Effect of Co^{2+} -substitution on the substrate specificity of phospholipase C from *Bacillus cereus* during attack on two membrane systems

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Phospholipid degradation by native phospholipase C from *Bacillus cereus* and enzyme forms where one or both of the Zn^{2+} prosthetic groups had been replaced with Co^{2+} was studied in human erythrocyte membranes (ghosts) and resuspended freeze-dried bovine brain myelin. The rate of total phospholipid degradation was 2–9-fold more rapid with erythrocytes than with myelin. With both membrane systems the activity decreased in the order $\text{ZnZn-enzyme} > \text{ZnCo-enzyme} > \text{CoCo-enzyme}$. For all three enzyme forms with either membrane system, phosphatidylethanolamine (or the ethanolamine-containing phosphoglycerides) and phosphatidylcholine were hydrolysed most rapidly and sphingomyelin least. The relative rate of sphingomyelin degradation was highest with the ZnCo-enzyme . In myelin at low ionic strength there seemed to be a core of phospholipid that was very resistant to degradation by native phospholipase C but which was much more accessible to the Co^{2+} -substituted forms. It is suggested that $\text{ZnCo-phospholipase C}$ has potential applications in membrane studies.

The phospholipolytic enzymes are commonly used in the study of biological membranes and phospholipid-dependent processes. In addition to their site of attack in a substrate molecule, their relative rates of hydrolysis of the various phospholipids are of special importance. Amongst the phospholipases, the phospholipase C (phosphatidylcholine cholinephosphohydrolase; EC 3.1.4.3) from *Bacillus cereus* is of particular interest since, in addition to being a tool in membrane research, the enzyme appears to have potential applications in the field of blood banking (Little & Rumsby, 1980; Little *et al.*, 1981) and also as a therapeutic agent to avoid post-traumatic intravascular coagulation (Golub *et al.*, 1962; Giercksky *et al.*, 1976). The enzyme is also unusual in that the substitution of its prosthetic Zn^{2+} by Co^{2+} appears to influence the substrate specificity so that the activity towards phosphatidylcholine is markedly decreased and that towards sphingomyelin is strongly enhanced (Otnæss, 1980; Little, 1981a). These latter studies were carried out mainly on deoxycholate micelles of pure phospholipids (phosphatidylcholine or sphingomyelin). Since the precise physical form of the substrate strongly influences the rate of hydrolysis by this enzyme (Little, 1977) the substrate specificity of the native and Co^{2+} -substituted enzyme towards the four major phospholipids in two

membrane systems, i.e. the membrane of lysed human erythrocytes and freeze-dried rehydrated bovine brain myelin, were examined.

Materials and methods

Crystalline phospholipase C was isolated from cultures of *Bacillus cereus* as described by Myrnes & Little (1980). Mono- and di-substituted Co^{2+} forms of the enzyme were prepared as described by Little (1981a). Erythrocyte membranes (ghosts) were prepared from fresh human blood by the method of Fröman *et al.* (1980). Myelin was isolated from bovine brain white matter as described by Rumsby *et al.* (1970) and freeze-dried.

Incubation procedure

Erythrocyte ghosts or myelin were suspended in 5 mM-Tris/HCl, pH 7.5, at concentrations of 4.8 and 9 μmol of lipid phosphorus/ml respectively. Resuspended myelin was allowed to rehydrate in 5 mM-Tris/HCl buffer for 1 h at room temperature before the addition of enzyme. Incubations with enzyme were carried out at 23°C.

Extraction and determination of phospholipids

During the course of the above incubation, portions of the mixture were removed and the lipid

was extracted therefrom by using the procedure described by Roelofsen *et al.* (1974) for use with phospholipase C-treated membranes. Individual phospholipids were determined after separation in two-dimensional t.l.c. on silica-gel H (Roelofsen *et al.*, 1974). Each lipid extract sample was analysed in duplicate. Phosphorus determinations were based on the method of Ames (1966) and each determination was made in duplicate.

Results and discussion

Rates of phospholipid degradation

The initial rates of hydrolysis of total phospholipid by native (ZnZn-) and the two Co²⁺-substituted (ZnCo- and CoCo-) forms of phospholipase C when attacking either erythrocyte ghosts or myelin were measured (Table 1). With both systems, the enzyme activity decreases in the order ZnZn-enzyme > ZnCo-enzyme > CoCo-enzyme. This is the same order as found when phosphatidylcholine/deoxycholate micelles or monomerically dispersed dihexanoylglycerophosphocholine serve as substrates (Little, 1981). For all enzyme forms, hydrolysis proceeds more rapidly with erythrocyte ghosts than with myelin. With native and ZnCo-phospholipase C, the difference in rates with the two membranes is a factor of 2–3, whereas with the CoCo-enzyme, erythrocyte ghosts are hydrolysed some 8–9-fold faster than is myelin. Isolated fresh myelin retains to a large degree its native multilamellar structure (Linington & Rumsby, 1980). Freeze-drying, however, disrupts this structure (Rumsby & Crang, 1977). Even so, this disrupted form of myelin is still a poorer substrate for the enzyme than is the erythrocyte ghost with its simple open bilayer structure. Indeed, erythrocyte ghosts seem to serve as an unusually

