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INFLUENCE OF CATIONIC AMPHIPHILIC DRUGS ON THE PHOSPHATIDYLCHOLINE HYDROLYSIS BY PHOSPHOLIPASE A_2

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Abstract—On chronic treatment certain amphiphilic drugs induce a generalized phospholipidosis. This drug side effect has been related to an inhibition of the lysosomal phospholipases due to the interaction of the drugs with phospholipids (PL). In the present experiments, the influence of the amphiphilic drugs ambroxol, imipramine, chloroquine and chlorphentermine on the hydrolysis of dipalmitoylphosphatidylcholine (DPPC) unilamellar liposomes by bee venom phospholipase A_2 (PLase A_2) was studied. Special emphasis was laid on the initial phase and temperature dependence. The activity of PLase A_2 was measured continuously with a spectrophotometric assay using cresol red as indicator. In most cases a lag-phase of different duration was observed before the enzyme exhibited its full activity. The duration of the lag-phase and the rate of hydrolysis in the second phase are inversely related. The temperature dependence of the hydrolysis reveals a maximum of activity near the phase transition of the bilayer and a gradually decreasing activity at lower and higher temperatures, respectively. The analysis of the influence of amphiphilic drugs reveals three types of interaction. (1) Imipramine and ambroxol shift the temperature activity profile towards lower temperatures without a substantial influence on the shape of the profile and on the maximal rate of hydrolysis. (2) Chloroquine inhibits the enzyme activity without any temperature dependence. (3) Chlorphentermine, the classical lipidosis inducing drug, exhibits a third type of interaction which seems to be a combination of the two former types.

Certain cationic amphiphilic drugs induce a disturbance of the PL metabolism which, at chronic administration in laboratory animals and even man, leads to an abnormally high accumulation of PL in the lysosomal compartment of many different cells (for review see refs 1 and 2). This side effect has been related to their interaction with PL. One of the consequences of this interaction is an impaired PL catabolism by phospholipases at least in lysosomes [3–5]. The interaction of amphiphilic drugs with PL is a well-known fact and has been studied mainly by means of spectroscopic techniques and DSC. For the group of phospholipidosis-inducing cationic amphiphiles the interaction with PL has also been proven by physiocochemical methods [6–9]. On the other hand, studies with PLase A_2 as a biochemical probe of PL bilayer alterations caused by phospholipidosis inducing amphiphiles are rather scarce and limited [3, 4, 10–12]. But this approach could be used as a model for the biochemical consequences of the PL bilayer alteration and may be related to the suggested mechanism of phospholipidosis induction.

In the present experiments the influence of four cationic amphiphilic drugs, ambroxol, imipramine, chloroquine and chlorphentermine, on the hydrolysis of DPPC bilayers by bee venom PLase A_2 with special emphasis on the initial phase and the temperature dependence of the interaction was studied. Under special conditions all compounds tested are inhibitory, but three distinct types of influence on enzyme activity were detectable.

MATERIALS AND METHODS

The following agents were used: imipramine hydrochloride (VEB Arzneimittelwerk Dresden, G.D.R.), ambroxol (*trans*-4-[(2-amino-3,5-dibrombenzyl)-amino]-cyclo-hexanol hydrochloride, Boehringer, Ingelheim, F.R.G.), chloroquine diphosphate (VEB Berlinchemie, Berlin, G.D.R.), chlorphentermine hydrochloride (a gift of H. Lüllmann, Kiel, F.R.G.) and DL-dipalmitoylphosphatidylcholine (Fluka, Buchs, Switzerland). The PLase A_2 was prepared from purified bee venom by Sephadex-G50 column chromatography [13]. It still contains about 20% melittin. All other chemicals were of analytical grade.

