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MECHANISMS OF INHIBITION OF PHOSPHOLIPASE A_2

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Abstract—Differential scanning calorimetry (d.s.c.) and assays of phospholipase A_2 activity were used as tools to distinguish between drugs which interact with the phospholipids and those which interact directly with the enzyme. Cholesterol lowered the transition temperature (*tc*) and reduced the heat absorbed at transition and inhibited phospholipase A_2 activity on liposomes prepared from dipalmitoyl-lecithin–cholesterol. Subsequent addition of filipin to these liposomes overcame the inhibitory effect. Cholesterol therefore inhibits phospholipase A_2 by interacting with the membrane phospholipids. Mepacrine and phentermine did not interact with dipalmitoyl-lecithin (DPL) as determined by d.s.c., but reduced the rate of hydrolysis induced by purified phospholipase A_2 by a direct interaction with the enzyme. The anaesthetics, ethrane, halothane and trichloroethylene, inhibited phospholipase A_2 more than 90 per cent and were found to interact with DPL to modify membrane fluidity and lower the transition temperature. However, they also appeared to interact directly with the enzyme because the inhibitory effect was not overcome either by assaying phospholipase A_2 at the new *tc* or by a ten-fold increase in Ca^{2+} concentration. The anti-inflammatory steroids hydrocortisone, dexamethasone and betamethasone did not affect the rate of hydrolysis of DPL liposomes induced by phospholipase A_2 even at 2:1 w/w steroid/lipid. Furthermore, these steroids were found to be without any effect on membrane fluidity as examined by d.s.c. and microviscosimetry.

In a great number of tissues the phospholipids are a major source of arachidonic acid (AA) for prostaglandin (PG) synthesis. Phospholipase A_2 is the enzyme responsible for the release of AA from the phospholipids and therefore plays a vital role in controlling PG synthesis. In view of its importance, it is not surprising that in recent years it has been the subject of intensive research. Different drugs including mepacrine [1], phentermine [2], some local anaesthetics [3, 4] and anti-inflammatory steroids [5, 6] have been reported to prevent fatty acid release in different tissues and it has been suggested that they act via inhibition of phospholipase A_2. We have therefore studied the mechanisms by which this enzyme can be inhibited using purified phospholipase A_2 in a model system and have investigated the mode of action of different drugs.

The physical state of the phospholipids is essentially important in determining their susceptibility to phospholipase A_2 activity [7, 8]. The lipids in the membrane may exist either in a rigid gel-like state where they are closely packed together and very little motion is possible, or they may exist in a liquid crystalline state where the molecules within the bilayer are further apart and considerable motion occurs. The transition between these two states is temperature-dependent and can be measured by d.s.c. This technique measures both the heat absorbed and the temperature at which this transition occurs. These are characteristic of the lipid under investigation and any molecule which interacts with the lipid can modify either the *tc* or the amount of heat absorbed [9].

Phospholipase A_2 can hydrolyze phosphatidylcholine containing saturated or unsaturated fatty acids maximally at the transition temperature [10]. Below or above this temperature the enzyme activity is considerably reduced. The coexistence of ordered and disordered regions of phospholipid occurring only at the *tc* may be responsible for the optimization of the hydrolysis rate. Irregularities in the packing of the lipid bilayer at this temperature may favour insertion of the enzyme into the bilayer.

Therefore, activity of phospholipase A_2 might be expected to modified either by drugs which interact directly with the enzyme or by molecules which interact with the lipids and modify their fluidity. It is possible to distinguish between these two mechanisms by measuring the rate of hydrolysis induced by phospholipid A_2 and by using d.s.c.

MATERIALS AND METHODS

The following agents were used: phospholipase A_2 from *Naja naja* venom, lyophilized powder and phospholipase A_2 from porcine pancreas, suspension in 3.2 M $(NH_4)_2SO_4$ (Sigma); filipin (Upjohn Company); cholesterol (Sigma); ethrane (Abbots Laboratories); halothane and trichloroethylene BP (I.C.I.); mepacrine BP (May & Baker); L-β-dipalmitoyl-α-lecithin (Fluca); dimethyl-hexatriene (Eastman–Kodak); dexamethasone sodium phosphate, hydrocortisone-21-sodium succinate and betamethasone sodium phosphate (Glaxo); phentermine (Duromine) was a gift from Riker.

