Effect of antituberculous calixarenes on phospholipase A₂ susceptibility and on fusion of phospholipid bilayers

Mahendra Kumar JAIN and Dattatreya V. JAHAGIRDAR
Department of Chemistry, University of Delaware, Newark, DE 19716, U.S.A.

(Received 1 October/10 December 1984; accepted 10 January 1985)

Some homologous calixarenes or polyoxyethylene ethers that are known to suppress or enhance experimental tuberculous infection (depending on their polyoxyethylene chain lengths) were examined for their effects on phospholipid bilayers. The effect of these solutes is seen at 0.5–50 p.p.m., and their effect depends upon their structure as well as that of the phospholipid substrate. The antituberculous compound HOC-12.5 (Macrocydon) inhibits susceptibility to pig pancreatic phospholipase A₂ action and to aggregation/fusion of the ternary co-dispersions of dimyristoyl phosphatidylcholine + 1-palmitoyl lysophosphatidylcholine + palmitic acid (50:11:11 molar proportions). In contrast, the protuberculous compound HOC-60 stimulates these effects. Differential scanning calorimetry suggests that these effects are probably due to modulation of the phase equilibrium in substrate bilayers by these polyethers.

The polyoxyethylene ethers or calixarenes (Gutsche, 1984) have been shown to have a variety of biological and biochemical effects including modification of experimental tuberculosis (Cornforth *et al.*, 1955; Hart, 1968) and modulation of the triacylglycerol lipase activity of macrophages (Hart & Payne, 1971), lysosomes (Stoffel & Trabbert, 1969), pancreas (Jorolan & Janicki, 1965) and plasma (Schotz *et al.*, 1957). One of the intriguing properties of these polymers is that their effects are dependent on their structural features, such as chain length and isomerism. For example, in the HOC series of polyoxyethylene ethers, shorter-chain compounds inhibit the triacylglycerol lipase activity of macrophages and have antituberculosis activity, whereas the longer-chain homologues stimulate the lipase activity and are protuberculous (Hart, 1968; Hart & Payne, 1971; Cornforth *et al.*, 1973).

In order to investigate the possible mode of action of these polyethers, we have studied the effects of the antituberculous compound HOC-12.5 and of the protuberculous compounds HOC-60, LOC-60 and LOC-75 on bilayers prepared from synthetic phospholipids. It is shown that these surfactants modify the properties of laterally phase-separated bilayers, such as their susceptibility to phospholipase A₂, fusion and aggregation of the vesicles, and the thermotropic phase-transition behaviour. A correlation is observed between the effect of these agents on tuberculous infection and

their ability to eliminate the laterally phase-separated domains in the bilayers. These observations suggest that calixarenes act on the boundaries of the domains, which are the common molecular locus in these various membrane processes (Jain & White, 1977; Jain, 1983).

Materials and methods

Phospholipids were purchased from Calbiochem. (In the present paper, for convenience, the terms dimyristoyl phosphatidylcholine, etc. are used to represent 1,2-dimyristoyl-*sn*-glycero(3)-phosphocholine, etc.) The polyoxyethylene ethers HOC-12.5 (Macrocydon), HOC-60, LOC-45, LOC-60 and LOC-75 were synthesized by Sir John Cornforth and his colleagues (Cornforth *et al.*, 1955, 1973) from a macrocyclic tetrahydric phenol ('HOC', a *p*-*t*-octylphenol-formaldehyde cyclic octamer) or its presumed tetramer or hexamer ('LOC') by reaction with monofunctional derivatives of poly(ethylene glycols); the compounds differ in their average number of ethylene oxide units per phenolic group (12.5, 60 etc.). Preparation of the ternary co-dispersions and the pH-stat titrimetric procedure for monitoring the kinetics of pig pancreatic phospholipase action is described elsewhere (Apitz-Castro *et al.*, 1982). Five different lipids were examined as substrates: (a) ternary co-dispersions containing dimyristoyl phosphatidylcholine + 1-palmitoyl lysophosphatidyl-