The activity of PLase A_2 was assayed spectrophotometrically using a pH indicator and was monitored continuously with the spectrophotometer Specol (VEB Carl Zeiss, Jena, G.D.R.) [14]. The incubation mixture contains: 1.0 mM DPPC as unilamellar liposomes, 12.5 mM glycine, 3.0 mM $CaCl_2$, 3.3×10^{-2} mM cresol red, and 160 mM KCl. The substrate liposomes were prepared by rapid injection of an ethanolic solution of DPPC (20 mg/ml) into a stirred solution of 160 mM KCl above the phase transition temperature (T_c) [15]. Before the initiation of the hydrolysis by adding 5 or 10 μl of a solution of 1 mg/ml purified bee venom in 160 mM KCl the pH of the incubation mixture was brought to 8.1–8.2. Usually, the amphiphilic drugs, 10 mM solutions in 160 mM KCl made alkaline with 1 M

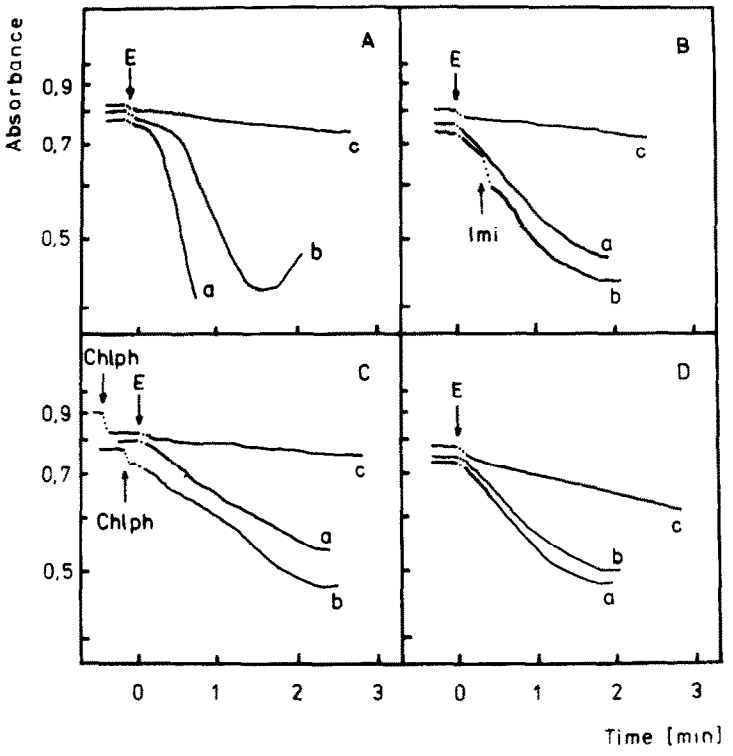

Fig. 1. Reaction progress curves for the hydrolysis of DPPC initiated by the addition of 5 μg (B, C) or 10 μg (A, D) enzyme (arrow). In (A) the reaction was performed at 41° in the absence (a) or in the presence of 0.25 mM (b) or 0.5 mM (c) ambroxol. In (B) the time dependence of imipramine inhibition at 41° is shown: curve (a) without drug; curve (b) 0.2 mM imipramine was added 20 sec after, and curve (c) 5 min before, the initiation of the enzyme reaction. In (C) the time dependence of the effect of chlorpentermine is shown at 41°: curve (a) without drug; curve (b) 0.2 mM chlorphentermine was added 5 sec before, and curve (c) 30 sec before, the initiation of the reaction. In (D) it is shown that preincubation at 41° with 0.2 mM chlorphentermine does not increase the inhibitor activity at 37° (b), in curve (a) the hydrolysis at 37° in the absence of chlorphentermine and in curve (c) the action of 0.2 mM chlorphentermine on the hydrolysis at 41° is shown for comparison.

NaOH, were added 5–10 min before the experiment began. In some experiments the inhibitors were added at fixed time intervals before or after initiation of the hydrolysis in order to study the influence of both drug and enzyme absorption on enzyme activity. The enzyme activity was calculated as μmoles H^+ released/min and the additional buffer capacity of the drugs was taken into account by measuring the decrease of extinction after successively adding 10 μl 0.01 M HCl to the incubation mixture.

