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On the Question of an Electrokinetic Requirement for Phospholipase C Action

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Summary. The phospholipase C of *clostridium welchii* (α toxin) has an absolute requirement for trace quantities of Ca^{2+}. It attacks pure phosphatidylcholine particles (smectic mesophases) having a close-packed bilayer structure only when their surface zeta potential is made positive by the addition of certain divalent ions (e.g., Ca^{2+}) to the aqueous phase or by the presence of low concentrations of long chain cations to the lipid. Alternatively, if the rotational freedom of individual phospholipid molecules is increased by the insertion of short *n*-alkanols (e.g., hexanol) into the bilayer or when a monolayer of the substrate at an air/water interface is expanded, enzymic hydrolysis can occur without any requirement for a net positive charge on the surface.

A major conclusion of a recent paper published in this Journal (Goldhammer, Jain & Cordes, 1975) was that the electrokinetic characteristics of a smectic mesophase of lecithin (liposomes) are unimportant in determining phospholipase activity, a conclusion entirely contrary to our own (Bangham & Dawson, 1958; Bangham & Dawson, 1962).

To establish the nature of the discordance, we have measured the electrophoretic mobility of the substrate in the systems used by Goldhammer *et al.* (1975), which were different from our own in that they always contained calcium. This mobility has been compared with the susceptibility of the substrate to enzymic hydrolysis by Phospholipase C (α toxin from *Clostridium welchii*).

Materials and Methods

Ovolecithin was prepared as previously described (Dawson, 1958). Hexadecylamine was a commercial preparation (Ralph N. Emanuel Ltd.), purified by the method of Goldhammer *et al.* (1975), melting point 45 °C. Partially purified *C. welchii* (*perfringens*) α toxin was used as a source of phospholipase as described by Klein, Miller, Kemp & Lasser (1975).

Incubation

An aliquot of phosphatidylcholine (5.4 μmole) in chloroform solution in ethanol was pipetted into 10 ml stoppered tubes. The solvent was removed under low pressure and the lipid residue then "vortexed" with 0.9 ml of 22 mM Tris-HCl buffer pH 7.2 containing hexanol, calcium chloride or EDTA as demanded by the experimental requirements. The reaction was started by adding 20 μg of the α toxin in 0.1 ml H_2O. Incubation was carried out at 25 °C and terminated by cooling rapidly and adding 0.2 ml of 5% serum albumin followed by 1.0 ml of ice-cold 10% perchloric acid. After centrifuging and filtering the water-soluble P released was determined as described by Bartlett (1959).

Electrophoretic mobilites

Mobilites were measured by direct observations of the substrate particles in the stationary layer of a cylindrical cell mounted horizontally in a water bath at 25 °C (Bangham *et al.*, 1958). To increase visibility the aqueous phase was increased from 1 ml to 7 ml compared with the enzyme incubation system although its ionic composition was kept constant. To avoid excessive contamination of electrode products and undesirable thermal effects in the electrophoresis cell, the power dissipated was limited to not more than 0.2 W.

Results

Previous results (Bangham & Dawson, 1962) showing that unsonicated phosphatidylcholine particles in the absence of added Ca^{2+} are only attacked by phospholipase C when a small proportion of a cationic amphipathic substance is added, have been confirmed. Both hexadecylamine (Fig. 1) and octadecylamine approached maximal stimulation when approximately 1.5–3% molar concentration of the base had been added to the phosphatidylcholine, and above this ratio the activity fell. The substrate particles showed a cationic mobility of about 0.7 $\mu sec^{-1}\ V^{-1}\ cm^{-1}$ at the point of maximal susceptibility to the enzyme (Fig. 2) which is of the same order of mobility as that of maximal activations previously achieved with stearylamine and docosanyl pyridinium bromide (Bangham & Dawson, 1962). The same order of charge was produced on pure phosphatidylcholine particles on the addition of 6 mM $CaCl_2$ to the system.

The addition of hexadecylamine in the presence of 6 mM Ca^{2+} produced no further increase in enzymic activity; rather this fell away (Fig. 1), as was also observed with excess hexadecylamine in the absence of added Ca^{2+}. An anionic amphipathic substance, dicetylphosphoric acid, produced by itself no activation of phosphatidylcholine hydrolysis, and it inhibited the activity produced by hexadecylamine.