good substrate for phospholipase C with initial rates of hydrolysis at pH 7.5 and 23°C exceeding those obtained in the standard assay system for this enzyme (crude egg phosphatidylcholine/deoxycholate micelles) at similar pH and temperature (cf. Little *et al.*, 1975). It should, however, be noted that certain univalent anions seem to inhibit this enzyme during its hydrolysis of the erythrocyte membrane and myelin phospholipid and that in 5 mM-Tris/HCl, pH 7.5, erythrocyte membrane phospholipids are hydrolysed some 5–6-fold more rapidly than when 0.15 M-NaCl is present (Aakre & Little, 1982). Previously we have reported briefly on the effect of native enzyme on myelin at 37°C in 0.154 M-Tris/HCl, pH 7.4 (Gwarsha *et al.*, 1979). Under these conditions, where the myelin concentration was the same as that used in the present work, the initial rate of phospholipid hydrolysis was approx. 13 nmol/min per µg of enzyme. In 5 mM-Tris/HCl, pH 7.5, the initial rate was 900 nmol/min per µg of enzyme at 23°C (Table 1) and 5400 nmol/min per µg of enzyme at 37°C (results not shown). Thus the lower buffer concentration/ionic strength causes a rate enhancement of 415-fold. Previous work (Aakre & Little, 1982) suggests that a direct effect on the enzyme by the Tris buffer and the higher ionic strength would account for a rate difference around 20-fold so that it would seem that changes in myelin structure caused by the very different ionic strengths are responsible for another 20-fold increase in the rate of phospholipid hydrolysis.

Hydrolysis of individual phospholipids

The hydrolysis of phosphatidylethanolamine (or ethanolamine-containing phosphoglycerides), phosphatidylcholine, phosphatidylserine and sphingomyelin together with total phospholipid during the attack by the three forms of phospholipase C on erythrocyte ghosts and on myelin was studied (Figs. 1–4). In all cases it is clear that phosphatidylethanolamine and phosphatidylcholine are the best phospholipid substrates, followed by phosphatidylserine, and that sphingomyelin is the poorest.

Hydrolysis by native phospholipase C

With native enzyme and erythrocyte ghosts, phosphatidylethanolamine and phosphatidylcholine disappear at identical rates. The enzyme can readily cause total hydrolysis of these phospholipids, whereas, under the conditions used, no detectable sphingomyelin hydrolysis occurred. These results are similar to those observed by Roelofsen *et al.* (1971). Previously we have observed that during the lysis by native phospholipase C of spherocytic erythrocytes from long-stored blood, sphingomyelin was fairly readily hydrolysed (Little & Rumsby, 1980). Such lytic studies required the use of enzyme concentrations up to 100-fold greater than those used in

Table 1. Initial rate of phospholipid hydrolysis in erythrocyte membranes and in myelin by native and Co²⁺-substituted phospholipase C

Erythrocyte membranes or myelin were incubated at 23°C in 5 mM-Tris/HCl (pH 7.5) with phospholipase C and the initial rates of hydrolysis of total phospholipid measured from the decrease in lipid phosphorus. Further details are given in the Materials and methods section.

Enzyme form	Initial rate of phospholipid hydrolysis (nmol of phospholipid hydrolysed/min per µg of enzyme)	
	Erythrocyte membranes	Myelin
ZnZn-enzyme	2270	900
CoZn-enzyme	115	64.3
CoCo-enzyme	72	8.23

the work shown in Fig. 1(a). Consequently, erythrocyte ghosts were also exposed to 50-fold higher enzyme concentrations than those used in Fig. 1(a). Under these conditions, hydrolysis of sphingomyelin was observed (Fig. 2). Clearly, therefore, sphingomyelin in erythrocyte membranes is a very poor substrate for native phospholipase C and is hydrolysed at considerably less than 0.1% of the rate at which phosphatidylethanolamine is degraded.

The previous seemingly anomalous finding that during the lysis of aged erythrocytes, sphingomyelin is apparently degraded by native phospholipase C at a rate similar to that at which phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine are degraded (Little & Rumsby, 1980) can be explained simply. Lysis of these cells requires very high enzyme concentrations, the phospholipids in unlysed cells being very much more resistant to this enzyme than phospholipids in ghosts. Once a cell is lysed, a situation equivalent to that studied in Fig. 2, i.e., the exposure of lysed erythrocytes (ghosts) to a high concentration of native phospholipase C, would result and hence also sphingomyelin degradation together with the very rapid hydrolysis of phosphatidylcholine, -ethanolamine and -serine. Consequently, monitoring phospholipid hydrolysis in cells undergoing lysis could lead to the mistaken conclusion that sphingomyelin is an almost equally good substrate for the native enzyme as are the other three phospholipids.

