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HYDROLYSIS OF PHOSPHATIDYLCHOLINE LIPOSOMES BY PHOSPHOLIPASES A_2

EFFECTS OF THE LOCAL ANESTHETIC DIBUCAINE

JAN C. WILSCHUT, JOKE REGTS, HENK WESTENBERG and GERRIT SCHERPHOF

Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, Groningen (The Netherlands)

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SUMMARY

(1) Dibucaine evokes a downward shift in the phase transition temperature of saturated phosphatidylcholines, while it also affects the pretransition.

(2) The binding of dibucaine to phosphatidylcholine liposomes increases sharply when the lipid is transformed from the gel phase to the liquid-crystalline phase.

(3) The activity of *Naja naja* phospholipase A_2 towards dimyristoyl phosphatidylcholine liposomes is either stimulated or inhibited by dibucaine, depending on whether the substrate is in the gel or the liquid-crystalline state, respectively, whereas the activity of pancreatic phospholipase A_2 is inhibited by the anesthetic irrespective of the physical state of the substrate. This observation is further substantiated by the results of studies on liposomes prepared from mixtures of dimyristoyl and dipalmitoyl phosphatidylcholine or dilauroyl and distearoyl phosphatidylcholine.

(4) The uptake of dibucaine by positively charged liposomes composed of phosphatidylcholine and stearylamine is considerably reduced in comparison with pure phosphatidylcholine liposomes. This decrease is paralleled by a reduction of the inhibitory and stimulatory effects of dibucaine on the hydrolysis of such liposomes by pancreatic and *Naja naja* phospholipase, respectively.

(5) The inhibitory action of dibucaine towards the pancreatic phospholipase is lowered by increasing $CaCl_2$ concentrations. This reduction is accompanied by a decreased uptake of anesthetic by the liposomes.

INTRODUCTION

The mechanism of action of local anesthetics is generally believed to originate in the interaction of these drugs with the lipids of the nerve membrane. A great deal of information on the interaction of local anesthetics with lipids has been obtained from studies with various lipid-water systems (for reviews see refs 1 and 2). Because

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

of the known involvement of Ca^{2+} in nerve conduction [3] and in view of the ability of local anesthetics to displace Ca^{2+} from membranes [4, 5], in most investigations the study of negatively charged lipids was emphasized, since these are known to bind substantial amounts of Ca^{2+} [6, 7], in contrast to neutral phospholipids such as phosphatidylcholine [8]. However, since the latter is the most abundant phospholipid in nearly all animal membranes, including nerve membranes [9], we thought it of interest to study the effect of a local anesthetic, dibucaine, on this particular lipid. The inhibition by local anesthetics of Ca^{2+}-requiring phospholipase A activities [10] provides an interesting approach to this problem. In addition, such experiments may contribute to the understanding of phospholipase-catalyzed hydrolysis of membranous phospholipids and thus of the lipid architecture of biological membranes.

It was clearly demonstrated by De Haas and coworkers that the Ca^{2+} requirement of pancreatic phospholipase A_2 involves an enzyme-level activation [11]. Inhibition of this phospholipase by dibucaine could imply, therefore, a direct interaction between enzyme and anesthetic. Such a mechanism of inhibition has been suggested previously [12]. However, recently we presented evidence for binding of the anesthetic to the substrate being involved in the mechanism of inhibition and we included some results of binding experiments with natural phosphatidylcholines [13].

In the present study we describe the effects of dibucaine upon phospholipase action on liposomes composed of synthetic saturated phosphatidylcholines. It is demonstrated that the amount of dibucaine taken up by the liposomes is dependent on the fluidity of the bilayer lipids. A relationship between inhibitory potency towards phospholipases and bilayer concentration of dibucaine could be established.

MATERIALS AND METHODS

Reagents

1,2-Dilauroyl-*sn*-glycero-3-phosphorylcholine (dilauroyl phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (dimyristoyl phosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (dipalmitoyl phosphatidylcholine) and 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (distearoyl phosphatidylcholine) were purchased from Calbiochem. No impurities were detectable by thin-layer chromatography. Gas chromatographic analysis of the fatty-acid composition revealed that dilauroyl, dimyristoyl and dipalmitoyl phosphatidylcholine were at least 99.5 % and distearoyl phosphatidylcholine 98 % pure in this respect. The lipids were used without further purification. Stearylamine was obtained from Merck. Dibucaine-HCl was a gift from Ciba AG. *Naja naja* venom (Sigma) was dissolved up to a concentration of 1 mg/ml in dilute Tris · HCl buffer (pH 7.0). The solution was heated in a boiling water bath for 5 min and centrifuged. The clear supernatant was used as phospholipase A_2 (EC 3.1.1.4) preparation. Porcine pancreatic phospholipase A_2 was a generous gift of Dr. A. J. Slotboom from the Laboratory of Biochemistry, University of Utrecht, The Netherlands.

