IN VITRO ACTIONS OF SOME ANTIBIOTICS ON PHOSPHOLIPASES

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The effects of some antibiotics on activities of phospholipase A_2, B and C were investigated *in vitro*. Tetracyclines, macrolides, chloramphenicol and carbenicillin inhibited the activity of *Crotalus adamanteus* phospholipase A_2 towards phospholipids of egg-yolk emulsions. When the ability to inhibit the activity of *Penicillium notatum* phospholipase B towards mixed micelles of phosphatidylcholine and Triton X-100 was investigated, polymyxin B was found to be inhibitory while chloramphenicol and carbenicillin were found to stimulate the activity of the phospholipase. The activity of *Bacillus cereus* phospholipase C towards the mixed micelles was inhibited by bleomycin, oleandomycin and chloramphenicol.

In a previous paper[1] we demonstrated that bleomycin and polymyxin B inhibited the activity of phospholipase C [EC 3.1.4.3] from *Clostridium perfringens*, while none of antibiotics tested exerted any effects on the activity of lysophospholipase[EC 3.1.1.5] from *Penicillium notatum*.

Other papers dealing with the effects of antibiotics on phospholipases report the following: (1) An inhibition of the synthesis of phospholipase C in *Bacillus cereus*[2], (2) an initiation of phospholipase A_2 activity in human platelets by the calcium-ion ionophore A23187[3], (3) an enhancement of bee venom phospholipase A_2 activity by the formation of a complex between polymyxin B and ovolecithin liposomes[4] and (4) the *in vivo* activation of phospholipase C from *Pseudomonas aeruginosa*[5] by polymyxin B.

Additional experiments have been conducted to elucidate the effects of several antibiotics on the activities of phospholipase A_2 [EC 3.1.1.4] from *Crotalus adamanteus*, phospholipase B* from *P. notatum* and phospholipase C from *B. cereus* and to compare these effects with those brought about by local anesthetics which interfere with the activity of phospholipase A_2 by interacting with the substrates[6].

Materials and Methods

Materials

The antibiotics and local anesthetics tested were as follows (the manufacturers in parentheses): sodium oxacillin, dihydrostreptomycin sulfate (Banyu), sodium cloxacillin (Meiji), disodium carbenicillin, oleandomycin phosphate, polymyxin B sulfate(Pfizer-Taito), minocycline hydrochloride, tetracycline hydrochloride (Lederle-Japan), chloramphenicol (Sankyo), mitomycin C (Kyowa Hakko), erythromycin, chlorpromazine hydrochloride (Shionogi), bleomycin hydrochloride (−Cu), pepleomycin sulfate (−Cu) and (+Cu) (Nippon Kayaku) and dibucaine hydrochloride (Teikoku). These compounds were obtained in pure and biologically active form. The bleomycin hydrochloride(−Cu) used was a mixture of structurally-analogous glycopeptides. Pepleomycin sulfate was a single glycopeptide and

* Throughout this paper the term phospholipase B will refer to a single enzyme catalyzing the complete deacylation of diacyl- and monoacylglycerophospholipids.

one component of the bleomycin. Erythromycin was dissolved with HCl at around pH 3.0 and immediately the pH was adjusted to the value indicated.

Phospholipase A_2 from *Cr. adamanteus* (Sigma Chemicals) was partially purified by heat treatment at pH 3.0[7]. Pure phospholipase B from *P. notatum*, generously supplied by Dr. T. OKUMURA of our laboratory, was prepared using the method described elsewhere[8]. Phospholipase C from *B. cereus* kindly donated by Dr. H. IKEZAWA, Faculty of Pharmaceutical Science, Nagoya City University, Japan, was almost homogeneous. Purified egg-yolk phosphatidylcholine was prepared as described previously[9]. [^{14}C]Phosphatidylcholine(1-acyl-2-[^{14}C]oleoyl-sn-glycero-3-phosphorylcholine) was prepared biosynthetically from [1-^{14}C]oleic acid with rat liver microsomes by a modification of the method of PUGH and KATES[10].

Phospholipase A_2 assays

(1) With egg-yolk emulsions as substrate. One egg-yolk was dispersed in 5 volumes of 0.9% NaCl. Five ml of this suspension and 7.5 ml of desired amount of antibiotic in 0.9% NaCl were pipetted into a thermostated vessel and the incubation mixture (14.5 ml) was adjusted to pH 7.00. The fatty acids liberated by the addition of 9 μg *Cr. adamanteus* phospholipase A_2 in 0.3 ml of 30 mM $CaCl_2$ were automatically titrated with 0.005 N NaOH at pH 7.00 and 25°C under a stream nitrogen, using a Radiometer pH-stat TTT 60 equipment.

