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Inhibition of *Bacillus cereus* phospholipase C by univalent anions

Signe-Elise AAKRE and Clive LITTLE
Institute of Medical Biology, University of Tromsø, P.O. Box 977, N-9001 Tromsø, Norway

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The rate of phospholipid hydrolysis in erythrocyte ghosts by *Bacillus cereus* phospholipase C was markedly decreased by the presence of NaCl at concentrations between 25 and 200 mM. The inhibition seemed to be due to Cl^- and was unaffected by the type of cation present. The larger univalent anions such as HCO_3^-, Br^-, Cl^-, NO_3^-, CNO^- and I^- seemed most effective, whereas the bivalent anion SO_4^{2-} was relatively ineffective at 0.1 M, as were acetate and formate. Tris buffers at 0.1 M caused marked inhibition. With bovine brain myelin, phospholipid hydrolysis by phospholipase C was also much more strongly inhibited by I^- and Cl^- than by SO_4^{2-} or acetate. NaCl inhibited the hydrolysis by the enzyme of the soluble substrate dihexanoylglycerophosphocholine, thereby suggesting that the inhibition did not arise simply from substrate effects.

Under non-denaturing conditions, phospholipase C (phosphatidylcholine cholinephosphohydrolase; EC 3.1.4.3) from *Bacillus cereus* can be readily inactivated/inhibited by removal or substitution of its Zn^{2+} prosthetic groups or by selective chemical modification of lysine, arginine, histidine or carboxylic acid groups in the enzyme (see review by Little, 1981*a*). In addition, Ottolenghi (1969) has reported that high levels of Na^+ and various polyamines inhibited phospholipase C, although in these cases substrate effects could not be ruled out. Certain antibiotics also seem to inhibit this enzyme (Sugatani *et al.*, 1979). We now report the inhibition of this enzyme by certain univalent inorganic anions.

Materials and methods

Crystalline phospholipase C was isolated from cultures of *Bacillus cereus* as described by Myrnes & Little (1980). Mono- and di-cobalt-substituted forms of the enzyme were prepared as described by Little (1981*b*). Erythrocyte ghosts were prepared from fresh human blood by the method of Fröman *et al.* (1980). 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine was synthesized by the method of Cubero Robles & Van den Berg (1969) as described by Little (1977) and also purchased from Calbiochem–Behring Corp., La Jolla, CA, U.S.A. Freeze-dried bovine brain myelin was generously donated by Kilani Gwarsha, Department of Biology, University of York, York, U.K. Egg glycerophosphocholine (egg lecithin; about 90% pure) was obtained from BDH Chemicals, Poole, Dorset, U.K.

Enzymes assays

With erythrocyte ghosts. Erythrocyte ghosts were resuspended in 5 mM-Tris/HCl, pH 7.5, together with various salts to a final concentration of 0.48 μmol of lipid phosphorus/ml. The incubation temperature was 23°C. Enzyme was added and the release of acid-soluble phosphate measured in portions removed from the reaction mixture at various times between 5 and 60 min. Enzyme activity was measured from the linear part of the phosphate-release–time curve.

With myelin. Freeze-dried bovine brain myelin was resuspended in 5 mM-Tris/HCl, pH 7.5, together with various salts and allowed to rehydrate for 1 h at room temperature with constant mixing. The final concentration of lipid phosphorus was 0.9 μmol/ml. Enzyme was added and the incubation carried out at 23°C with portions being removed from the mixture at various times from 5 to 60 min and the release of acid-soluble phosphate measured.

With dihexanoylglycerophosphocholine. These assays were performed by continuous titration in a pH-stat at pH 7.6 and 23°C. The substrate concentration was 2 mM and 0.02 M-NaOH was used as titrant.

Acid-soluble phosphate was determined by the method described by Ames (1966). All assays were made in duplicate or triplicate.

Results and discussion

The rate of phospholipid degradation in red cell ghosts by phospholipase C in 5 mM-Tris/HCl buffer,

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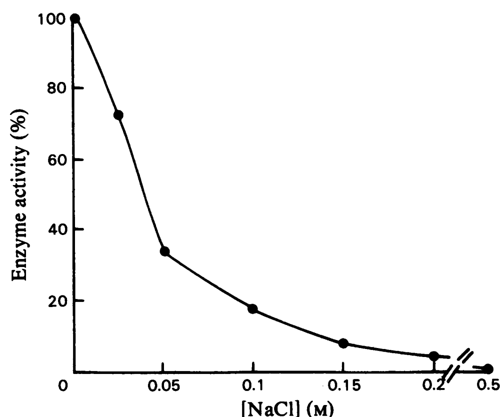

Fig. 1. *Effect of NaCl concentration on the relative activity of phospholipase C against phospholipid in erythrocyte ghosts*

Phospholipase C (0.04 μg/ml) was incubated at 23°C with erythrocyte ghosts in 5 mM-Tris/HCl (pH 7.5) containing various concentrations of NaCl and the rates of release of acid-soluble phosphate measured. The reaction rate in the absence of NaCl was taken as 100%.

Table 1. *Inhibition by NaCl of phospholipase C hydrolysis of dihexanoylglycerophosphocholine*

Enzyme activity was measured at pH 7.6 and 23°C against 2 mM-dihexanoylglycerophosphocholine in the presence of different concentrations of NaCl.

[NaCl] (M)	Enzyme activity (μmol of substrate hydrolysed/min per mg of enzyme)
0.025	3583
0.07	2000
0.125	1418
0.25	480
0.5	208

pH 7.5, was found to be lowered markedly by the presence of NaCl. Marked inhibition is discernible at lower concentrations of NaCl with 50% inhibition occurring at 0.04 M, whereas at 0.5 M near total inhibition occurs (Fig. 1). Similar results were obtained with two different preparations of erythrocyte ghosts.