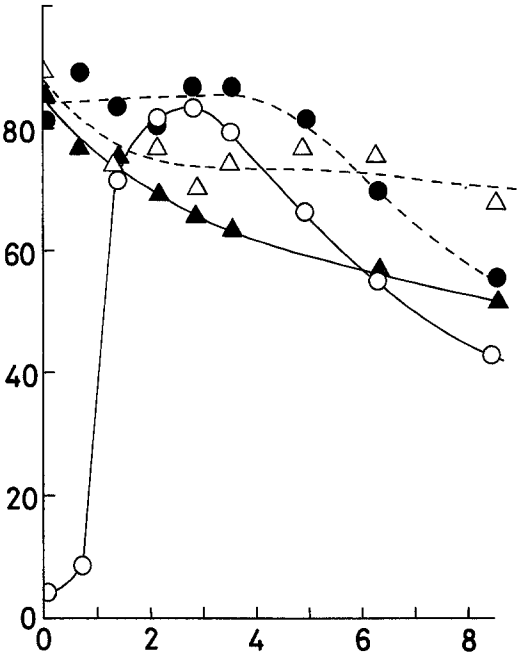

Fig. 1. Hydrolysis of phosphatidylcholine by *C. welchii* phospholipase C as a function of Hexadecylamine concentration. The phosphatidylcholine and hexadecylamine were mixed and the solvent solution taken to dryness. The incubation of this substrate with enzyme, etc., was carried out as described in the Experimental. Concentration of phosphatidylcholine, 5.4 μM/ml. ○ = No calcium or hexanol added: Incubation time 40 min; ● = Hexanol (0.04) added: Incubation time 40 min; ▲ = Calcium (6 mM) added: Incubation time 40 min; △ = Calcium (6 mM) and hexanol (0.04 M) added: Incubation time 15 min. *Ordinate:* Water soluble P liberated (μg). *Abscissa:* Molar ratio of hexadecylamine to phosphatidylcholine as a percentage

Pure phosphatidylcholine particles in the presence of hexanol (40 mM) and in the absence of added Ca^{2+} were hydrolysed by phospholipase C; addition of hexadecylamine had no stimulating effect but the fall of activity above 3% molar base was observed (Fig. 1). Hexanol (40 mM) plus calcium (6 mM) produced a very rapid hydrolysis of pure phosphatidylcholine particles as found by Jain and Cordes (1973), and in agreement with Goldhammer *et al.* (1975), the further addition of hexadecylamine produced no stimulation of activity.

Since recent evidence has suggested that very low concentrations of Ca^{2+} may be obligatory for phospholipase C action (Klein *et al.*, 1975), we have examined the effect of EDTA on the enzymic activity towards phosphatidylcholine particles elicited by hexadecylamine or hexanol in the absence of added Ca^{2+}. In each instant addition of EDTA above a concentration of 10^{-6} M produced a dramatic reduction in activity

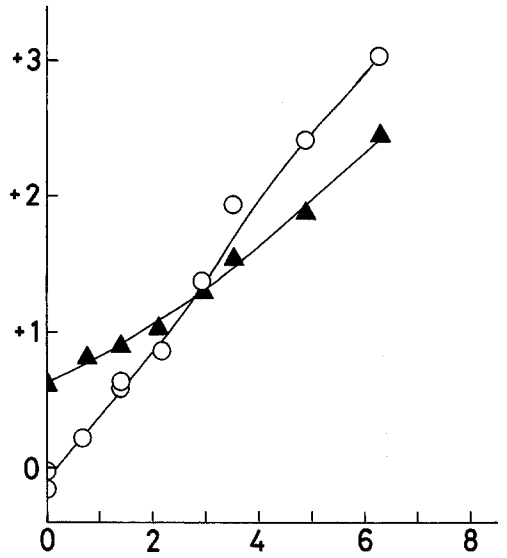

Fig. 2. Electrophoretic mobility of lecithin + hexadecylamine particles with and without added Ca^{2+}. ○ = No calcium added; ▲ = $CaCl_2$ (6 mM) added. *Ordinate:* Anodic mobility in $\mu sec^{-1}\ V^{-1}\ cm^{-1}$. *Abscissa:* Molar ratio of hexadecylamine to phosphatidylcholine as a percentage