Preparation of liposomes. Dipalmitoyl-lecithin is a saturated phospholipid and when it is dispersed in solution and heated above the *tc* (41°), it forms multibilayer liposomes.

Multibilayer liposomes were prepared in 0.1 M Tris-buffer pH 7.2 containing 1mM $CaCl_2$. The preparations containing 1 mg DPL per ml of buffer were heated above the *tc* (41°) and whirlimixed.

Preparation of cholesterol–dipalmitoyl-lecithin

liposomes. Mixtures of cholesterol and DPL were dissolved in benzene/methanol (95:5 v/v) and lyophilized. The liposomes were then prepared as described above.

Hydrolysis of dipalmitoyl-lecithin liposomes. The liposome suspension was incubated at the *tc* (41°) with purified phospholipase A_2 from pig pancreas or *Naja naja* venom in a volume of 1 ml. The reaction was terminated by the addition of 2 ml methanol and 15 mM EDTA. The mixture was evaporated to dryness, the lipids dissolved in chloroform/methanol (2:1 v/v) and chromatographed on silica gel H plates in chloroform/methanol/water (65:25:4 v/v/v). The lysophospholipids and remaining DPL were scraped off the plates and quantified by phosphate assay [11].

A novel method was also used for assaying the enzyme activity spectrophotometrically. These measurements were carried out in a Pye Unicam SP 1800 spectrophotometer using a wavelength of 340 nm. The liposomes were incubated in buffer and maintained at 41° during the whole experiment. The addition of phospholipase A_2 induced a rapid decrease in absorbance which corresponded to the hydrolysis of phospholipids when assayed in parallel by chromatographic and phosphorous techniques. A comparison of the results obtsined from the two methods is illustrated in Figs. 1a and b. This new method has the advantage of being able to determine the initial reaction rate.

Differential scanning calorimetry. Samples were sealed in Perkin–Elmer volatile sample capsules and examined on the differential scanning calorimeter DSC-2. A scan speed of 20° per min was used for heating and cooling runs over the temperature range 280–350 K. Peak areas were estimated by weighing the area under the curve and enthalpies calculated using indium as a standard for temperature and power calibration. After the scans, the sample pans were opened and the total amount of lipid determined by phosphate assay. The accuracy of DSC-2 under the range used is ±0.2 per cent.

Microviscosimetry. Microviscosimetry is an alternative method of measuring membrane fluidity and has been used in the present experiments with fat cell ghosts. Fluorescence polarization measurements were performed with a Perkin–Elmer MPF3-L spectrometer using an excitation wavelength of 366 nm, an emission wavelength of 460 nm and 10 nm slits. Corrections were made for light scattering and for the polarization produced by the emission monochromator. Diphenylhexatriene was used as the fluorescent probe.

Fat cell ghosts. Fat cell ghosts were prepared from rabbit isolated fat cells according to the method of Rodbell [12].