(2) With radioactive substrate. The incubation medium contained 5 mM [^{14}C]phosphatidylcholine, 30 mM Triton X-100, 25 mM Tris·HCl buffer/10 mM $CaCl_2$, 0.1 mM EDTA (pH 7.4), desired amount of antibiotic and 2 μg *Cr. adamanteus* phospholipase A_2 in a total volume of 0.4 ml. After incubation at 30°C for 10 minutes, the enzyme reaction was stopped and the hydrolytic activity determined as described previously[9].

Phospholipase B assay

The incubation medium contained 5 mM egg-yolk phosphatidylcholine, 30 mM Triton X-100, 25 mM acetate buffer (pH 4.0), desired amount of antibiotic and 0.07 μg *P. notatum* phospholipase B in a total volume of 0.4 ml. After incubation at 30°C for 10 minutes, the enzyme reaction was stopped by adding 1.0 ml CH_3OH and 0.5 ml $CHCl_3$ followed by an additional 0.5 ml each of $CHCl_3$ and H_2O. After centrifugation, 1.0 ml of CH_3OH - H_2O layer was washed once with 0.5 ml $CHCl_3$. The amount of glycerophosphorylcholine in the CH_3OH - H_2O layer liberated from the phosphatidylcholine was determined by a modified method of the KING's one[11]. When the radioactive substrate was used, a hydrolytic activity was determined according to the method of described above.

Phospholipase C assay

The incubation medium contained 5 mM egg-yolk phosphatidylcholine, 30 mM Triton X-100, 25 mM malate buffer (pH 6.4), desired amount of antibiotic and 0.4 μg *B. cereus* phospholipase C in a total volume of 0.4 ml. After incubation at 30°C for 10 minutes, the enzyme reaction was terminated as described in the phospholipase B assay. The amount of phosphorylcholine in the CH_3OH - H_2O layer which was liberated from the phosphatidylcholine was determined by a phosphorus analysis[11]. When the radioactive substrate was used, the amount of [^{14}C]diacylglycerol in the $CHCl_3$ layer which had been liberated from phosphatidylcholine was determined previously[9], with the exception that the areas in the thin-layer chromatograms which corresponded to the substrate and product were scrapped off. In the thin-layer chromatography, the first solvent, $CHCl_3$ - CH_3OH - H_2O (65: 35: 8, v/v) was run to the middle of the plate and dried , and then the second solvent, $CHCl_3$ - CH_3COCH_3 (96: 4, v/v) was run to the top with the same direction.

The enzyme reaction, unless stated otherwise, was initiated by the addition of enzyme after preincubation with the substrate and the antibiotic. Each specific activity towards mixed micelles of phosphatidylcholine and Triton X-100 was 34 μmol min^{-1} (mg protein)$^{-1}$ for *Cr. adamanteus* phospholipase A_2, 446 μmol min^{-1} (mg protein)$^{-1}$ for *P. notatum* phospholipase B and 114 μmol min^{-1} (mg protein)$^{-1}$ for *B. cereus* phospholipase C. Reaction rates were linear within the times of incubation and protein concentrations used. Protein concentrations were determined by the method of LOWRY *et al.*[12] with crystalline bovine serum albumin as the standard.

Results and Discussion

Table 1 shows the inhibitory effect of several antibiotics (which are in widespread clinical use) on phospholipase A_2-catalyzed hydrolyses of egg-yolk emulsions. Minocycline hydrochloride and tetracycline hydrochloride (10 mM) caused 100% inhibition of phospholipase A_2 activity. This inhibition could be fully reversed by the addition of 10 mM Ca^{2+}. Because it has been reported that tetracyclines have the chelating action[13], this inhibitory action of tetracyclines may be explained by the removal of Ca^{2+} which is needed for *Cr. adamanteus* phospholipase A_2. Such macrolides as erythromycin and oleandomycin phosphate were effective in inhibiting phospholipase A_2 activity. IC_{50} of erythromycin was 7.8 mM which was almost the same as the substrate concentration (7.2 mM phospholipids) found

Table 1. Effects of antibiotics (10 mM) on hydrolyses of egg-yolk emulsions by *Cr. adamanteus* phospholipase A_2.