choline+palmitic acid in 50:11:11 molar proportions; (b) dilauroyl phosphatidylethanolamine+1-palmitoyl lysophosphatidylcholine+palmitic acid in 50:11:11 molar proportions; (c) dimyristoyl phosphatidylmethanol; (d) dimyristoyl phosphatidic acid; and (e) egg-yolk phosphatidylcholine. All substrates were used as sonicated dispersions. Lipids (c) and (d) were used in aqueous solutions containing $15\ \mu\text{M}$ - CaCl_2 ; all other lipids were used with 10mM - CaCl_2 . Lipid (e) was activated with 20mM -hexan-1-ol. Typically, the lipids were dissolved in chloroform and the solvent was evaporated *in vacuo*. The dried film was suspended in an aqueous buffer and sonicated in a bath-type sonicator for 15–20 min until a clear suspension was obtained. In order to minimize aggregation, all lipid dispersions were stored at 55°C during their use. The composition of the aqueous solution for the preparation of the ternary co-dispersions was the same as used for the subsequent experiments. For phospholipase A_2 kinetics the aqueous phase consisted of 100mM -KCl and $15\ \mu\text{M}$ - or 10mM - CaCl_2 at pH 8.0. Specific activities for pig pancreatic phospholipase in the five lipid dispersions described above under the conditions used here were for lipid (a) 520, for lipid (b) 1040, for lipid (c) 2232, for lipid (d) 185 and for lipid (e) $250\ \mu\text{mol}/\text{min}$ per mg of protein. For fusion (Elmaharani & Blume, 1982) and thermotropic phase-transition studies (Jain & Wu, 1977) the buffer consisted of 100mM -KCl in 100mM -Tris at pH 8.0. Unless stated otherwise all studies were done at 30°C .

Results

The action of phospholipase A_2 on its substrate depends on the quality of the interface (Slotboom *et al.*, 1982), and therefore it is of interest to study the effect of the polyethers on the bilayer substrate. We have examined the effect of the polyethers on the kinetics of action of pig pancreatic phospholipase A_2 on the ternary co-dispersions as substrate (Apitz-Castro *et al.*, 1982). As shown in Fig. 1, Macrocydon inhibits and HOC-60 stimulates the steady-state rate and the extent of phospholipase A_2 -catalysed hydrolysis of dimyristoyl phosphatidylcholine in the ternary co-dispersions. Similar behaviour is shown by all known inhibitors of phospholipase A_2 (Jain & Jahagirdar, 1985). As shown in Fig. 2, these effects are concentration-dependent. The effect of these compounds is seen at as low as 2 p.p.m., and a complete inhibition of phospholipase activity is observed by about 500 p.p.m. of Macrocydon, LOC-45 and LOC-60, whereas HOC-60 stimulates the phospholipase activity at all concentrations tested. In a few cases only, the effect is monotonic. The effect of LOC-75 is biphasic, i.e. at low concentration it stimulates

Fig. 1. Reaction progress curves for the pig pancreatic phospholipase A_2 -catalysed hydrolysis of dimyristoyl phosphatidylcholine in the ternary co-dispersions with 1-palmitoyl lysophosphatidylcholine and palmitic acid (50:11:11 molar proportions)

The reaction mixture (5 ml) contained 100mM -KCl and 10mM - CaCl_2 and 500 ng of pig pancreatic phospholipase A_2 at pH 8.0 and 30°C . Curve A was obtained in the presence of 0.1% (w/v) HOC-60, curve B in the absence of any additive, curve C in the presence of 0.1% Macrocydon, and curve D in the presence of 0.02% LOC-60.

and at higher concentration it inhibits the action of phospholipase A_2 (Fig. 2). Such biphasic effects are a manifestation of non-ideal mixing of these solutes in bilayers, and depend on both the composition and the nature of the bilayer and the additive. The inhibition and stimulation depends not only on the concentration but also on the nature of the substrate. Whereas HOC-60 stimulates and Macrocydon inhibits the hydrolysis of all the substrates, the effect of the LOC series is very sensitive to the nature of the substrate. In some cases the biphasic effect is accentuated (e.g. LOC-75 with dimyristoyl phosphatidylmethanol), whereas in other cases it is completely suppressed (e.g. Macrocydon with dimyristoyl phosphatidylcholine or dimyristoyl phosphatidylmethanol).