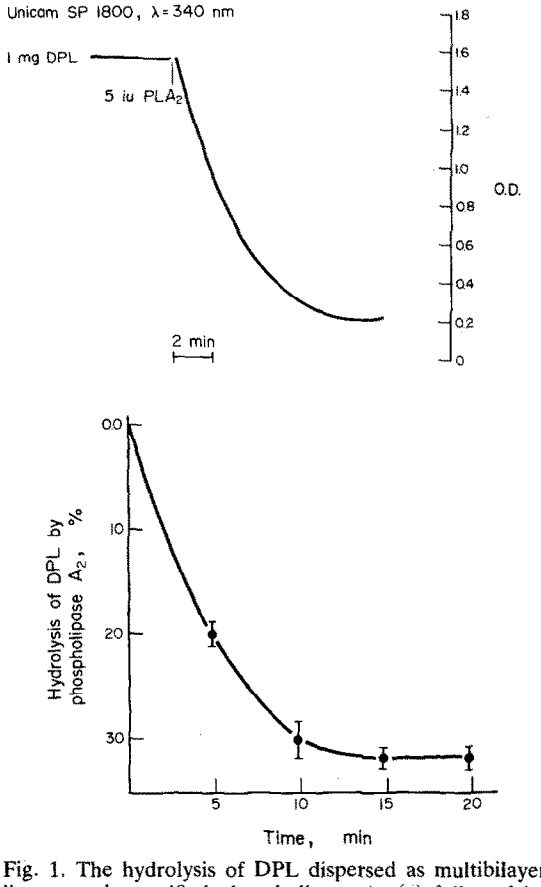

Fig. 1. The hydrolysis of DPL dispersed as multibilayer liposomes by purified phospholipase A_2 (a) followed by recording absorbance changes at 340 nm, (b) followed by chemical assay of digestion products.

RESULTS

Effects of drugs on hydrolysis of dipalmitoyl-lecithin liposomes. When DPL liposomes were incubated at the *tc* of 41° with phospholipase A_2 (5 i.u.), hydrolysis of the phospholipids occurred. In the presence of mepacrine (0.2–1 mg mepacrine/mg DPL) or phentermine (0.2–1.0 mg phentermine/mg DPL) there was a concentration-related inhibition of hydrolysis as shown in Fig. 2. Figure 2 illustrates that a concentration of 0.8 mg mepacrine or 1 mg phentermine/mg DPL caused 75 per cent inhibition.

Dipalmitoyl-lecithin liposomes containing cholesterol, a compound known to interact with phospholipids, also inhibited hydrolysis. Figure 3 shows that cholesterol/DPL mixtures (0.03–0.12 mg cholesterol/mg DPL) decreased the rate of hydrolysis as the concentration of cholesterol increased. At 0.12 mg cholesterol/mg DPL, 95 per cent inhibition occurred.

The general anaesthetics, ethrane (enflurane),

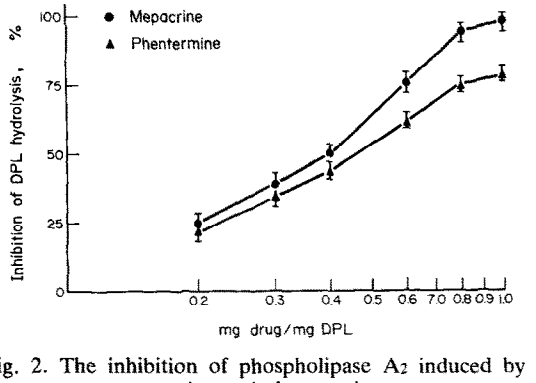

Fig. 2. The inhibition of phospholipase A_2 induced by mepacrine and phentermine.

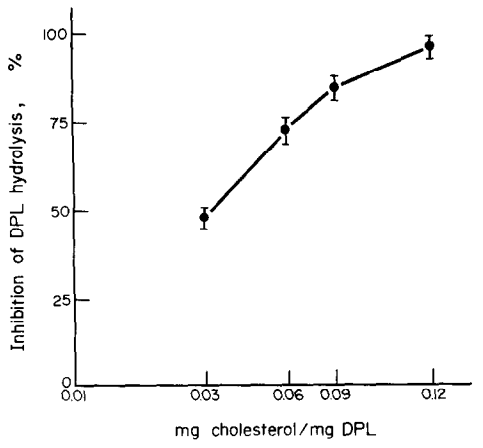

Fig. 3. The effect of cholesterol on DPL hydrolysis induced by phospholipase A_2.

halothane and trichloroethylene also caused a concentration-related reduction of hydrolysis as shown in Fig. 4. Below 0.2 μl anaesthetic/mg DPL the inhibition was negligible, while with 1μl anaesthetic/mg DPL all three anaesthetics inhibited phospholipase A_2 activity by more than 80 per cent.