Preparation of liposomes

Chloroform solutions of lipids were taken to dryness in vacuo. Residual lipids were dispersed in 0.1 M Tris · HCl buffer (pH 7.0) by shaking with glass beads. The suspensions were sonicated for 1–2 min with a Branson Sonifier. The manipula-

tions were carried out at a temperature above the phase transition temperature of the lipids.

Phase transition measurements

Phase transition temperatures of the lipids were determined by measuring apparent light absorbances of liposomal suspensions at 400 nm as a function of temperature [14]. These studies were performed with a Uvichem H 1620 spectrophotometer equipped with a thermostatted cell holder. The temperature in the cuvettes was estimated with a thermocouple.

Binding studies

Nonsonicated lipid dispersions were equilibrated with 1 mM dibucaine and subsequently centrifuged for 45 min at $15\,000 \times g$ in a Sorvall RC-2B centrifuge at the desired temperature. The supernatants were carefully removed. The liposome pellets were dissolved in a chloroform/methanol mixture. The absorbances at 325 nm of both supernatants and dissolved pellets were estimated as a measure of the amount of dibucaine taken up by the liposomes [13].

Alternatively, the uptake of anesthetic by liposomes was investigated by means of equilibrium dialysis with a Dianorm apparatus (Innovativ Medizin Ltd, Zurich, Switzerland) positioned in a thermostatted water bath. Teflon cells were used with two 1-ml compartments separated by a Spectrapor-2 dialysis membrane. The liposome suspension was transferred to one of the cell compartments and buffer solution to the other. Both compartments were 1 mM with respect to dibucaine. The cells were rotated at a constant speed of 10 rev/min. After a minimum of 7 h (the time required to obtain equilibrium at the lowest temperature) the dibucaine concentration in both compartments was determined by measuring the absorbance at 325 nm after suitable dilution with a chloroform/methanol mixture.

Incubation of liposomes with phospholipase A

Incubations were carried out in 0.1 M Tris · HCl buffer (pH 7.0) at a lipid concentration of 0.75 mM. $CaCl_2$ was added to a concentration of 1.0 mM and dibucaine as indicated. After preincubation during 10–15 min at the desired temperature the reaction was initiated by adding phospholipase A. EDTA was added up to 5 mM to terminate the reaction. Controls, incubated with 5 mM EDTA, did not show any detectable lipid degradation.

Lipid analyses

Incubation mixtures were extracted according to the procedure of Bligh and Dyer [15]. Phosphatidylcholine and lysophosphatidylcholine were separated by thin-layer chromatography on silicagel HF (Merck) with chloroform/methanol/20 % (w/v) ammonia (67 : 33 : 5, by vol.) as a developing solvent. The lipids were visualized with iodine vapor and/or by illumination with ultraviolet light. The relevant spots were scraped off and eluted with chloroform/methanol mixtures. Lipid contents were estimated by phosphorus determination as described by Chen et al. [16] after destruction of the lipid according to the procedure of Ames and Dubin [17]. For gas chromatographic analysis the lipids were transesterified in methanol/sulfuric acid (95 : 5, v/v) for 2 h at 70 °C. After extraction with pentane the methylated fatty acids were

analyzed with a Hewlett-Packard 402 gas chromatograph. A known aliquot of heptadecanoic acid (Merck) was added as a standard prior to methanolysis. Absolute amounts of fatty acids were calculated from the chromatograms by triangulation, the peak of the standard fatty acid being used as a reference.