The stimulatory or inhibitory effect of calixarenes is by modification of the interaction of phospholipase A_2 with the substrate interface. A direct

Fig. 2. Initial rate of hydrolysis of the lipid dispersions by pig pancreatic phospholipase A_2 as a function of inhibitor concentration expressed in parts per million in the aqueous phase (p.p.m. on a w/v basis)

The lipid dispersions are (clockwise): dimyristoyl phosphatidylmethanol (DMMe), dilauroyl phosphatidylethanolamine (DLPE) + 1-palmitoyl lysophosphatidylcholine (LPC_{16}) + palmitic acid (FA_{16}), egg-yolk phosphatidylcholine (egg PC) activated with 20 mM-hexan-1-ol, dimyristoyl phosphatidylcholine (DMPC) + 1-palmitoyl lysophosphatidylcholine (LPC_{16}) + palmitic acid (FA_{16}), and dimyristoyl phosphatidic acid (DMPA). In each case diacylphospholipid concentration is $75 \mu\text{M}$. In each part of the Figure the inhibitors are HOC-60 (I), LOC-75 (II), LOC-60 (III), LOC-45 (IV) and Macrocydon (V). The concentration scale for LOC-60 is 10-fold lower than that shown. The initial rate of hydrolysis was measured by pH-stat titration at pH 8.0. The reaction was initiated by adding $0.5 \mu\text{g}$ of phospholipase A_2 . The reaction medium contained $15 \mu\text{M}$ - CaCl_2 for the anionic phospholipids and $10 \mu\text{M}$ in all other cases. The data points are omitted for clarity.

effect of the calixarenes on the enzyme can be ruled out by the following observations (results not shown). First, calixarenes do not modify the fluorescence properties of the enzyme. Furthermore, the specific activity of the pig pancreatic phospholipase A_2 on the soluble monomeric substrate 2,3-bis(hexanoylthio)propylphosphocholine (Volwerk *et al.*, 1979) is not influenced by Macrocydon or HOC-60.

The effects of polyoxyethylene ethers on the phospholipase-catalysed hydrolysis of ternary co-dispersions are confined to the enzyme bound to the bilayer because only this form is catalytically active. This effect on the reaction progress curve could be either due to a change in the equilibrium concentration of the bound enzyme, or due to its in-

hibition by direct interaction. One of the measures of the lack of binding of the enzyme to dimyristoyl phosphatidylcholine bilayers is that the reaction progress curve exhibits a long latency phase, which depends upon all the factors that regulate the molar fractions of lysophosphatidylcholine and fatty acid in the substrate bilayers (Aritz-Castro *et al.*, 1982). Similarly, activation of phospholipase A_2 action is observed with lysophosphatidylcholine added to preformed dimyristoyl phosphatidylcholine vesicles (Jain & DeHaas, 1983). Thus, as shown in Fig. 3, the steady-state hydrolysis of pure dimyristoyl phosphatidylcholine vesicles is reached in about 8 min, but in the presence of freshly added lysophosphatidylcholine the latency period is less than 30 s (curve 3 in Fig. 3). The effects of HOC and

Fig. 3. Reaction progress curves for the hydrolysis of dimyristoyl phosphatidylcholine (1.5 mg/5 ml) vesicles by pig pancreatic phospholipase A_2 (E; 1.5 μ g/5 ml) added at time = 0

The reaction mixture also contained 0.012% Macrocydon (M) or 0.012% HOC-60 (H) for profiles 2, 4 and 5. 1-Palmitoyl lysophosphatidylcholine (L; 0.1 mg/5 ml) was added as indicated. The temperature was 25°C. Other conditions were as given in the legend to Fig. 1.

Macrocydon are particularly revealing; in the presence of either of these calixarenes the hydrolysis of dimyristoyl phosphatidylcholine vesicles could not be initiated until after 30 min (curve 2). However, after the addition of lysophosphatidylcholine the hydrolysis is initiated immediately in the presence of HOC-60 (curve 5), whereas in the presence of Macrocydon it is delayed to 30 min or more (curve 4). These observations show that Macrocydon inhibits the action of phospholipase A_2 in the pre-steady-state phase of the reaction, and that HOC-60 does not do so.