On the other hand, the glucocorticoids hydrocortisone, dexamethasone and betamethasone failed to influence the hydrolysis of DPL liposomes induced by phospholipase A_2 from pig pancreas or *Naja naja* venom even in concentrations as high as 2:1 (w/w) steroid/lipid.

Effect of drugs on the transition temperature of dipalmitoyl-lecithin. When the interaction of mepacrine and phentermine with DPL liposomes was studied by d.s.c., even concentrations of 400 μg compound/mg DPL had no effect on the transition temperature (41.5°) or enthalpy (36.6 kJ mole^{-1}) of DPL. In contrast, addition of cholesterol to DPL inhibited the transition between the gel and liquid crystalline state. Cholesterol 30–130 μg/mg DPL (i.e. the same range of concentrations found to inhibit phospholipase A_2) decreased the heat absorbed at the transition, as shown previously by Ladbrooke *et al* [13]. They also found that no transition occurred in the presence of equimolar ratios of lecithin/cholesterol. It is known that the polyene antibiotic filipin forms an association with cholesterol and restores the co-operative phase transition of the phosphatidyl-choline molecules [14]. In the present experiments, filipin (in equimolar concentrations filipin/cholesterol) was found to restore 95 per cent of the phospholipase A_2 activity.

All three anaesthetic agents studied, at a concentration of 1 μg/ml DPL, lowered *tc*, as illustrated in Fig. 5. Ethrane, halothane and trichloroethylene interacted with the phospholipids, lowering the phase transition by 9, 15 and 30°, respectively and increased the width of the transition. The activity of phospholipase A_2 in the presence of these anaesthetics was not restored when the enzyme was assayed at the new *tc*s. Mountcastle *et al.* [15] have suggested that these anaesthetics decrease the degree of co-operativity of the phospholipid molecules at the *tc*. This property might explain their inhibitory effect. Another possibility is a direct inhibition of phospholipase A_2 by these molecules.

It has been suggested that some local anaesthetics inhibit phospholipase A_2 by competing for Ca^{2+} [3, 4]. This, however, is not the case for general anaesthetics. In the present experiments, even when the Ca^{2+} concentration was increased 10-fold (10 mM), the effect of these anaesthetics was not reversed.

An attempt was also made to use d.s.c to explain the earlier finding that glucocorticoids inhibit AA release from fat cell ghosts [6].

Glucocorticoid–lipid interactions were studied by d.s.c. and at concentrations of the three steroids up to 100 μg/ml DPL (i.e. a much higher concentration than that used in biological studies), no effect on *tc* (41.5°) or enthalpy (36.6 kJ.mole^{-1}) of DPL.

A direct study of the effect of glucocorticoids on fat cell ghosts was carried out using the technique of microviscosimetry. Fluorescence polarization measurements at 25° on fat cell ghosts using diphenylhexatriene as a fluorescent probe showed that incu-

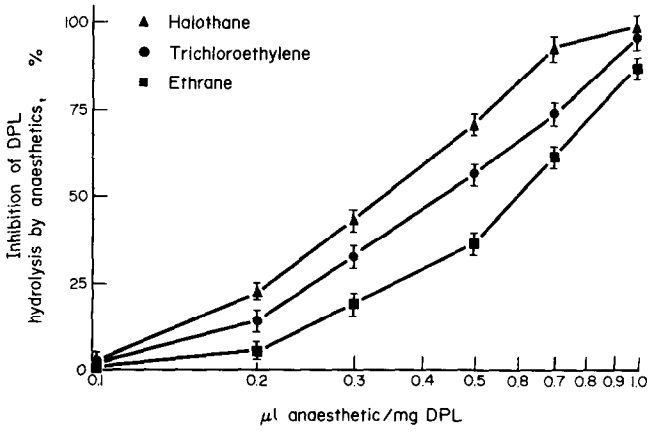

Fig. 4. The effect of different general anaesthetics on DPL hydrolysis induced by phospholipase A_2.

