J. Pharm. Pharmac., 1978, **30**, 608–612 *Received March* 30, 1978

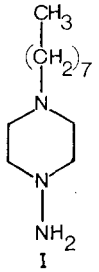
Phospholipase inactivation induced by an amino-piperazine derivative: a study at the lipid - water interface

F. DEFRISE-QUERTAIN, P. CHATELAIN AND J. M. RUYSSCHAERT*

Laboratoire de Chimie Physique des Macromolécules aux Interfaces, Université Libre de Bruxelles, Boulevard du Triomphe 5, C.P. 206/2, 1050 Bruxelles, Belgium

1-Amino-4-octylpiperazine, AP 22, an antiviral agent causes lipid accumulation in nervous tissue cultures. A physicochemical membrane model was used to demonstrate the formation of a lipid-AP 22 complex hindering phospholipase A_2 action. A well defined amphiphilic balance seems essential to explain the mode of action of the drug. The hydrophilic group prevents enzyme-substrate complex formation whereas the hydrophobic group allows the penetration in the lipid layer and determines the stability of the drug-lipid complex. This stability of the drug-lipid association has a direct influence on phospholipase A_2 activity but does not affect phospholipase C activity. No inactivation of phospholipase A_2 due to a drug-enzyme interaction could be detected.

Various hydrophilic drugs with different pharmacological effects have the same side action: an abnormal accumulation of lipid material in cells (Hruban, Slesers & Hopkins, 1972; Lüllmann, Lüllmann-Rauch & Wassermann, 1973). Electron microscopy of treated cells reveals the formation of lipid cytoplasmic inclusions with multilamellated structure (myeloid bodies). Two main factors have been proposed to explain drug-induced phospholipidosis. First amphiphilic drugs can inhibit phosphatidate phosphohydrolase which is concerned with the synthesis of glycerolipids. This inhibition could modify the lipid pattern and lead to an accumulation of anionic phospholipids (Michell, Allan & others, 1976). Subsequently, lipid breakdown can be modified either by direct inactivation of phospholipase by the drugs or by formation of a drug-lipid complex which hinders the enzymic activity (Lüllman & others, 1973).



I

AP 22 (1-amino-4-octylpiperazine, I) is an antiviral agent for which phospholipidosis properties were demonstrated in mouse nervous tissue cultures (Dubois-Dalcq, Buyse & others, 1973). This drug inhibits the growth of virus but is toxic for nervous cells, probably as a consequence of a lipid accumulation. This can be due to a change in the biosynthesis and/or to a modification of the degradation according to the hypotheses above.

The purpose of the present work is to demonstrate that, in a physicochemical model of biological membranes, AP 22 can inhibit the enzymic degradation. The phospholipids will be spread at the air-water interface in the close-packed state and the enzymes (phospholipase A_2 or phospholipase C) dissolved in the aqueous phase. Surface pressure and surface radioactivity measurements allow the investigation of the two possible modes of action of AP 22 that is the direct inactivation of the phospholipases or the formation of a drug-phospholipid complex which inhibits the enzymic cleavage.

MATERIALS AND METHODS

Phosphatidylserine (Mann Research Laboratories) and dipalmitoyl-DL-α-phosphatidylcholine (Sigma Chemical Company) were spread from a chloroform–methanol solution (3:1) using an Agla microlitre Syringe unit. n-Octylamine was a Merck-Schuchardt product. The phospholipase A_2 EC 3.1.1.4 (*Vipera Russelli*), enzymic activity 14·5 units mg^{-1}, and the phospholipase C EC.3.1.4.3 (*Clostridium perfringens*), enzymic activity 1 to 2 units mg^{-1}, were purchased from Sigma Chemical Company and Koch-Light Laboratories Ltd, respectively. Buffered solutions (tris/HCl 10^{-2} M, pH 7·4) were used to prepare the subphase. The Ca^{2+} concentration was fixed at 10^{-2}M. AP 22 synthetized by R. Cricchio (Gruppo-

* Correspondence.

Lepetit Laboratories, Milan, Italy) (Cricchio, Arioli & Lancini, 1975) was generously supplied by Dr N. Van Thieghem (Laboratory of Microbiology and Immunology, Free University of Brussels). AP 22 was tritiated in pure tritium gas (Dorfman & Wilzbach, 1959) using a silent electric discharge (Kummer, Ruysschaert & Jaffé, 1973). The tritiated AP 22 has a specific actvity of 140 μCi mg^{-1} and showed the same adsorption isotherm as the non-tritiated product.