RESULTS

Temperature-dependent phase changes in aqueous dispersions of homogeneous saturated phosphatidylcholines can be observed by turbidity measurements [14, 18, 19]. Fig. 1 shows the apparent light absorbance at 400 nm as a function of temperature for dimyristoyl phosphatidylcholine liposomes in the absence as well as in the presence of dibucaine. At a concentration of 1 mM the anesthetic lowers the phase transition temperature from 24 to 19 °C. The absorbance shift at the lower temperature, which probably reflects the pretransition as observed with differential scanning calorimetry [20], is also affected by dibucaine. In its presence the pretransition was no longer detectable in the temperature range studied. Similar studies with dipalmitoyl phosphatidylcholine also revealed a decrease of the phase transition temperature and a diminution of the lower-temperature absorbance shift. Apparently, dibucaine affects the structure of the lipid phase by loosening the interaction between the phosphatidylcholine molecules. This suggests that the anesthetic penetrates the lipid bilayer. Further evidence for interaction of dibucaine with dimyristoyl phosphatidylcholine liposomes was obtained by binding experiments, as presented in Fig. 2. At temperatures below the phase transition, which in the presence of dibucaine occurs at 19 °C, approx. 60 nmol of dibucaine are bound per μmol of phosphatidylcholine. This amount increases up to about 140 nmol at temperatures above the transition temperature.

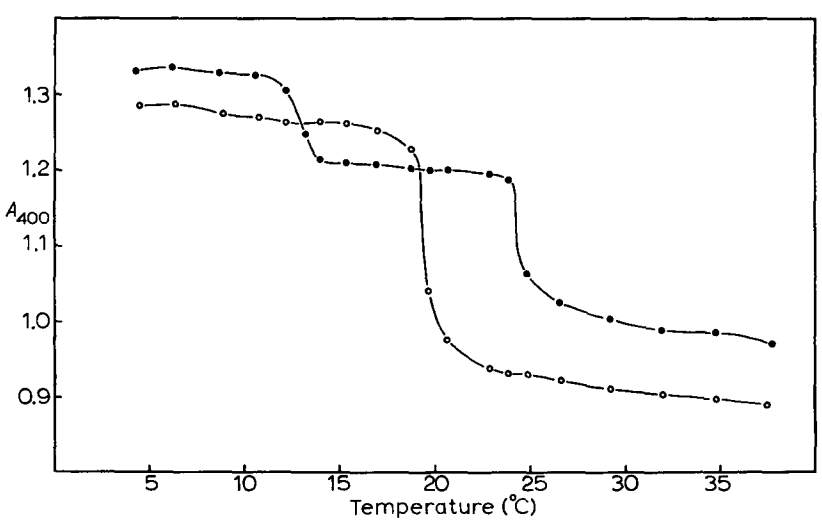

Fig. 1. Apparent light absorbance at 400 nm of a liposomal suspension of dimyristoyl phosphatidylcholine in 0.1 M Tris · HCl (pH 7.0) as a function of temperature in the absence (●) or presence (○) of dibucaine. Lipid concentration, 0.75 mM. Dibucaine concentration, 1.0 mM.

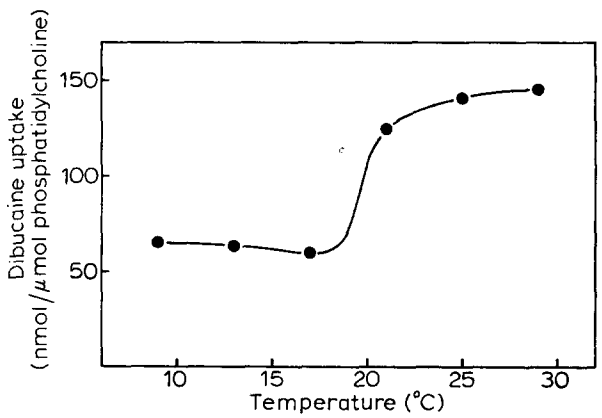

Fig. 2. Binding of dibucaine to dimyristoyl phosphatidylcholine liposomes as a function of temperature. The centrifugation technique, as described under Materials and Methods, was applied. Lipid concentration during equilibration, 1.5 mM. Dibucaine concentration, 1.0 mM. Similar results were obtained by equilibrium dialysis, at several lipid concentrations.

The presence of dibucaine in the substrate membrane influences the hydrolytic activity of phospholipase A_2. The degradation of dimyristoyl phosphatidylcholine by *Naja naja* phospholipase A (Fig. 3.) appeared to be greatly enhanced by dibucaine at temperatures below the transition temperature. At higher temperatures a strong inhibition of the enzyme activity was observed. The pancreatic phospholipase displayed a different behaviour. Recently Op den Kamp et al. reported that saturated phosphatidylcholines are degraded by the pancreatic enzyme only at temperatures close to the phase transition temperatures of the lipid [21, 22]. Our results are grossly in agreement with those reports, as we also found optimal activity of the pancreatic enzyme towards dimyristoyl phosphatidylcholine near the transition temperature of