Drug	Remaining activity (%)
No addition	100
Sodium cloxacillin	95
Sodium oxacillin	80
Disodium carbenicillin	80
Minocycline hydrochloride	0
Tetracycline hydrochloride	0
Chloramphenicol	61
Dihydrostreptomycin sulfate	145
Mitomycin C	88*
Oleandomycin phosphate	56
Erythromycin	44

* This value was obtained at 7.5 mM drug concentration.

Fig. 1. Effect of antibiotic concentrations on hydrolyses of egg-yolk phospholipids by *Cr. adamanteus* phospholipase A_2.

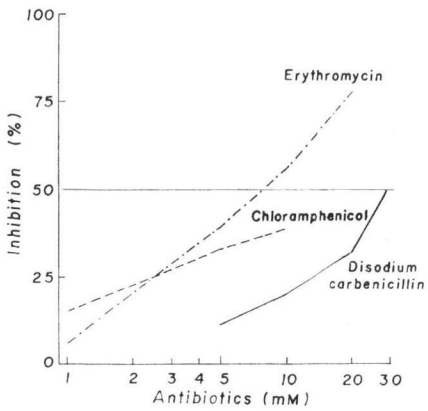


Table 2. Effects of antibiotics (5 mM) on hydrolyses of egg-yolk phosphatidylcholine by *Cr. adamanteus* phospholipase A_2, *P. notatum* phospholipase B and *B. cereus* phospholipase C.

Drug	Remaining activity (%)		
	Phospholipase A_2 (*Cr. adamanteus*)	Phospholipase B (*P. notatum*)	Phospholipase C (*B. cereus*)
No addition	100	100	100
Chloramphenicol	84	141	77
Disodium carbenicillin	99	129	107
Erythromycin	110	94	96
Oleandomycin phosphate	81	103**	61**
Bleomycin hydrochloride (−Cu)	100	76	46
Polymyxin B sulfate	98	88	107
Chlorpromazine hydrochloride*	64	59	63
Dibucaine hydrochloride*	110	75	68

* These local anesthetics were compared with antibiotics.

** With radioactive substrate.

The molecular weights of bleomycin hydrochloride and polymyxin B sulfate were calculated as 1,400 and 1,200, respectively.

in the egg-yolk emulsions (Fig. 1). Antibiotics such as erythromycin, oleandomycin phosphate, chloramphenicol and disodium carbenicillin which have different therapeutic effects inhibited phospholipase A_2 activity. They all belong to amphiphilic drugs the structures of which contain a hydrophobic region.

Table 2 shows the effects of these amphiphilic antibiotics and copper-free bleomycin hydrochloride (−Cu) on the activities of phospholipase A_2, B and C towards mixed micelles of phosphatidylcholine and a nonionic detergent Triton X-100. On the activity of phospholipase A_2, disodium carbenicillin (50 mM), erythromycin, bleomycin hydrochloride (−Cu) and polymyxin B sulfate had no effect, while chloramphenicol and oleandomycin phosphate were slightly inhibitory. This observation indicates that there is no direct inhibition of phospholipase A_2 activity by erythromycin and disodium carbenicillin.

In regards to the inhibition of phospholipase B activity, polymyxin B sulfate (IC_{50}, 22.5 mM) was only slightly effective as compared with chlorpromazine hydrochloride (IC_{50}, 7.3 mM) (Fig. 2). On the other hand, disodium carbenicillin (50 mM) and chloramphenicol (20 mM) exerted 1.5-fold and 1.9-fold stimulation, respectively (Fig. 2). These antibiotics showed no effect on the deacylation of monoacylglycerophospholipids by this enzyme as described previously[1], but inhibited or stimulated the deacylation of diacylglycerophospholipids.