Surface pressure measurements were made on a Cahn RG electrobalance according to the Wilhelmy method. A platinum plate was used. The surface radioactivity was measured with a gas flow counter (Kummer & others, 1973). All the experiments were carried out at a temperature of 23° without stirring. The surface concentration of adsorbed labelled AP 22 was calculated from its specific activity and from the counter efficiency. Test for statistical significance utilised Student's *t*-test.

RESULTS

Fig. 1 is a typical display of the hydrolysis occurring in a dipalmitoyl-DL-α-phosphatidylcholine monolayer in the presence of an AP 22-phospholipase C mixture. If this mixture is injected in the aqueous phase, an increase in surface pressure due to AP 22 adsorption on the monolayer is observed initially. After 4 min, the surface pressure begins to decrease. This fall in surface pressure is associated with the enzymic cleavage of the lipids. Equilibrium is reached after 100 min. The main characteristics on the enzymic hydrolysis by phospholipase C in the presence and absence of AP 22 are given in Table 1. As already described for another phospholipase C

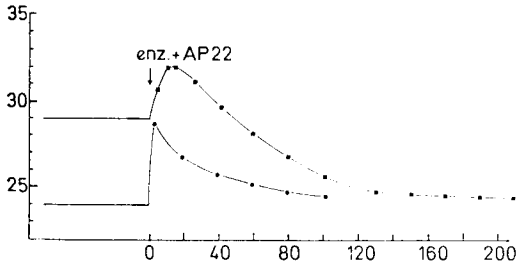


FIG. 1. Surface pressure evolution after injection of a phospholipase—AP 22 mixture under a dipalmitoyl-DL-α-phosphatidylcholine monolayer. ● Phospholipase C. The substrate, enzyme and AP 22 concentrations are fixed at 2·35 mg m^{-2}, 0·20 and 3·7 mg litre^{-1} respectively. ■ Phospholipase A_2. The substrate, enzyme and AP 22 concentrations are fixed at 2·50 mg m^{-2}, 0·25 and 3·7 mg litre^{-1} respectively. All experiments were at 23° using a tris HCl 10^{-2} M buffer, pH 7·4, Ca^{2+} 10^{-2} M. Ordinate: π (dynes cm^{-1}; mN m^{-1}). Abscissa: Time (min).

Table 1. *Influence of AP 22 on the hydrolysis of a dipalmitoyl-DL-α-phosphatidylcholine monolayer by phospholipases.*

Experiments	Phospholipase C Lag period* min	Phospholipase C Final surface pressure dynes cm^{-1} (mN m^{-1})	Phospholipase A_2 Lag period* min	Phospholipase A_2 Final surface pressure dynes cm^{-1} (mN m^{-1})
Injection of enzyme	4 ± 0·49	16·5	17 ± 3·15	15·8
Injection of an AP 22 enzyme mixture	4 ± 1·58	24·2	17 ± 3·50	24·6
Injection of enzyme under the AP 22-lipid complex	4 ± 1·20	24·6	45 ± 11·09	26

Experimental conditions: see Fig. 1.
* Mean ± s.e.m., $P < 0{\cdot}01$.

B. cereus (Demel, Guerts van Kessel & others, 1975), the hydrolysis begins after a lag period; however, because the same phenomenon is observed with and without AP 22, it can be concluded that the drug does not inhibit enzyme activity. The final surface pressure is different with and without AP 22 dissolved in the aqueous phase, but this effect is clearly due to the adsorption of AP 22 on the monolayer during the hydrolysis. Indeed, if, at the end of the control experiment (action of phospholipase C alone), AP 22 was injected in the aqueous phase, the surface pressure reached, at equilibrium, the final surface pressure obtained in experiments with AP 22. This observation allows the conclusion that there is no direct inhibition of phospholipase C activity by AP 22.

In the same manner, the possibility of direct inhibition of phospholipase A_2 by AP 22 was investigated. The characteristics of the hydrolysis of a dipalmitoyl-DL-α-phosphatidylcholine monolayer by phospholipase A_2 with and without AP 22 in the subphase are given in Fig. 1 and Table 1. Again, after the injection of an AP 22-phospholipase A_2 mixture under the monolayer, the surface pressure begins to decrease after the same lag period.

With the AP 22-phospholipase A_2 mixture (Table 1), the final surface pressure (at the equilibrium) reaches a higher value. As with phospholipase C, this phenomenon is a consequence of the AP 22 adsorption.

The hypothesis of a direct inhibition of phospholipase A_2 and C can thus be excluded.