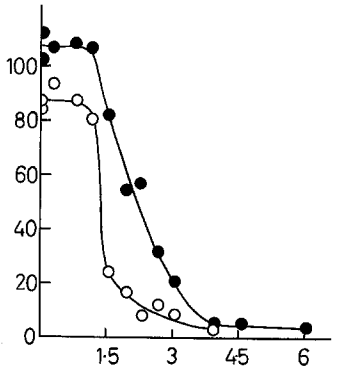

Fig. 3. Inhibition of hexanol and hexadecylamine stimulated phospholipase C by EDTA. ● = Hexanol (0.04 M) added; ○ = Hexadecylamine (2.7% molar) added to phosphatidylcholine. *Ordinate:* Water soluble P liberated in μg. *Abscissa:* Ethylenediamine tetracetate concentration ($\mu M \times 10^{-6}$)

(Fig. 3). This inhibition was reversed by Ca^{2+} but not by Mg^{2+} addition. It can be calculated that in the present system approximately 2 *n* atoms of Ca are sufficient to allow effective stimulation by the addition of cationic amphipathic molecules or hexanol. This can be contrasted to the thousandfold higher levels of calcium needed to produce the optimal interfacial charge density for enzymic activity.

Discussion

Quite apart from the fact that no one, to our knowledge, has ever claimed an electrostatic dependency for the A_2 phospholipase of bee or snake venoms (Dawson, 1968), it would appear that the experimental systems used by Goldhammer *et al.* (1975), for the *C. welchii* enzyme, were electrostatically favorable anyway; for example, 6 mM Ca^{2+} was used in all their experiments, contributing in our measurements a net positive mobility of 0.6 $\mu sec^{-1}\ V^{-1}\ cm^{-1}$ (Fig. 2). As shown here and previously (Bangham & Dawson, 1962), this density of positive charges is sufficient to ensure a near maximal hydrolysis of a phosphatidylcholine particle whose surface is not diluted with hexanol. In contrast, and in confirmation of our earlier results, if no calcium was added to the system, the phosphatidylcholine particles were isoelectric and not attacked by phospholipase C, (Figs. 1 and 2). As before, Fig. 1 clearly shows that as positively charged amphiphiles are progressively added to the net uncharged lecithin both the susceptibility to hydrolysis and the electrophoretic mobility increase (Fig. 2). Furthermore negatively charged amphiles were, again, without effect.

The inhibitory effect of excess hexadecylamine (i.e., above 3% molar on the enzymic activity in the absence of added Ca^{2+} [Fig. 1]) is likely to be due to an unfavorable effect of excess charge density on the formation of the enzyme-substrate complex. We have consistently observed that high charge densities are inhibitory to phospholipases exhibiting electrokinetic requirements (Dawson, 1968) and this may be due to an irreversible or reversible denaturation of the enzyme on the highly charged surface. The observation that hexadecylamine addition inhibits the enzyme attack in the presence of 6 mM Ca^{2+} (Fig. 1) is probably due to the counter ion binding of the Ca^{2+} with phosphate groups causing a density of positive groups which is already optimal (Fig. 2) so that the further addition of protonated amine groups to the surface causes an inhibitory excess.

A new observation arising from this controversy is that the *C. welchii* Phospholipase C will hydrolyse lecithin in the presence of hexanol alone, i.e., without added Ca^{2+} or any positive amphiphile (Fig. 1); but as was shown by Klein, Miller, Kemp, and Laser (1975), trace amounts of Ca^{2+} are absolutely necessary (Fig. 3). This presumably acts as an essential co-enzyme in the enzyme-substrate interaction and is independent of any role of Ca^{2+} in producing the correct interfacial charge density required for activity. From what is already known about the

partitioning of straight-chain alcohols, including hexanol, into the bilayers of lecithin liposomes (Hill, 1975) it would be reasonable to assume that at an aqueous concentration of 0.04 M, the lecithin molecules would be very well separated by inserted hexanol molecules. The rotational freedom of the lecithin would, as a result, be substantially increased, analogous to the expansion of a monolayer shown, by Bangham and Dawson (1962), to be likewise susceptible to enzymic attack without added Ca^{2+} or positive amphiphile.

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