris-buffered saline and changes in protein accessibility at the external surface after phospholipase C treatment were evaluated using lactoperoxidase-catalyzed iodination which was carried out as previously described [14].

Since phospholipase C treatment of erythrocytes causes some haemolysis, the phospholipase C concentrations used in the present study were relatively low, of the order of 1 μg/ml, and the reaction time did not exceed 10 min. Using these conditions, haemolysis was reduced to less than 2%, thereby maintaining the integrity of the membrane and thus assuring that only the outer surface was accessible to lactoperoxidase. Although the determination of lipid phosphorus, based on membrane protein, varied somewhat from experiment to experiment, the extent of phospholipid hydrolysis measured as total phosphorus in chloroform/methanol membrane extracts, was $<10\%$ when haemolysis was $<1\%$ (three experiments, not shown). Despite this relatively small degree of phospholipid hydrolysis there was a marked effect on iodination. For example, in a typical experiment iodide incorporation was increased by 57% at a very low phospholipase C concentration (0.1 μg/ml) and then reduced by 80% at 1.0 μg/ml. A composite graph of a number of experiments (Fig. 1) shows the extent of iodination as a function of the degree of haemolysis. Increases in iodination were observed, but only at very low phospholipase C concentrations which produced virtually no haemolysis. At phospholipase C concentrations which caused 0.5% haemolysis, iodination decreased markedly. That the stimulation of iodide incorporation at low phospholipase C concentrations and the marked reduction of iodide incorporation at higher phospholipase C concentrations were not due to the presence of trace quantities of proteolytic enzymes in commercial preparations of phospholipase C was evidenced by the following:

(i) Similar results to the foregoing were obtained if precaution was taken

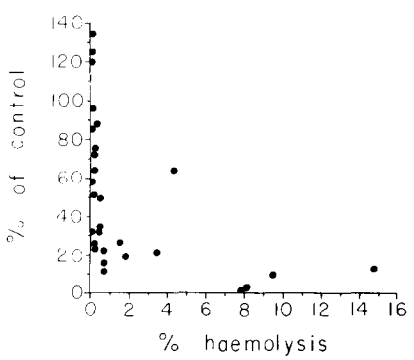

Fig. 1. Extent of iodination as a function of the degree of haemolysis.

to eliminate possible proteolytic activity in phospholipase C, i.e. by heat inactivation at 95°C for 15 min [13], albeit at higher phospholipase C concentrations due, presumably, to some heat inactivation of the lipolytic enzyme as well.

(ii) The detection of protein loss due to phospholipase C treatment was tested by iodinating intact cells with ^{125}I prior to phospholipase C treatment and reiodinating with ^{131}I following phospholipase C treatment. As shown in Fig. 2, only the ^{131}I incorporation is reduced; the ^{125}I incorporation remains constant. Thus phospholipase C treatment does not reduce the amount of protein normally accessible at the external surface but only decreases the accessibility of the proteins to lactoperoxidase catalyzed iodination. It is

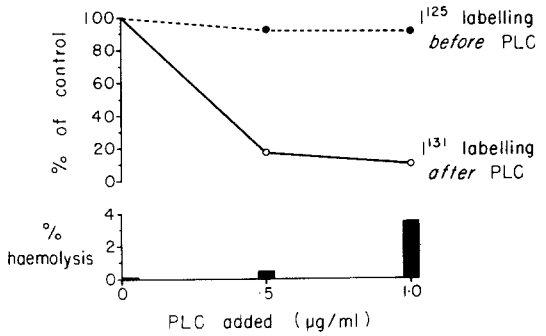

Fig. 2. Effect of phospholipase C (PLC) treatment on protein.

important to note that, as in earlier experiments with resealed red cell ghosts [15], reiodination (with ^{131}I) of previously labelled (with ^{125}I) red cells, gave the same labelling distribution on SDS gels (control, not shown). Moreover, we have not been able to detect labelling patterns for exposed protein different from the Coomassie Blue staining profile of the bulk uniodinated corresponding peptides [14]. Thus we have assumed that iodinated and non-iodinated peptide behave similarly.