The variation of the surface pressure ($\Delta\pi$) induced by the adsorption of AP 22 on the phosphatidylserine and the dipalmitoyl-DL-α-phosphatidylcholine monolayers as a function of the phospholipids surface concentration (C_s) is shown in Fig. 2. In the absence of lipids, the adsorption is high since AP 22 is surface-active.

The importance of the amphiphilic balance is shown in Fig. 3. In the case of an acidic bulk phase, the completely ionized molecule is only weakly adsorbed. At basic pH, the unionized prevalently hydrophobic molecule shows a maximum adsorption.

At the lipid–water interface, injection of AP 22 produces an increase of surface pressure at low lipid surface concentration ($C_s < 1$ mg m^{-2}) and a regular decrease at higher lipid surface concentration (Fig. 2). Results in Fig. 4 show clearly that an increase of phospholipid surface concentration causes a regular decrease of the number of AP 22 molecules adsorbed in the plane of the interface. Thus the increase of the surface pressure noted in Fig. 2 isn't due to a variation in the AP 22 adsorption but rather to a specific interaction between the hydrocarbon chain of AP 22 and phospholipids molecules lying flat on the surface. If the surface concentration of the phospholipids increases ($C_s > 1$ mg m^{-2}), AP 22 surface concentration decreases in response to the surface pressure.

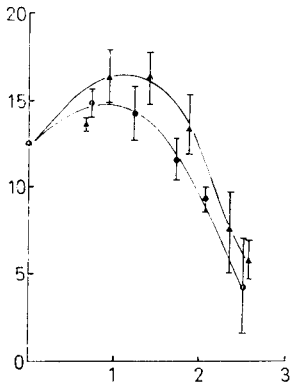


FIG. 2. AP 22 adsorption. Evolution of the surface pressure ($\Delta\pi$, dynes cm^{-1}; mN m^{-2}) (ordinate) as a function of the phospholipid surface concentration (C_s, mg m^{-2}) (abscissa) (●: dipalmitoyl-DL-α-phosphatidylcholine; ▲: phosphatidylserine). AP 22 bulk concentration: 3·7 mg litre^{-1}. Buffer: tris HCl 10^{-2} M, pH 7·4, Ca^{2+} 10^{-2} M.

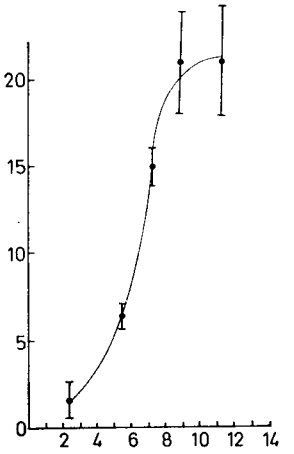


FIG. 3. AP 22 adsorption. AP 22 surface concentration in function of the pH of the subphase. AP 22 bulk concentration: 3·7 mg litre^{-1}. Ordinate: π (dynes cm^{-1}; mN m^{-1}). Abscissa: Subphase pH.

The net charge of the phospholipid monolayer influences significantly the AP 22 adsorption. The variation of surface pressure ($\Delta\pi$) (Fig. 2) and the surface concentration of AP 22 (Fig. 3) in presence of phosphatidylserine, a negatively charged lipid (Rojas & Tobias, 1965) are always higher than those observed with the dipalmitoyl-DL-α-phosphatidylcholine, a neutral lipid. At pH 7·4, the importance of the electrostatic interaction is particularly clear for condensed monolayers. Indeed, the surface concentration of AP 22 molecules adsorbed on a negatively charged film is twice that on a zero net charge film. The stoicheiometry of the dipalmitoyl-DL-α-phosphatidylcholine-AP 22 complex is one AP 22 molecule for two phospholipids. The importance of electrostatic interactions in the binding of cationic

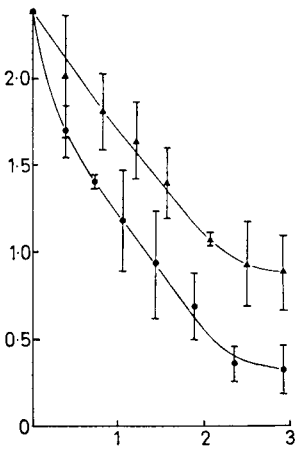


FIG. 4. AP 22 adsorption. Evolution of the AP 22 surface concentration in function of the phospholipid surface concentration (●: dipalmitoyl-DL-α-phosphatidylcholine; ▲: phosphatidylserine). AP 22 bulk concentration: 3·7 mg litre^{-1} Buffer: tris HCl 10^{-2} M, pH 7·4, Ca^{2+} 10^{-2} M. Ordinate: C_s AP 22 (mg m^{-2}). Abscissa: C_s lipids (mg m^{-2}).

drugs to acidic lipids has been proposed recently to explain the development of lipidosis (Matsuzawa, Yamamoto & others, 1977). Indeed, it has been suggested that phosphatidate interacts with positively charged drugs and that this new substrate is not attacked by phosphatidate phosphohydrolase.