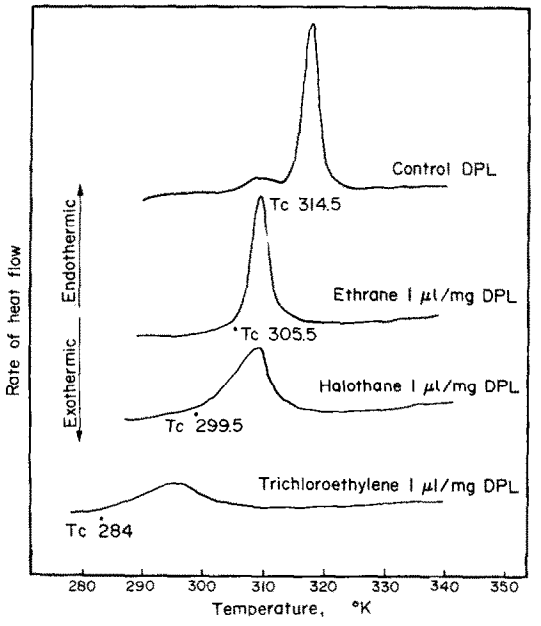

Fig. 5. The effect of anaesthetics on the DPL gel to liquid-crystalline phase transition.

bation with different concentrations of dexamethasone from 5 to 100 μg/ml did not alter the measured value of *P* (the fluorescence polarization) found to be 0.228 for control and 0.225 for steroid treated preparations (mean of three experiments).

DISCUSSION

The present findings have demonstrated the importance of membrane fluidity with regard to phospholipase activity. Irregularities in lipid packing in the bilayer of liposomes strongly enhance enzyme activity and these packing faults exist at the transition temperature of the lipid. At this temperature, lipids in both the liquid crystalline and the gel phase coexist. It appears likely that penetration of the enzyme into the interphase is facilitated at the border of the domains of "frozen lipid" [10, 16].

Purified phospholipase A_2 has been widely used in a number of different studies on membrane structure and physical properties. The asymetric distribution of the phospholipid classes across the membrane of the erythrocyte [17] and the micro-organism *Acholeplasma laidlawii* [18] have been studied using this enzyme. It has also proved a useful tool in the studies on membrane physical properties, e.g. membrane surface pressure [7], membrane sidedness [19] and metabolism of membrane lipid [20].

The hydrolysis of phospholipids by phospholipase A_2 has thus become a very suitable tool for the investigation of the physical properties of the plasma membrane, its lipid organization and its function as a permeability barrier.

Interest in phospholipase A_2 has grown considerably in recent years in view of its importance with regard to PG synthesis. Arachidonic acid is released from the phospholipids by phospholipase A_2 before being converted into prostaglandins. The spectrophotometric assay of phospholipase A_2 which we have presented in this paper is rapid and simple and would be ideally suited for the screening of drugs which might modify phospholipase activity.

Parallel studies of drug–phospholipid interactions using d.s.c. provide further insight into the mechanism of action of phospholipase inhibitors. The present experiments show that agents which rigidify the membrane, such as cholesterol, or those which fluidize the membrane, such as anaesthetics, inhibited phospholipase action. Other drugs, e.g. mepacrine and phentermine, directly inhibited the enzyme without interacting with the membrane phospholipids. In biological systems, however, other regulatory mechanisms might also be present.

Cholesterol was found to decrease the heat absorbed at the transition and to inhibit the hydrolysis rate induced by phospholipase in the same concentration range. Furthermore, this activity was reversed by filipin. It seems likely, therefore, that cholesterol inhibits the phospholipase by interacting with the phospholipids. Some local anaesthetics have been reported to inhibit endogenous phospholipase A_2 [3, 4]. Similar molecules have been found to interact with the phospholipids and modify their fluidity [21]. In the present study, the actions of the general anaesthetics, ethrane, halothane and trichloroethylene, were found to be more complex. They lowered the *tc*, decreased the heat absorbed at transition and inhibited the hydrolysis. In addition, when hydrolysis was carried out at the new *tc*, the enzyme was still inhibited, indicating some other action. This was not competition for Ca^{2+}, since an increase in Ca^{2+} did not reverse the inhibition. It appears, therefore, that the three anaesthetics not only interact with the membrane phospholipids but influence the enzyme activity either by direct interaction or by reducing the cluster size of the phospholipids at this temperature [15].