The inhibitory effect of Macrocydon and the stimulating effect of HOC-60 have also been shown on the triacylglycerol lipase activity from macrophages (Hart & Payne, 1971) and could have been due to a direct effect of these agents on the enzyme, or they could have modified the substrate interface so as to modulate the incorporation and/or the turnover of the enzyme. The present observation that these homologous compounds exert different effects on two diverse catalytic functions suggests that their primary effect is on the lipid/water interface, at which both of these enzymes are catalytically functional. The effect of premixed lysophosphatidylcholine and fatty acid (Apitz-Castro *et al.*, 1982) or of freshly added lysophosphatidylcholine (Elmaharani & Blume, 1982; Jain & DeHaas, 1983) on the bilayer is to modify the bilayer organization. Since these changes in the bilayer organization also facilitate aggregation/fusion of vesicles, we have examined the effects of the polyethers on this process in order to decide whether their effects are on the enzyme or on the bilayer.

Fig. 4. Progress curve for the change in turbidity of the ternary co-dispersions of dimyristoyl phosphatidylcholine + 1-palmitoyl lysophosphatidylcholine + palmitic acid (50:11:11 molar proportions) alone (curve B) and in the presence of 0.05% HOC-60 (curve A) and of 0.025% Macrocydon (curve C)

Other conditions were as given in the legend to Fig. 1.

By monitoring the change in turbidity, it has been shown previously that the freshly added lysophosphatidylcholine stimulates the rate of fusion of dimyristoyl phosphatidylcholine vesicles in the vicinity of their phase-transition temperature at 23°C (Elmaharani & Blume, 1982). Similarly, the ternary co-dispersions undergo fusion/aggregation at a rate that is several hundred times faster than that observed with the dispersions of dimyristoyl phosphatidylcholine alone. This is probably because the additive-induced organizational defects in the bilayers of the vesicles are the sites for inter-vesicular interactions required for fusion/aggregation. It appears that the same defects also function as the binding sites for phospholipase A_2 (Jain *et al.*, 1982). This hypothesis is further substantiated by the fact that the several other inhibitors of fusion are also inhibitors of phospholipase action (Jain *et al.*, 1984). Indeed, as shown in Figs. 4 and 5, Macrocydon inhibits, whereas HOC-60 promotes, aggregation of fusion both in the ternary co-dispersions (Fig. 4) and in the dimyristoyl phosphatidylcholine vesicles modified with freshly added lysophosphatidylcholine (Fig. 5). This observation is particularly important since it provides direct evidence for the action of these calixarenes on the putative organizational defects in bilayers.

Further support for a direct effect of Macrocydon and HOC-60 on the bilayer organization comes from the thermotropic phase-transition studies. As shown in Fig. 6, the phase-transition

Fig. 5. Progress curves for the change in the turbidity of dimyristoyl phosphatidylcholine vesicles (5 mg/2.5 ml) in the absence (curve B) and in the presence of 0.0125% HOC-60 (curve A) or 0.0125% Macrocydon (curve C).

The reaction was initiated by the addition of 1-palmitoyl lysophosphatidylcholine (0.5 mg/2.5 ml). The temperature was 25°C. Other conditions were as given in the legend to Fig. 4. Under these conditions dimyristoyl phosphatidylcholine vesicles alone showed no change in turbidity.

Fig. 6. Phase-transition profile for the ternary co-dispersions (curve C) in the presence of HOC-60 (curve B) and Macrocydon (curve A).

Each mixture contained dimyristoyl phosphatidylcholine (2 mg), 1-palmitoyl lysophosphatidylcholine (0.4 mg) and palmitic acid (0.2 mg) and the additive (2 mg) in 30 μ l of buffer at pH 8.0. The heating scans were made in sealed pans on a Mettler DTA 2000B in the range 1–60°C at sensitivity of 100 μ V and scan rate of 1°C/min. Equilibration of samples (at 4°C) over long time periods had little effect on the shape of these profiles.

profiles of the ternary co-dispersions are broad and complex, indicative of co-existing phase-separated domains in the bilayers (Jain & DeHaas, 1981; Jain *et al.*, 1982). In the presence of Macrocydon the transition profile is not seen at all, demonstrating that the phase-separated domains are not present in these bilayers. In contrast, dispersions containing HOC-60 retain the phase-transition characteristics, suggesting that the phase-separated domains are somewhat modified yet still persist in the HOC-60-modified bilayers.