When AP 22 is injected under a monolayer of dipalmitoyl-DL-α-phosphatidylcholine, equilibrium pressure is reached after 2 h. If then phospholipase C is added in the aqueous phase (Fig. 5), there results a decrease in surface pressure after the same 4 min lag seen when phospholipase C is added without AP 22 dissolved in the aquous phase.

The conclusion is different with phospholipase A_2. Indeed, with AP 22 in the lipid layer, the degradation begins after 45 min (Fig. 5). This delay cannot be attributed to the increase of surface pressure (4 dynes cm^{-1}; mN m^{-1}), due to the adsorption of AP 22. Indeed, the same lag period (17 min) is obtained for phospholipid hydrolysis at 29 and 33 dynes cm^{-1} (mN m^{-1}).

DISCUSSION

The inhibition of the enzymic reaction can be due to the presence of a voluminous hydrophilic group on the AP 22 molecule. This aminopiperazine group would hinder the enzyme–substrate complex formation. The importance of the hydrophilic group dimension appears if AP 22 is compared with the properties of n-octylamine (Fig. 6). This molecule has the same hydrophobic chain as AP 22; only the hydrophilic group is reduced to an amine group. When mixed films of n-octylamine-dipalmitoyl-DL-α-phosphatidylcholine (molar ratio 2:1) were spread at the air–water interface, the process of lipid hydrolysis by phospholipase A_2 was not modified.

Fig. 6a and b show a schematic picture of the AP 22 and n-octylamine molecule in their lipid environment. According to Demel's definition (Demel, 1968), it must be admitted that AP 22 penetrates the

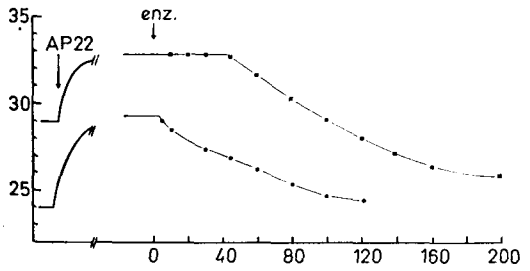


FIG. 5. Hydrolysis of a dipalmitoyl-DL-α-phosphatidylcholine monolayer by phospholipases after adsorption of AP 22. Experimental conditions: see Fig. 1.

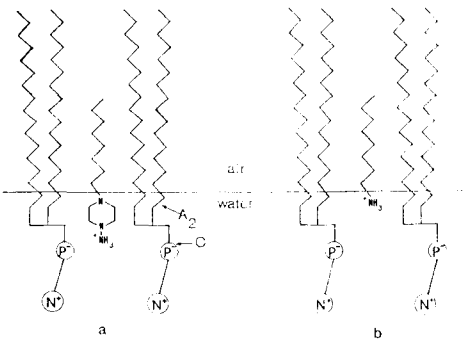


FIG. 6. Schematic picture of the complex. (b) n-Octylamine-dipalmitoyl-DL-α-phosphatidylcholine. (a) AP 22-dipalmitoyl-DL-α-phosphatidylcholine. A_2 →: action of phospholipase A_2. C →: action of phospholipase C.

lipid layer even in a close-packed state. Because phospholipase C cleaves the lipid molecule between the phosphate and glycerol groups, the hydrophilic part of AP 22 immersed in the aqueous phase doesn't hinder the enzyme–substrate formation. This absence of inhibition was not observed with phosphatidate phosphohydrolase. Indeed, the removal of the phosphate group from the phosphatidate by the enzyme is inhibited by the amphiphilic cationic drugs which interact with phosphatidate. In these conditions, it seems likely that the neutralized phosphatidate is no longer recognized as a substrate and this inactivation leads to the accumulation of anionic lipids (Bowley, Cooling & others, 1977). However, for phospholipase A_2, the enzymic cleavage occurs at the level of the β-ester linkage. Clearly a large hydrophilic group (Fig. 6a) will inhibit the enzymic action by steric hindrance.