RESULTS

As shown in Fig. 1 (Ab) the decrease of extinction after adding PLase A_2 is a triphasic process. After a lag-phase at a low rate of change a period of higher rate follows. This high rate eventually reversed after a certain time interval by an increase in turbidity. In agreement with Jain *et al.* [12], this increase in turbidity depends on temperature and on the proportion between substrate, enzyme and inhibitor concentration, and seems to be due to vesicle fusion. The enzyme activity during the second phase of hydrolysis appears to decrease exponentially. This is caused by the decrease of the substrate concentration which is not in excess because of the constraints of the assay system as well as by the increase in turbidity. A third influence on enzyme activity might be the drop of the pH (0.1 units of extinction per 0.1 unit of pH) during hydrolysis. In order to eliminate all these factors, we used the initial rate of hydrolysis of the second phase as a measure of activity. This activity and the duration of the lag-phase are the two most useful parameters to characterize the interaction of the enzyme with the bilayer and to study the influence of amphiphilic compounds. As shown in Fig. 2 the two parameters are inversely correlated and characteristically depend on temperature. In unmodified vesicles the enzyme exerts its highest activity near to T_c of DPPC without any detectable lag-phase. At both lower and higher temperatures an increasing duration of the lag-phase and a decreasing activity is observed. These results are in general agreement with the observations made by Op den Kamp *et al.* [16] and Upreti and Jain [17] using pancreatic PLase A_2 and by Goormaghtigh *et al.* [18] using bee venom PLase A_2. The enzyme activity at a given temperature is proportional to the enzyme concentration of 1–20 μl/ml purified bee venom. The duration of the lag-phase is inversely related to the amount of enzyme. At higher enzyme concentrations (>10 μg/ml) the lag-phase is difficult to evaluate.

The analysis of the influence of amphiphilic drugs

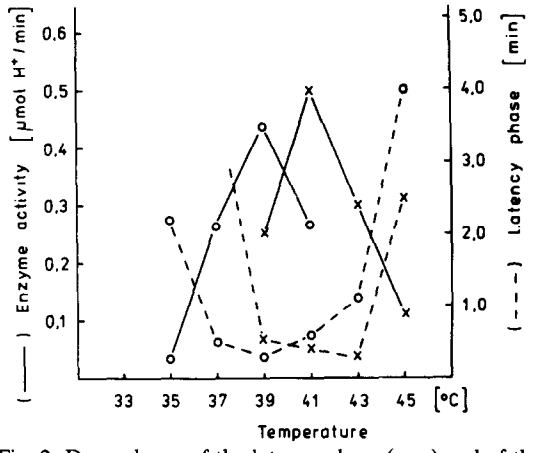

Fig. 2. Dependence of the latency phase (---) and of the enzyme activity (——) of 10 μg PLase A_2 on temperature of incubation in the absence (×) or presence of 0.25 mM ambroxol (○).

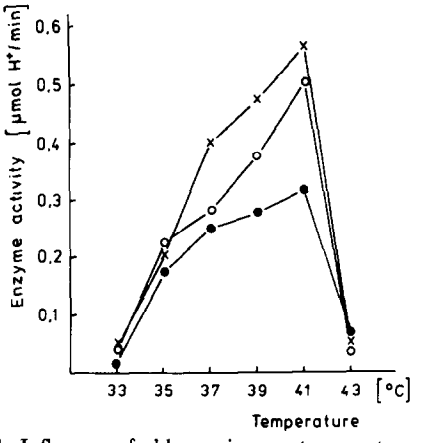

Fig. 4. Influence of chloroquine on temperature dependence of enzyme activity. The concentration of PLase A_2 was 10 μg/ml and that of chloroquine 0 mM (×), 0.2 mM (○) and 0.5 mM (●).

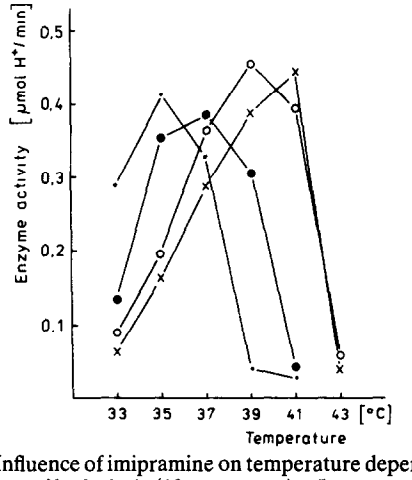

Fig. 3. Influence of imipramine on temperature dependence of the rate of hydrolysis (10 μg enzyme). The concentration of imipramine was 0 mM (×), 0.1 mM (○), 0.2 mM (●) and 0.5 mM (·).