The effect of calixarenes on the phase-transition properties of the ternary co-dispersions depends on their relative molar fraction in the sample. Although we have not measured the partition coefficients of calixarenes in bilayers, we expect that more than 98% of the solute is localized in the ternary co-dispersions (cf. Jain *et al.*, 1978). The effect of these solutes depends on their molar fraction, and the initial effect of Macrocydon at low molar fractions is on the peak at about 23°C, i.e. on the dimyristoyl phosphatidylcholine domain. The morphology of the ternary co-dispersions, as observed in freeze-fracture electron micrographs, remains unchanged in the presence of HOC-60 or Macrocydon at above 300 p.p.m. However, preliminary fluorescence experiments (Jain *et al.*, 1982) suggest that Macrocydon decreases the binding of phospholipase A₂ to the ternary co-dispersions containing a dialkyl ether analogue of diacyl phosphatidylcholine. Such an effect is not seen with HOC-60, suggesting that the apparent decrease in binding is not due to an inner-filter effect. Unfortunately, such measurements could not be used to obtain the binding parameters because at high calixarene concentrations the inner-filter effects are quite large.

Discussion

The most surprising observation reported in this paper is that calixarenes can specifically modulate the properties of a bilayer at very low concentrations, at which the gross morphology of the substrate is not modified. An effect of a solute at 5 p.p.m. means that its molar fraction in the bilayer is well below 0.03. The origin of the specificity of the effect of these polyethers is also intriguing. Taken together, the observations reported in the present paper show that Macrocydon eliminates the phase separation in the ternary co-dispersions, whereas HOC-60 does not. The hypothesis that the organizational defects in the ternary co-dispersions are the common locus of action of phospholipase A₂, and of the aggregation/fusion process, adequately accounts for the opposite effects of these two surfactants. The observation that their effects are on the phase properties of bilayers is consistent with their similar effects on triacylglycerol lipase activity (Hart & Payne, 1971), where these additives could modify the lipase-binding sites on the triacylglycerol dispersions. The effects of LOC-45, LOC-60 and LOC-75 are intriguing. The biphasic effect of LOC-75 on phospholipase activity as well as stimulation of the hydrolysis of phosphatidic acid by LOC-60 (Fig. 2) suggest that the effect of these compounds is on the bilayer organization. If the effect were directly on the enzyme, we would have seen a monotonic response. The exact mechanism

of their action is not yet apparent. Several classes of lipid-soluble solutes exhibit biphasic effects as a function of concentration (Jain & Cordes, 1973; Jain & Wu, 1977), as well as of the chain lengths as manifested in the cut-off effect (Hart & Payne, 1971; Lee, 1976; Jain *et al.*, 1978; Pringle & Miller, 1979; Rowe, 1982; Kamaya *et al.*, 1984). Such biphasic effects or cross-over are rather specific in the sense that they depend not only on the nature of the solute but also on the composition of the bilayer. This is because they are manifestations of the phase properties, probably the non-ideal mixing of the lipid additives in bilayers.

It remains to be established whether the anti-tuberculous effect of Macrocydon is due to a direct modification of the membrane interface or if it is due to the metabolic products induced by the inhibition of triacylglycerol lipase, phospholipase A₂ or any other protein whose activity depends on the presence of laterally-phase-separated domains or domain boundaries in bilayers (Jain & White, 1977; Jain, 1983). Both phospholipase A₂ (Flower & Blackwell, 1976) and acylglycerol lipases (Bell *et al.*, 1979) have been implicated in the mobilization of arachidonic acid. The correlation between anti-tuberculous action and inhibition of phospholipase A₂ is seen only with phosphatidic acid as substrate. Such a selectivity of the effect on the nature of phospholipid on which action of phospholipase A₂ is seen makes it a likely locus of action. Phosphatidic acid is an obligatory intermediate in the phosphoinositol cycle. The role of phosphatidic acid in the release of arachidonate and the putative role of arachidonate metabolites in the regulation of anti-microbial action within macrophages would be consistent with such a role of these polyethers. Thus the phosphoinositol cycle of the host could play a role in the host-microbe interaction.