These results agree with those obtained with another drug, AC 3579 (2-*N*-methylpiperazinomethyl-1,3-diazafluoranthen 1-oxide), which inhibits completely the hydrolysis of dipalmitoyl-DL-α-phosphatidylcholine monolayers by phospholipase A_2 (Chatelain, Berliner & others, 1976). But the capacity to inhibit the enzymic activity depends on the amphiphilic balance of the compound. Indeed, if for AC 3579 and AP 22 molecules the hydrophilic parts are rather similar, differences do appear in the hydrophobic moieties. Indeed, the AC 3579 hydrophobic group is much larger. This last characteristic probably allows a strong anchorage of the drug molecule to the lipid layer and consequently a very stable drug-lipid complex results. Because the AP 22 chain is shorter, the complex AP 22-lipid is less stable and the phospholipase A_2 inactivation less complete. In this case only a delay in the lipid hydro-

lysis will be observed. These results support the postulated mechanism of a lipid accumulation due to an inhibition of the lipid degradation (Seydel & Wassermann, 1973, 1976; Laurent, Hildebrand & Thys, 1975).

The global mechanism of inhibition is rather similar to this observed with phosphatidate phosphohydrolase, an enzyme concerned with the synthesis of glycerolipids rather than with their degradation. Indeed, in this case, it seems likely that interaction of amphiphilic amines with phosphatidate inhibits also the phosphatidate phosphohydrolase probably resulting from a change in the physicochemical properties of the phosphatidate. This modification could include the rearrangement and the modification of the electrical potential of the lipid layer. Again two parts in the drug molecule enhance the interaction: a large hydrophobic region allowing the anchorage in the lipid layer and a positively charged hydrophilic group (Bowley & others, 1977).

Finally, even if preliminary results (N. Van Thieghem, personal communication) showed that incorporation of labelled precursor (^{32}P orthophosphate) into total lipids was identical in controls and AP22 treated cells, the possibility of phosphatidate phosphohydrolase inhibition leading to an abnormal accumulation of anionic lipids cannot be excluded.

Acknowledgements

The authors are most grateful to Dr Van Thieghem for providing the aminopiperazine derivative AP 22. One of us (P. C.) thanks I.R.S.I.A. for financial assistance in this research.

REFERENCES

Bowley, M., Cooling, J., Burditt, S. L. & Brindley, D. N. (1977). *Biochem. J.*, **165**, 447–454.

Chatelain, P., Berliner, C., Ruysschaert, J. M. & Jaffé, J. (1976). *Biochim. biophys. Acta*, **419**, 540–548.

Cricchio, R., Arioli, V. & Lancini, V. G. (1975). *Farmaco, Edn Sci.*, **30**, 605–619.

Demel, R. A. (1968). *J. Am. Oil Chem. Soc.*, **45**, 305–312.

Demel, R. A., Guerts van Kessel, W. S. M., Zwaal, R. F. A., Roelofsen, B. & Van Deenen, L. L. M. (1975). *Biochim. biophys. Acta*, **406**, 97–107.

Dorfman, L. M. & Wilzbach, K. E. (1959). *J. phys. Chem.*, **63**, 799–801.

Dubois-Dalcq, M., Buyse, M., de Bruyne, J., Van Thieghem, N. & Thiry, L. (1973). *Expl Cell Res.*, **77**, 303–311.

Hruban, Z., Slesers, H. & Hopkins, E. (1972). *Lab. Invest.*, **27**, 62–70.

Kummer, J., Ruysschaert, J. M. & Jaffé, J. (1973). *Berichte von VI. Internationalen Kongress für Grenzflächenaktive Stoffe*, Band II, 1, 285–294, München: Carl Hauser.

Laurent, G., Hildebrand, J. & Thys, O. (1975). *Lab. Invest.*, **32**, 580–584.

Lüllmann, H., Lüllmann-Rauch, R. & Wassermann, O. (1973). *Dt. med. Wschr.*, **98**, 1616–1625.

Matsuzawa, Y., Yamamoto, A., Adachi, S. & Nishikawa, M. (1977). *J. Biochem*, **82**, 1369–1377.

Michell, R. H., Allan, D., Bowley, M. & Brindley, D. N. (1976). *J. Pharm. Pharmac.*, **28**, 331–332.

Rojas, E. & Tobias, J. M. (1965). *Biochim. biophys. Acta*, **94**, 394–404.

Seydel, J. K. & Wassermann, O. (1973). *Naunyn-Schmiedebergs Arch. Pharmac.*, **279**, 207–211.

Seydel, J. K. & Wassermann, O. (1976). *Biochem. Pharmac.*, **25**, 2357–2364.