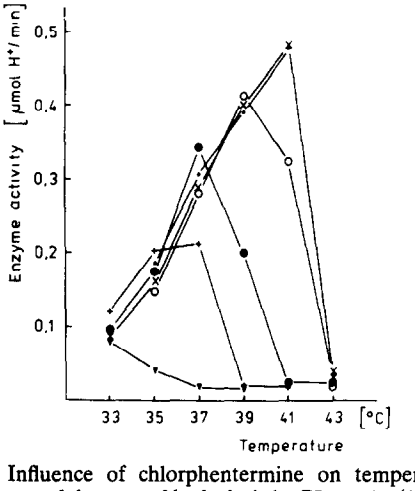

Fig. 5. Influence of chlorphentermine on temperature dependence of the rate of hydrolysis by PLase A_2 (10 μg). The concentration of chlorphentermine was 0 mM (×), 0.1 mM (·), 0.2 mM (○), 0.35 mM (●), 0.7 mM (+) and 1.75 mM (▽).

on the temperature dependence of the enzyme activity reveals three types of interaction. As shown in Figs 2 and 3 the most characteristic feature of both imipramine and ambroxol interaction is the shifting of the temperature activity profile towards lower temperatures. The shape of the profile and the maximal rate was not substantially influenced. Figure 2 shows for ambroxol that the lag-phase temperature profile is shifted towards lower temperatures too. An interesting phenomenon was observed when imipramine or ambroxol was added at the beginning of the active hydrolysis near to T_c. In these experiments the hydrolysis proceeds at an unchanged rate (Fig. 1 Bb) in contrast to the strong inhibition caused by the same amount of drug added 5–10 min before the initiation of the hydrolysis (Fig. 1 Bc). The reason for this effect may be that the lag-phase is due to an inhibited enzyme absorption [17, 19].

Chloroquine exhibited a second type of influence. As shown in Fig. 4 this compound inhibits the enzyme activity without any effect on its temperature dependence.

Chlorphentermine, the classical lipidosis-generating amphiphilic pharmacon, exhibited a third type of influence. The most prominent feature is a strong inhibition at T_c and a gradually decreasing efficacy at lower temperatures (Fig. 5). When chlorphentermine was added during the second phase of active hydrolysis the rate did not change. In contrast to the similar behavior of imipramine (Fig. 1 Bb) and ambroxol this drug is also ineffective when added a few seconds before initiation of hydrolysis (Fig. 1 Cb). Obviously, this effect is due to a slow absorption of the drug by the substrate. As shown in Fig. 1 (Cc), the addition of chlorphentermine 30 sec before the enzyme is sufficient for drug absorption and thus for acting inhibitorily. In Fig. 1 Cb, it is also shown that the drug is without inhibitory activity over the entire experimental period. Similar to ambroxol and imipramine, chlorphentermine is not inhibitory when

absorbed after enzyme addition. In a further experiment the possibility was tested whether the low inhibitory effectiveness of chlorphentermine at lower temperatures is due to an inhibited drug binding by the bilayer which is in the rigid gel phase (Fig. 1 D). After 2 min preincubation with the drug at T_c the temperature was decreased and the enzyme was added. There is no decreased inhibitory effect after that preincubation. Therefore, the complex inhibitory activity of chlorphentermine is not due to an inhibited drug absorption.

DISCUSSION

The fact that PLase A_2 hydrolyses a substrate organized in a lipid–water interface influences its kinetic behaviour very strongly. All models which describe the action of PLase A_2 take into account this complex situation (for a review see ref. 20), but depending on the special experimental approach one or the other fact is emphasized. In our experimental approach we wanted to study the inhibition of PLase A_2 by amphiphilic drugs as a model of its potency to induce phospholipidosis [1] as well as the influence of these drugs on the PL bilayer as a cause of further influences on lipid metabolism [21–24]. For this purpose, models which stress the quality of the bilayer are most suitable. In the concept proposed by Jain *et al.* [17, 25, 26] and others [16, 18], the border region between two lipid phases or other organisational defects within the bilayer are thought to be preferred sites of enzyme absorption and action. In these models the hydrolysis of bilayer substrates by PLase A_2 is dependent on the process of absorption of the enzyme by the bilayer as well as on kinetic properties of the enzyme. Both processes depend on the physical state of the bilayer and might be influenced by drugs.

In the present study three types of influence of amphiphilic drugs on PLase A_2 were found. Ambroxol and imipramine belong to the first type. From the experiments it seems to be clear that these two drugs influence the PLase A_2 activity by decreasing the T_c of the bilayer. In fact, the effect of imipramine on the temperature profile of the PLase A_2 activity is very similar to its action on the phase transition profile as evaluated by DSC [9]. A correspondence of the temperature activity profile of PLase A_2 and the DSC diagrams of the hydrolyzed PL has been shown for porcine pancreatic PLase A_2 [16, 18], for bee venom PLase A_2 [17] and for platelet PLase A_2 [27]. The similar areas and shapes of the temperature activity profiles (Figs 2 and 3) suggest that the hydrolytic potency of the PLase A_2 is not substantially influenced by imipramine and ambroxol.