Using myelin, native enzyme lowered the content of ethanolamine-containing phosphoglycerides more rapidly than it did the phosphatidylcholine. As for

erythrocyte ghosts, sphingomyelin in myelin was very resistant to hydrolysis. However, one apparent difference between the two membrane systems is that, whereas with erythrocyte ghosts, the native

Fig. 2. Phospholipid hydrolysis in erythrocyte ghosts at a high concentration of ZnZn-phospholipase C

Erythrocyte ghosts were incubated with 5 μ g of native phospholipase C/ml and phospholipid hydrolysis was determined and expressed as a percentage of untreated controls. For the reaction conditions and definition of symbols, see the Materials and methods section and the legend to Fig. 1.

Fig. 1. Phospholipid hydrolysis in erythrocyte ghosts and myelin by ZnZn-phospholipase C

Erythrocyte ghosts and myelin were incubated at 23°C in 5 mM-Tris/HCl (pH 7.5) and phospholipid hydrolysis determined as described in the Materials and methods section. The results are expressed as percentages of untreated controls. Symbols: Δ , sphingomyelin; $\square$, phosphatidylserine; $\blacktriangle$, ethanolamine-containing phosphoglycerides; $\circ$, phosphatidylcholine; $\bullet$, total phospholipid. (a) Erythrocyte ghosts incubated with 0.1 μ g of enzyme/ml; (b) myelin incubated with 0.7 μ g of enzyme/ml.

enzyme can readily hydrolyse the entire content of phosphatidylcholine and phosphatidylethanolamine, together with most or all of the phosphatidylserine (Fig. 1a; Roelofsen *et al.*, 1971), in myelin there appears to be a resistant core comprising in addition to all the sphingomyelin, around 10% of the original phosphatidylethanolamine, 20% of the phosphatidylcholine and 60% of the phosphatidylserine.

Hydrolysis by Co^{2+} -substituted phospholipase C

The main feature seen when either membrane system was incubated with ZnCo-phospholipase C

was a significant rate of hydrolysis of sphingomyelin. With the CoCo-enzyme, sphingomyelin degradation was observed in myelin but not under the present conditions in erythrocyte ghosts. Further comparison of the relative rates of hydrolysis of the phospholipids by the three enzyme forms shows only relatively small differences. Thus, in erythrocyte ghosts, the rate of phosphatidylserine hydrolysis relative to that of phosphatidylcholine seems greater with ZnCo-phospholipase C than with the ZnZn-enzyme. In addition, whereas the phosphatidylcholine and phosphatidylethanolamine content of

Fig. 3. *Phospholipid hydrolysis in erythrocyte ghosts and myelin by ZnCo-phospholipase C* Erythrocyte ghosts and myelin were incubated with ZnCo-phospholipase C and phospholipid hydrolysis was determined and expressed as a percentage of untreated controls. For the reaction conditions and definition of symbols, see the Materials and methods section and the legend to Fig. 1. (a) Erythrocyte ghosts incubated with 1 µg of ZnCo-phospholipase C/ml; (b) myelin incubated with 7 µg of ZnCo-phospholipase C/ml.

Fig. 4. *Phospholipid hydrolysis in erythrocyte ghosts and myelin by CoCo-phospholipase C* Erythrocyte ghosts and myelin were incubated with CoCo-phospholipase C and phospholipid hydrolysis was determined and expressed as a percentage of untreated controls. For the reaction conditions and definition of symbols, see the Materials and methods section and the legend to Fig. 1. (a) Erythrocyte ghosts incubated with 1 µg of CoCo-phospholipase C/ml; (b) myelin incubated with 35 µg of CoCo-phospholipase C/ml.

erythrocyte ghosts disappear at very similar rates in the presence of the native enzyme, with both Co²⁺-substituted forms, the former phospholipid appears to be clearly the better substrate. Effects equivalent to these are not apparent from the studies using myelin. However, one interesting effect of Co²⁺-substituted enzyme on myelin is that the previously mentioned inaccessible core of phospholipids seen with native phospholipase C is no longer clearly apparent (*cf.* Figs. 3*b* and 4*b* with Fig. 1*b*). Possibly, therefore, this core owes its existence in some way to sphingomyelin and when this is degraded the residual amounts of the other phospholipids become available to the enzyme. Previously we have reported briefly the effect of ZnZn-phospholipase C on both freshly isolated and resuspended freeze-dried myelin in 0.15 M-Tris/HCl buffer, pH 7.4, at 37°C (Gwarsha *et al.*, 1979, 1980). The maximum extents of phospholipid degradation were 40% and 60% respectively. The latter value agrees well with that obtained under the conditions used in the present work (see Fig. 1*b*).

The above results were repeated on two different batches of erythrocyte ghosts and two different batches of myelin. Two different batches of enzyme were used and several different preparations of metal-substituted enzyme without affecting the above pattern of results.

Based on the present work it would seem that Co²⁺-substituted phospholipase C, like its native form, is suitable for use in membrane studies. The strongly enhanced sphingomyelinase activity of the ZnCo-form could under certain circumstances be of value. Furthermore, this form is readily produced by the direct dialysis of native enzyme against free Co²⁺ (Little, 1981*a*) and is only slightly less stable than the very stable native enzyme (Little, 1981*b*). The CoCo-enzyme is probably less useful in that it has a

lower overall activity than the other two enzyme forms and is fairly unstable (Little, 1981*b*).

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