THE HYDROLYSIS OF SPHINGOMYELIN BY PHOSPHOLIPASE C FROM *BACILLUS CEREUS*

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Received 20 March 1980
Revised version received 10 April 1980

1. Introduction

Phospholipase C from *Bacillus cereus* contains 2 Zn^{2+}/molecule [1] and catalyzes the hydrolysis of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine in artificial [2,3] and biological membranes [4–6] while sphingomyelin was found not to be a substrate for the enzyme.

In the experiments presented here, the enzyme can be inactivated by *o*-phenanthroline and reactivated by Co^{2+} in such a way that also sphingomyelin micelles become a substrate. Phospholipase C with such altered specificity does not cause lysis of freshly isolated human or bovine erythrocytes.

Such a change of the substrate specificity is of particular interest since phospholipases are widely used tools in membrane studies.

2. Materials and methods

2.1. *Phospholipase C from* Bacillus cereus

ATCC 10987 AB 1 was purified as in [7], and the enzyme preparation was homogeneous by disc gel electrophoretic analysis [2]. The enzyme (0.3–0.4 mg/ml) was stored in veronal-buffered saline containing 1 mM $ZnCl_2$. Prior to use, free Zn^{2+} were removed by passing 1 ml enzyme solution through a column (10 × 0.6 cm) of Bio-Rad Chelex-100 chelating resin equilibrated with veronal-buffered saline without $ZnCl_2$. An enzyme aliquot was inactivated by incubating at 4°C for 2 h with an equal volume of 10 mM *o*-phenanthroline (Sigma, Chemical Co., St Louis, MO). Enzyme without *o*-phenanthroline was incubated with H_2O under identical conditions.

2.2. *Preparation of substrate*

Sonicated vesicles were prepared with mannitol-buffer as described elsewhere [2]. Sphingomyelin (bovine brain, Koch-Light, Colnbrook) and phosphatidylcholine (L-dipalmitoyl, Koch-Light) at 1 mg phospholipid/ml were sonicated 12 × 15 s while kept on ice.

2.3. *Treatment of phospholipids with phospholipase C*

Sonicated vesicles (1.5 ml) were incubated with 10 μl 100 mM $ZnCl_2$ or $CoCl_2$ for 5 min at 37°C. The reaction was initiated by the addition of phospholipase C (final conc. 0.2–2.5 μg phospholipase C/ml). The reaction was quenched after 10 or 30 min by the addition of 50 μl concentrated perchloric acid, and the reaction vessels were left on ice for 2 h or overnight. Following filtration through Millipore filters (0.22 μm), 500 μl samples were digested [2] and phosphate measured [8]. Values reported are the means of duplicate assays.

2.4. *Treatment of erythrocytes with phospholipase C*

Human and bovine erythrocytes were washed 3 times with veronal-buffered saline. Aliquots (2 ml) of 2.5% erythrocyte suspension were mixed with 2–5 μg phospholipase C and 20 μl 100 μM $CoCl_2$ or $ZnCl_2$. The samples were incubated 1 h at 37°C, alternatively, for 30 min at 23°C, followed by a 10 min incubation on ice and another incubation (20 min) at 23°C. The cells were removed by centrifugation and the degree of hemolysis determined [4].

3. Results and discussion

Phospholipase C is a zinc metalloenzyme [1,9,10]

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Table 1
Hydrolysis of phospholipid vesicles by phospholipase C (*Bacillus cereus*)

Pretreatment with *o*-phenanthroline (5 mM)	Metal ion (0.6 mM)	Phospholipid hydrolyzed[a] (%)	
		Phosphatidylcholine	Sphingomyelin
–	–	54	0
–	Zn^{2+}	56	0
–	Co^{2+}	42	0
+	–	11	0
+	Zn^{2+}	56	0
+	Co^{2+}	48	16

[a] Incubation (30 min at 37°C) of phosphatidylcholine or sphingomyelin vesicles with phospholipase C (2.5 μg/ml final conc.)

and may be inactivated by *o*-phenanthroline. The inactivation is reversed by the addition of Zn^{2+} or Co^{2+} [1].

The activity towards sonicated phosphatidylcholine vesicles is fully restored by the addition of $ZnCl_2$ while the reactivation observed with $CoCl_2$ is somewhat lower (table 1). The addition of $CoCl_2$ to native enzyme results in a similar decrease in activity towards phosphatidylcholine, suggesting that $CoCl_2$ may have a slight inhibitory effect under these conditions.

No hydrolysis of sphingomyelin was obtained by native phospholipase C in the absence or presence of metal ions. Following reactivation of *o*-phenanthroline treated enzyme with $CoCl_2$, the enzyme hydrolyzed sphingomyelin and the rate of hydrolysis was ~30% of the phosphatidylcholine hydrolysis (table 1). No sphingomyelin was hydrolyzed when the enzyme was reactivated by Zn^{2+}. When lower enzyme concentrations were used (0.2–0.6 μg phospholipase C/ml), no hydrolysis of sphingomyelin by Co^{2+} was observed, in agreement with [2].

These data indicate that phospholipase C from *Bacillus cereus* may hydrolyze sphingomyelin in sonicated vesicles under certain conditions. The enzyme also may hydrolyze sphingomyelin in aged erythrocytes [11] and erythrocyte ghosts [12]. The sphingomyelin hydrolyzing activity is not likely due to contamination by other phospholipases, since sphingomyelin hydrolysis has been obtained when crystallized phospholipase C was used [11].

When Co^{2+}-reactivated *o*-phenanthroline treated enzyme was added to suspensions of human or bovine erythrocytes, no hemolysis was observed. The change of substrate specificity observed with sphingomyelin vesicles is probably not accompanied by a similar hydrolysis attack on sphingomyelin in fresh erythrocytes, although there might be a limited degradation of phospholipids without hemolysis.

The hydrolysis of sphingomyelin by *B. cereus* phospholipase C under conditions where either the native membrane substrate has been altered [11,12], or the enzyme has been altered by the replacement of Zn^{2+} with another metal ion, opens new possibilities for the regulation of the specificity of phospholipid hydrolysis by phospholipases. Work with phospholipase A_2 (*Naja naja naja*) has shown that substrate specificity reversal may occur depending on the structure and composition of the substrate [13,14]. Studies of membrane structure based on the action of added phospholipases, should be interpreted with more caution.

Acknowledgement

This work was supported by the Norwegian Research Council for Science and the Humanities.

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