This work was supported by the National Institutes of Health (Grant 29703). We thank Dr. P. D'Arcy Hart of the National Institute of Medical Research for bringing this problem to our attention and for providing the various calixarenes. Phospholipase A₂ from pig pancreas was kindly provided by Professor G. H. DeHaas, and dimyristoyl phosphatidylmethanol was provided by Professor F. Ramirez.

References

- Apitz-Castro, R., Jain, M. K. & DeHaas, G. H. (1982) *Biochim. Biophys. Acta* **688**, 349–356
- Bell, R. L., Kennerly, D. A., Stanford, N. & Majerus, P. W. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3238–3241
- Cornforth, J. W., Hart, P. D'A., Nicholls, G. A., Rees, R. J. W. & Stock, J. A. (1955) *Br. J. Pharmacol.* **10**, 73–86
- Cornforth, J. W., Morgan, E. D., Potts, K. T. & Rees, R. J. W. (1973) *Tetrahedron* **29**, 1659–1667
- Elmeharani, K. & Blume, A. (1982) *Biochemistry* **21**, 521–526
- Flower, R. J. & Blackwell, G. J. (1976) *Biochem. Pharmacol.* **25**, 285–291
- Gutsche, C. D. (1984) *Top. Curr. Chem.* **123**, 1–47
- Hart, P. D'A. (1968) *Science* **162**, 686–689
- Hart, P. D'A. & Payne, S. N. (1971) *Br. J. Pharmacol.* **43**, 190–196
- Jain, M. K. (1983) in *Membrane Fluidity in Biology* (Aloia, R., ed.), vol. 1, pp. 1–37, Academic Press, New York
- Jain, M. K. & Cordes, E. H. (1973) *J. Membr. Biol.* **14**, 101–118
- Jain, M. K. & White, H. B. (1977) *Adv. Lipid Res.* **15**, 1–60
- Jain, M. K. & Wu, N. M. (1977) *J. Membr. Biol.* **34**, 157–201
- Jain, M. K. & DeHaas, G. H. (1981) *Biochim. Biophys. Acta* **642**, 203–211
- Jain, M. K. & DeHaas, G. H. (1983) *Biochim. Biophys. Acta* **736**, 157–162
- Jain, M. K. & Jahagirdar, D. V. (1985) *Biochim. Biophys. Acta* in the press
- Jain, M. K., Gleeson, J., Upreti, A. & Upreti, G. C. (1978) *Biochim. Biophys. Acta* **509**, 1–8
- Jain, M. K., Egmond, M. R., Verheij, M. H., Apitz-Castro, R., Dijkman, R. & DeHaas, G. H. (1982) *Biochim. Biophys. Acta* **688**, 341–348
- Jain, M. K., Streb, M., Rogers, J. & DeHaas, G. H. (1984) *Biochem. Pharmacol.* **33**, 2541–2553
- Jorolan, E. P. & Janicki, B. W. (1965) *Proc. Soc. Exp. Biol. Med.* **120**, 313–316
- Kamaya, H., Matubayashi, N. & Ueda, I. J. (1984) *J. Phys. Chem.* **88**, 797–800
- Lee, A. G. (1976) *Biochemistry* **15**, 2448–2453
- Pringle, M. J. & Miller, K. W. (1979) *Biochemistry* **18**, 3314–3319
- Rowe, E. S. (1982) *Mol. Pharmacol.* **22**, 130–136
- Schotz, M. C., Scanu, A. & Page, I. H. (1957) *Am. J. Physiol.* **188**, 399–402
- Slotboom, A. J., Verheij, H. M. & DeHaas, G. H. (1982) in *Phospholipids*, vol. 4 (Hawthorne, J. N. & Ansell, G. B., eds.), pp. 359–434, Elsevier Biomedical, Amsterdam
- Stoffel, W. & Trabbert, U. (1969) *Hoppe-Seyler's Z. Physiol. Biochem.* **350**, 836–844
- Volwerk, J. J., Dedieu, A. G. R., Verheij, H. M., Dijkman, R. & De Haas, G. H. (1979) *Rec. Trav. Chim. Pays-Bas* **98**, 214–220