In regards to the effect on phospholipase C activity, chloramphenicol, oleandomycin phosphate and bleomycin hydrochloride (−Cu) were inhibitory (Table 2). Due to the incubation of *B. cereus* phospholipase C without polymyxin B sulfate in 0.1 M malate buffer (pH 6.4) at 30°C, the activity of the enzyme decreased slowly (85% remaining activity for 30-minute incubation). But when polymyxin B sulfate was present, the activity remained 100% (1.5-fold activity as compared without it for a 30-minute incubation). However in a previous paper[1] polymyxin B sulfate was shown to inhibit the activity of phospholipase C from *Cl. perfringens*. These effects with cationic amphiphile polymyxin B sulfate may be due to the difference of the charges of enzyme proteins, *i.e.*, anionic polypeptide (pI 5.2~5.6) from *Cl. perfringens* and cationic polypeptide (pI 8.0~8.1) from *B. cereus*, since the activity of *P. notatum* phospholipase B, anionic polypeptide (pI 4.0) was inhibited by this drug. Oleandomycin

Fig. 2. Effect of antibiotic concentrations on hydrolysis of egg-yolk phosphatidylcholine by *P. notatum* phospholipase B. The molecular weights of bleomycin hydrochloride and polymyxin B sulfate were calculated as 1,400 and 1,200, respectively.

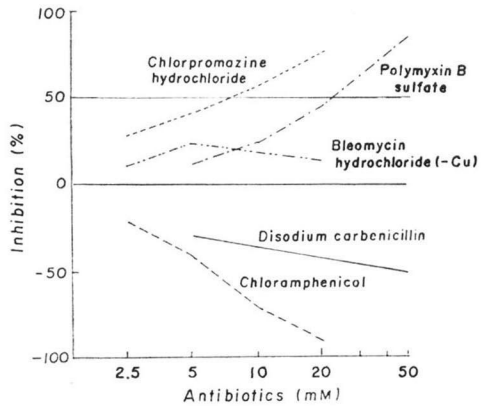
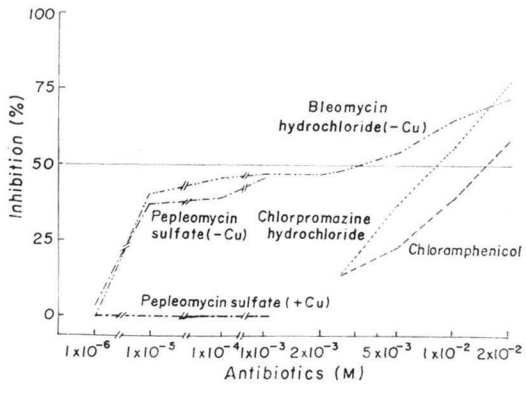


Fig. 3. Effect of antibiotic concentrations on hydrolysis of egg-yolk phosphatidylcholine by *B. cereus* phospholipase C. The molecular weights of bleomycin hydrochloride and pepleomycin sulfate were calculated as 1,400.



phosphate and chlorpromazine hydrochloride (5 mM) caused about 50% inhibition, while erythromycin was not inhibitory (Table 2). Fig. 3 shows that IC_{50} of chloramphenicol and chlorpromazine hydrochloride were 14.9 mM and 8.1 mM, respectively, while bleomycin hydrochloride (−Cu) at a concentration between 0.1 to 5 mM inhibited phospholipase C activity by 50%. Bleomycin hydrochloride (−Cu) had no effect on the activities of *Cr. adamanteus* phospholipase A_2 and *P. notatum* phospholipase B, but inhibited the activities of phospholipase C from *B. cereus* and *Cl. perfringens* (Table 2 and Reference 1). Preincubation of bleomycin hydrochloride (−Cu) (1.7 mM) and *B. cereus* phospholipase C (16 μg protein/ml) for 20 minutes caused 100% inhibition. However in the presence of 1 mM $Zn(CH_3COO)_2$, the enzyme activity was not affected at all by 1 mM bleomycin hydrochloride (−Cu). In addition copper-free pepleomycin sulfate, one component of the bleomycin, showed the similar result to bleomycin hydrochloride (−Cu), while pepleomycin sulfate copper complex did not inhibit phospholipase C activity (Fig. 3). This observation suggests that zinc atom found in the metalloenzyme, phospholipase C, may participate in the inhibiting action of basic glycopeptides bleomycin hydrochloride(−Cu).

As suggested by MICHELL *et al.*[14] and DEFRIZE-QUERTAIN *et al.*[15], some of the therapeutic actions or side-effects of amphiphilic cationic drugs such as chlorpromazine hydrochloride may be brought about by their interaction with the phospholipids of the biomembranes. Similarly, the amphiphilic antibiotics tested may modify the physicochemical properties of the phospholipids used as substrate and thus modulate the activity of phospholipase A_2, B and C. On the other hand, the inhibitory action of tetracyclines and bleomycin hydrochloride (−Cu) may be due to the removal of Ca^{2+} and zinc atom from the system, each of which is essential for the activities of phospholipase A_2 and C, respectively.

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