It is also unlikely that the masking of the red cell membrane surface is due to proteins, other than the phospholipase, present in the commercial phospholipase C preparation. This conclusion is based on the observation that omission of Ca^{2+} during phospholipase C treatment markedly reduced the effect on surface labelling, i.e. a slight increase (9.3%) in labelling was observed with 1.0 μg/ml and a 51% decrease with 4 μg/ml phospholipase C, in contrast to the results shown in Fig. 2 and Fig. 3.

The effects of phospholipase C on the various membrane proteins were examined by SDS gel electrophoresis to determine if all the peptides which are iodinated at the external surface are similarly affected. As shown in Fig. 3, there was a reduction in iodination of all the major classes of peptides usually iodinated at the external surface [14].

The effects of perturbation of the lipid bilayer by phospholipase C on the accessibility of the intrinsic proteins, band III and PAS I, were quite dramatic. Even at low degrees of phospholipid hydrolysis (<1%) there was a large reduction of iodide incorporation into these two components (Fig. 3)

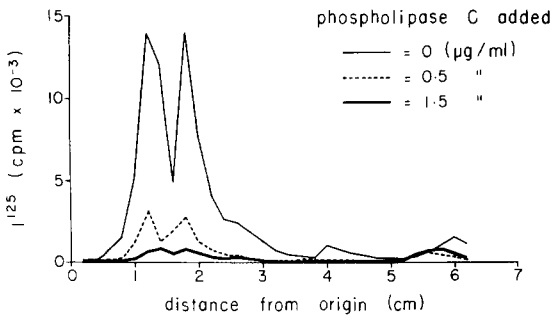

Fig. 3. Effect of phospholipase C pre-treatment on iodination.

due to a loss of accessibility of the proteins rather than a loss of protein per se. The fact that these two components are both glycoproteins and thus hydrophilic and yet are inaccessible at the external surface following phospholipase C treatment is relevant to the argument whether hydrophilic regions of a protein must extend into the intracellular or extracellular space on thermodynamic grounds. It is unlikely that the reduced protein iodination is a result of competition by lipid for iodination since the effect observed on total iodide incorporation was, in fact, due only to effects on protein labelling. Thus, on SDS gels, the radio-iodination in the lipid region, only slightly labelled in the controls, was not changed by phospholipase C.

These effects of phospholipase C may be relevant to the phenomenon described by Lunny and Ashwell [16] who found that phospholipase C reduced binding of asialoglycoproteins of rat liver membranes. The reduction in binding was not caused by a release of glycoprotein from the binding site but by a masking of the binding site. Using freeze-fracture to study the membrane perturbation by phospholipase C, Limbrick and Knutton found that following treatment of ghosts by phospholipase C from *Cl. welchii* the number of membrane associated particles was reduced without loss of protein [17].

The degree of phospholipid hydrolysis as measured by total phosphorus in chloroform/methanol extracts was approximately 10% at the highest phospholipase concentration used. This measurement may slightly underestimate the degree of phospholipid hydrolysis since Allan et al. have demonstrated that following treatment of intact human erythrocytes with phospholipase C from *Cl. perfringens*, there is an increase in phosphatidate content [18].

It is possible that the loss of accessibility of proteins at the external surface is caused by a masking of proteins by the fatty acid chains of the products formed by phospholipid C hydrolysis. Despite the low level of phospholipid hydrolysis, 80 to 90% of the iodinatable surface proteins were masked. Although it is possible that the phospholipids in close proximity to the intrinsic proteins are more susceptible to the phospholipases than the bulk of the lipids due to the perturbing influence of the proteins, Mavis et al. [19] showed that the rate at which enzymes requiring lipids were deactivated by phospholipase C treatment of *E. coli* membranes was similar to the rate of phospholipid hydrolysis suggesting that the bulk of the phospholipid and the phospholipid associated with protein are equally susceptible to phospholipase C. It remains possible that products of hydrolysis such as the diglycerides,

remain associated with the membrane [20], diffuse to form clusters with the intrinsic proteins which are part of the intramembraneous particles [21], and thereby mask them. The possibility that internalization of the membrane of intact red cells following treatment with phospholipase C, as observed by Allan et al. [18], gives rise to the reduced accessibility of proteins at the external surface is unlikely since the reduction in iodination was as great as 90% in most of our experiments.

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