Chloroquine exhibits a quite different effect on PLase A_2 activity. The drug inhibits the enzyme activity in a dose dependent manner. This is in agreement with the results of Jain *et al.* [12], who studied pig pancreatic PLase A_2 and with the results of Matsuzawa and Hostetler [4] who found that chloroquine is a potent inhibitor of lysosomal phospholipases. The temperature dependence of the activity is not influenced, especially the maximum of the hydrolytic activity is not shifted. This indicates a low potency of chloroquine to decrease T_c, which has also been shown in DSC experiments by Kursch *et al.* [9]. Whether chloroquine affects the DPPC hydrolysis by a direct influence on the enzyme or by an indirect influence via specific interaction with the PL bilayer is not clear. Studies on the temperature dependence of cholesterol action on PLase A_2 activity revealed the same type of inhibition [16, 27]. The addition of cholesterol makes the PL bilayer more rigid, a property that also has been attributed to chloroquine. Therefore, chloroquine is believed to inhibit PLase A_2 by a similar mechanism via interaction with the PL bilayer.

Chlorphentermine, the most frequently examined phospholipidosis inducing drug, exerts a more complex influence on DPPC hydrolysis. This third type is supposed to be a combination of the two types described above. This means that chlorphentermine shifts the temperature maximum of the hydrolytic activity by decreasing the T_c and inhibits the enzyme in a similar way as chloroquine or cholesterol do. Both effects have been demonstrated independently [3, 9].

The experiments described here support the hypothesis that the lipidosis inducing activity of drugs could be related to their ability to inhibit the PLase A_2 activity. They also show that this inhibition is caused by interaction of the amphiphilic drug with the PL bilayer. A strong proportionality between these *in vitro* findings and those observed *in vivo* might not be expected because drug metabolism and pharmacokinetics have to be considered in the latter case. Furthermore, the different conditions of hydrolysis have to be taken into account. *In vivo*, the PL are hydrolyzed by lysosomal phospholipases at an acidic pH. The pH of the medium is important for the action of cationic amphiphiles because the absorption of the drug depends on its charge [28]. However, anionic PL of natural membranes diminish these differences. Imipramine and chlorphentermine with a pK_a of 9.5 and 10.0, respectively, are almost completely protonized in our incubation medium. Ambroxol has a pK_a of about 8.0 and, therefore, only half of the molecules carry a positive charge. Chloroquine has two pK_a values (8 and 10) and carries one or two positive charges, respectively (50/50). Certainly, the differences in ionization play an essential role *in vitro*. *In vivo* this appears to be of minor importance because the drugs are completely ionized at the acidic pH and because of the content of anionic PL of natural membranes.

We realize that bee venom PLase A_2 and lysosomal enzymes which are responsible for the degradation of PL *in vivo* have different properties, e.g. with respect to their pH optimum and Ca^{2+} dependence. But most important for the evaluation of the effect of the cationic amphiphilic drugs appears to be the common property of the enzymes to be sensibly regulated by the physical state of the aggregated substrate. Regardless of their source most studied PLase A_2 show this behaviour [17, 20, 25, 27, 29]. Strong evidence that the lysosomal phospholipases of the A-type, and even C-type, are also dependent on the physical state of their substrate comes from studies made by Hostetler [5]. It has been shown that many structurally unrelated drugs which possess

a common cationic amphiphilic nature inhibit lysosomal phospholipases of rat liver. Because different enzymes have different requirements to the physical state of the PL the quantity and even the quality [30] of the effect of amphiphiles may depend upon the source of the enzyme.

Jain *et al.* [31] studied the influence of a series of small molecules including some amphiphilic compounds on the phase behaviour of DPPC by DSC and showed that at least four different types of influence exist. They related these types to the different position of the solute within the bilayer; but they also speculated that a change in the phase behaviour would involve domain boundary effects which are possibly more important for the biological effect. PLase A_2 as a biochemical probe may be a useful tool for the study of such biologically important membrane alterations.

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