The normal substrates for phospholipases are, of course, in the form of fairly large and physically complex insoluble structures such as cell membranes or phospholipid micelles. The precise physical form of the substrate can have a very marked effect on the rate at which it is hydrolysed by a phospholipase. Consequently the inhibitory effect of NaCl on phospholipase C was also examined by using a soluble substrate, dihexanoylglycerophosphocholine at a substrate concentratin of 2 mM, which is well below the critical micelle concentration (de Haas *et al.*, 1971). The data in Table 1 confirm that the enzyme activity towards a soluble/monomolecular-dispersed substrate is decreased by the presence of NaCl and hence does not arise simply from a substrate effect.

Table 2. *Inhibition of phospholipase C by various salts*

Enzyme activity was measured using erythrocyte ghosts at 23°C and pH 7.5 as described in the Materials and methods section. Where necessary, the pH of the salt solution was adjusted to 7.5. The activity in 5 mM-Tris/HCl, pH 7.5, was taken as 100%.

Salt	Enzyme activity (%)
None	100
0.04 M-Na_2SO_4	100
0.1 M-Sodium formate	85
0.1 M-Na_2SO_4	80
0.1 M-NaF	76
0.1 M-Sodium acetate	70
0.1 M-Tris/H_2SO_4	23
0.1 M-NaBr	21
0.1 M-LiBr or 0.1 M-KCl	20
0.05 M-$CaCl_2$	19
0.1 M-NaCl	18
0.1 M-$NaHCO_3$	17
0.05 M-$Ca(NO_3)_2$	14
0.1 M-$NaNO_3$	10
0.1 M-Tris/HCl	7
0.1 M-KCNO	6
0.1 M-KI	4

A wide range of salts was tested for inhibitory effects on phospholipase C activity towards erythrocyte ghosts (Table 2). That the inhibition is not simply an ionic-strength effect is demonstrated by the failure of 0.04 M-Na_2SO_4 to inhibit. A small inhibition was noted at 0.1 M-Na_2SO_4, but concentrations of 0.5 to 1 M were needed to cause inhibition similar to that in 0.05–0.1 M-NaCl (not shown). At similar Cl^- concentrations, LiCl, KCl, NaCl and $CaCl_2$ had very similar inhibitory properties. In addition, both $Ca(NO_3)_2$ and $NaNO_3$ were powerful inhibitors so that the nature of the anion rather than the cation would appear crucial for the inhibition. However, Tris/H_2SO_4 was an effective inhibitor and Tris/HCl even more so. It is possible that Tris itself can inhibit, a view consistent with the fact that Tris appears able to interact with phospholipase C in some way and displace the enzyme from certain types of affinity gel (Gerasimene *et al.*, 1977).

The inhibitory effects of the halides increased generally with the size of the anion ($F^- < Cl^- \approx Br^- < I^-$). The organic anions formate and acetate were fairly ineffective.

Thus phospholipase C appears to be particularly sensitive to inhibition by relatively large inorganic univalent anions such as I^-, NO_3^-, HCO_3^- and CNO^-. The latter ion is a known chaotropic agent.

However, in view of the high conformation stability of this enzyme (Little, 1978; Little & Johansen, 1979), it is unlikely that at 0.1 M, enzyme denaturation is involved in the inhibitory effects of CNO^-. Metal substitution strongly affects phospholipase C activity and the effect of 0.1 M-NaCl on the activities against erythrocyte ghosts of CoZn-phospholipase C and CoCo-phospholipase C was also investigated and compared with that on native ZnZn-phospholipase C. Although these three enzyme forms have very different catalytic activities, there was no significant difference in their sensitivity to inhibition by NaCl (results not shown).

In addition to erythrocyte ghosts and dihexanoylglycerophosphocholine, myelin was also used as a substrate for phospholipase C and the effects of certain salts on the enzyme activity measured. It was found that at the same ionic strength (0.1), the rate of total phospholipid hydrolysis decreased in the order Na_2SO_4 > sodium acetate > NaCl > KI (the ratio of the rates was 1:0.75:0.4:0.07 respectively), thus confirming the results obtained with erythrocyte ghosts. However, with the crude egg-yolk extract/deoxycholate substrate used for routine assays of phospholipase C (Little *et al.*, 1975) no changes in enzyme activity were noticed when the 0.15 M-NaCl in the medium was replaced by 0.06 M-Na_2SO_4. Similar results were obtained using a sonicated suspension of egg glycerophosphocholine in the absence of deoxycholate. Possibly, therefore, the extent of this salt inhibition effect may in some way be substrate-dependent, but further studies are needed to clarify this point.

The mechanism whereby these univalent anions inhibit phospholipase C is not clear. However, the enzyme contains two Zn^{2+} whose removal or substitution diminishes or abolishes catalytic activity (Little & Otnaess, 1975). It is possible that these anions inhibit by interacting with the Zn^{2+} in the enzyme. Support for this view comes from recent ^{113}Cd n.m.r. studies that show that I^- perturbs the resonance from this isotope when added to phospholipase C samples where $^{113}Cd^{2+}$ has replaced Zn^{2+} in the enzyme (Aalmo *et al.*, 1982).

Phospholipase C is a widely used tool in membrane studies. In view of the present findings, it would seem that the nature and concentration of the anions present in the various reaction systems should be chosen with care and that the commonly used medium, buffered physiological saline, may not necessarily permit optimal activity of this enzyme.

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