IN VIVO ACTIVATION BY POLYMYXIN B OF PHOSPHOLIPASE C FROM *PSEUDOMONAS AERUGINOSA*

Sir:

Our previous papers[1,2] indicated that polymyxin B (PLB) markedly induced the degradation of phospholipids in *Pseudomonas aeruginosa* AMS 6073 and that this phenomenon was caused by the *in vivo* activation of phospholipase which liberated fatty acids from the phospholipids. In the course of the work, we also detected diglycerides (DG) as products from exogeneously added phosphatidylethanolamine (PE) in the phospholipase assay system[2]. The present paper describes that phospholipase C (EC 3.1.4.3) is also activated *in vivo* by PLB.

The activity of the phospholipase C was assayed by the formation of 1,2-diacylglycerol from the substrate, PE. Enzyme fractions from control- and PLB- (or colistin-) treated cells were prepared as described in the previous paper[2]. The substrate, ^{14}C-PE exclusively labeled in the fatty acid moieties, was also prepared as described earlier[2] according to the procedure of DOI *et al.*[3] The standard reaction mixture contained 0.25 ml of the enzyme solution, 0.25 ml of 0.1 M Tris-HCl buffer (pH 8.0) and 0.125 ml of 800 μM ^{14}C-PE in a total volume of 0.75 ml. The reaction mixture was incubated at 40°C with shaking for 60 minutes. After incubation, the reaction was terminated by adding 4.5 ml chloroform - methanol (2: 1) solution. The lipid materials in the reaction mixture were recovered by the modified extraction procedure of BLIGH & DYER[4]. After the chloroform phase was evaporated to dryness, the resulting residue was dissolved in a small volume of chloroform - methanol (2: 1) and spotted on a pre-coated thin-layer plate (Silica gel 60, E. Merck, Darmstadt Germany: Code No. 5724). The identity of PE, DG and free fatty acid (F-FA) was established by comparing the Rf values of the spots with those of appropriate standards after development with two different solvent systems: chloroform - methanol - water (65: 25: 4) and petroleum ether - diethylether - acetic acid (80: 30: 1). Standard DG were prepared by hydrolysing purified PE with authentic phospholipase C from *Bacillus cereus* (Sigma Chemical Company, St. Louis, Mo., U.S.A.). After the autoradiography, areas corresponding to each spot were scraped off from the thin-layer plate. Their radioactivities were counted by a liquid scintillation spectrometer (Packard Tri-Carb 3385, Packard Instrument Company, Inc.) using a toluene scintillation fluid.

Table 1 indicates that the enzyme from PLB treated-cells degraded ^{14}C-PE to F-FA and DG. Colistin, another polymyxin-group antibiotic, also induced the degradation of ^{14}C-PE to the same extent as PLB. The degradation with untreated enzyme was of much lower order. It is evident that phospholipase C as well as F-FA liberating phospholipase(s) are activated by PLB and colistin *in vivo*.

In the previous paper,[2] we showed that an activation of F-FA liberating phospholipase(s) was observed with the cells that were incubated with PLB (5 μg/ml) only for 5 minutes. We performed a similar type of experiment with phospholipase C. The results were shown in Table 2. As reported previously, F-FA liberating phospholipase(s) was optimally activated in the cells that were incubated with PLB for only 5 minutes. By contrast, a longer time of incubation was required for the activation of phospholipase C. This result may be due to different locations of the enzymes. It has been already known that phospholipase C exists mainly as an extracellular enzyme in *Pseudomonas aureo-*

Table 1. Degradation of ^{14}C-PE to DG and F-FA by enzyme fractions from control and PLB (or colistin) treated cells

Treatment	Enzyme concentration (mg protein/ml)	D.P.M. (% of total)		
		Substrate remaining	Reaction product	
		PE	F-FA	DG
None	3.72	54,400 (89.9)	3,100 (5.1)	3,000 (5.0)
PLB-treated (5 μg/ml)	2.08	24,500 (40.2)	14,800 (24.4)	21,500 (35.4)
Colistin-treated (5 μg/ml)	2.56	27,500 (41.0)	16,100 (24.0)	23,400 (35.0)

Table 2. Effect of incubation time with PLB on activation of ^{14}C-PE degradation

PLB treatment of enzyme (min.)	Enzyme concentration (mg protein/ml)	Substrate remaining	Reaction product	
		PE (D.P.M.)	DG (D.P.M.)	F-FA (D.P.M.)
0	2.5	56,800	2,900	600
5	2.3	49,600	4,700	6,500
10	2.3	39,600	16,900	1,600
15	2.7	38,600	16,100	2,000
30	2.8	27,300	25,600	2,600
60	2.6	25,000	32,600	3,200

* When the optical density of the culture reached 0.3, PLB (5 μg/ml, final concentration) was added to the culture. Cells were harvested by centrifugation at times indicated and enzyme fractions were prepared from cells as described previously[2].

Table 3. Effect of CM on the activation of ^{14}C-PE degradation

Treatment of cells	Enzyme concentration (mg protein/ml)	% of total radioactivity		
		Substrate remaining	Reaction products	
		PE	DG	F-FA
None	2.0	92.8	5.9	1.3
PLB	2.0	66.2	30.5	3.3
CM	2.0	96.2	2.7	1.1
CM+PLB	2.0	81.1	16.4	2.5

At the optical density of 0.3, the culture was divided into two fractions, one of which was incubated with CM (200 μg/ml) for 30 minutes. They both incubated fractions were again divided into two protions. PLB (5 μg/ml) was added to the one portion of each fraction and all cultures were further incubated for 60 minutes. After incubation, the cells were harvested by centrifugation. Enzymes were prepared as described earlier[2].

faciens[5,6] and phospholipase A in *P. aeruginosa*[7] exists as an outer membrane-bound enzyme.

There are some data that suggest phospholipase A in *Bacillus subtilis*,[8] *Escherichia coli*[9], and bacteriophage T4[10] exists in a "latent" form similar to bacterial lytic enzymes. Consequently, we examined whether or not the activation of phospholipase C by PLB occurred in the presence of 200 μg/ml of chloramphenicol (CM: Sankyo Co., Ltd., Tokyo). This concentration of CM completely inhibited the protein synthesis in this organism. As shown in Table 3, activation of phospholipase C induced by PLB occurred even in the presence of CM. A similar result was obtained with the F-FA liberating phospholipase(s). The induced activities of both enzymes were, however, less than those obtained in the absence of CM. These results indicate that both phospholipases exist primarily in latent forms.

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