In 1961, glucocorticoids were first reported to stabilize biological membranes [22] and we have therefore studied glucocorticoid–phospholipid interaction by d.s.c. and microviscosimetry to determine whether this stabilization is due to a direct interaction with the membrane phospholipids. Our studies using even higher concentrations of glucocorticoids than those used in biological systems failed to reveal any effect of these drugs on membrane fluidity. A stabilization of the membrane by glucocorticoid–phospholipid interactions can therefore be excluded.

Another possible stabilization mechanism of these drugs could be via a direct inhibition of phospholipase A_2 activity. However, the present investigation has shown that the rate of hydrolysis of DPL by purified phospholipase A_2 from pig pancreas or *Naja naja* venom was not altered in the presence of hydrocortisone, dexamethasone or betamethasone up to 2:1 (w/w) steroid/lipid.

The inhibition of phospholipase A_2 in biological systems by anti-inflammatory steroids does not therefore appear to be either via direct interactions of the steroids with the enzyme or by steroid–phospholipid interactions. A further mechanism presently under investigation must therefore be involved to explain their action.

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REFERENCES

1. B. B. Vargaftig and N. Dao Hai, *J. Pharm. Pharmac.* **24**, 159 (1972).
2. M. Fry, Ph.D thesis. University of London (1977).
3. G. L. Scherphof., A. Scarpa and A. Van Toorenenbergen, *Biochim. biophys. Acta* **270**, 226 (1972).
4. M. Waite and P. Sisson, *Biochemistry* **11**, 3098 (1972).
5. G. J. Blackwell, R. J. Flowers, F. P. Nijkamp and J. R. Vane, *Br. J. Pharmac.* **62**, 79 (1978).
6. G. P. Lewis, P. J. Piper and C. Vigo, *Br. J. Pharmac.* **67**, 393 (1979).
7. R. A. Demel, W. S. M. Geurts van Kessel, R. F. A. Zwaal, B. Roelofsen and L. M. Van Deenen, *Biochim, biophys. Acta* **382**, 169 (1975).
8. B. de Kruyff and R. A. Demel, *Biochim. biophys. Acta* **339**, 57 (1974).
9. D. Chapman, *Q. Rev. Biophys.* **8**, 87 (1975).
10. J. A. F. Op den Kamp, J. de Gier and L. L. M. Van Deenen, *Biochim. biophys. Acta* **345**, 253 (1979).
11. E. J. King, *Biochem. J.* **26**, 292 (1932).
12. M. Rodbell, *J. biol. Chem.* **249** 5744 (1967).
13. B. D. Ladbrooke, R. M. Williams and D. Chapman, *Biochim. biophys. Acta* **150**, 333 (1968).
14. J. A. F. Op den Kamp, M. T. H. Kavert and L. L. M. Van Deenen, *Biochim. biophys. Acta* **382**, 169 (1975).
15. D. B. Mountcastle, R. L. Biltonen and M. J. Halsey, *Proc. natn. Acad. Sci. U.S.A.* **75**, 4906 (1978).
16. C. D. Linden, K. L. Wright, H. M. McConnell and C. F. Fox. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2271 (1973).
17. R. F. A. Zwaal, B. Roelofsen, P. Confurius and L. L. M. Van Deenen, *Biochim. biophys. Acta* **406**, 83 (1975).
18. E. M. Bever, S. A. Singal, J. A. F. Op den Kamp and L. L. M. Van Deenen, *Biochemistry* **16**, 1290 (1977).
19. R. Sundler, A. W. Alberts and P. R. Vagelos, *J. biol. Chem.* **253**, 5299 (1978).
20. M. Gan-Elepano and J. F. Mead, *Biochim. biophys. Res. Commun.* **83**, 247 (1978).
21. M. W. Hill, *Biochim. biophys. Acta* **356**, 117 (1974).
22. G. Weissman and J. T. Dingle, *Expl Cell. Res.* **25**, 207 (1961).