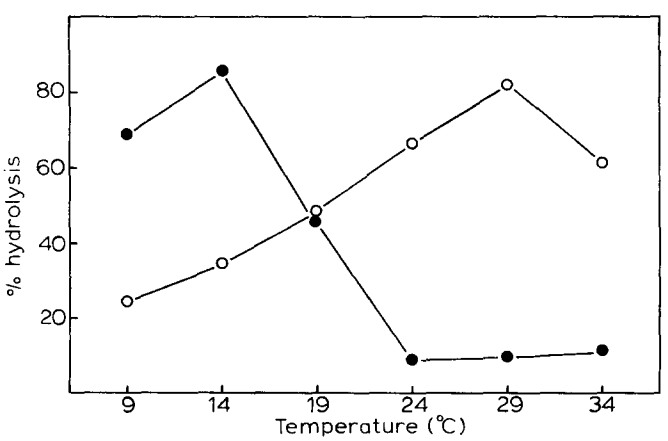

Fig. 3. Hydrolysis of dimyristoyl phosphatidylcholine liposomes in 0.1 M Tris · HCl (pH 7.0) by *Naja naja* phospholipase A_2 at different temperatures in the absence (○) and presence (●) of dibucaine. Concentrations: lipid, 0.75 mM; $CaCl_2$, 1.0 mM; dibucaine, 1.0 mM; phospholipase, 10 μg/ml. Incubation time, 60 min.

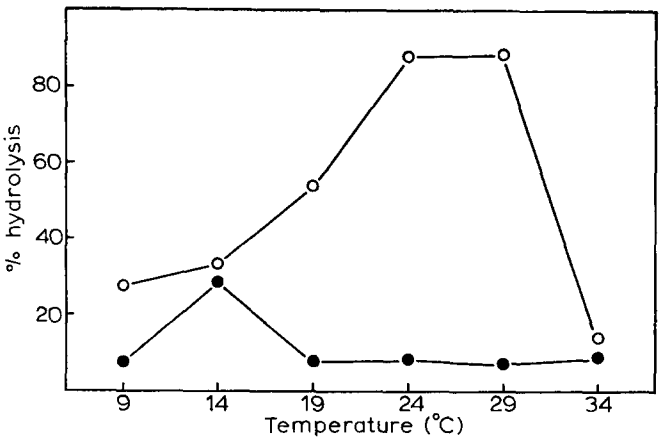

Fig. 4. Hydrolysis of dimyristoyl phosphatidylcholine liposomes by pancreatic phospholipase A_2 at different temperatures in the absence (○) or presence (●) of dibucaine. Phospholipase, 3 μg/ml. For further experimental details see legend to Fig. 3.

24 °C. However, we observed that the temperature range in which considerable hydrolysis still occurs is relatively wide and, particularly, reaches rather low values, as is demonstrated in Fig. 4. At a concentration of 1 mM, dibucaine inhibits dimyristoyl phosphatidylcholine hydrolysis by pancreatic phospholipase at all temperatures studied, the extent of inhibition being remarkably low at 14 °C.

At temperatures below the phase transition of the substrate the effects of dibucaine upon the two phospholipases seem to be different, the snake venom enzyme being stimulated and the pancreatic enzyme being inhibited. In order to further substantiate this observation we investigated the effect of the anesthetic upon the action of the phospholipases on liposomes prepared from mixtures of saturated phosphatidylcholines. If such phosphatidylcholines differ by only two carbon atoms in fatty acid chain length they are completely miscible in the gel phase as well as

TABLE I

EFFECT OF DIBUCAINE ON THE PHOSPHOLIPASE-CATALYZED DEGRADATION OF DIMYRISTOYL PHOSPHATIDYLCHOLINE/DIPALMITOYL PHOSPHATIDYLCHOLINE AND DIMYRISTOYL PHOSPHATIDYLCHOLINE LIPOSOMES

Liposomes were prepared from a mixture of dimyristoyl and dipalmitoyl phosphatidylcholine (molar ratio 1 : 1) or from dimyristoyl phosphatidylcholine alone. Incubations were carried out at the temperatures indicated under the conditions as described in the legends of Figs. 3 and 4, except that half of the amount of phospholipase was added to the incubations at 32° C.

Phosphatidylcholine species	Temp. (°C)	Pancreatic phospholipase			*Naja naja* phospholipase		
		% Hydrolysis		Inhibition (%)	% Hydrolysis		Inhibition (%)
		Control	+Dibucaine		Control	+Dibucaine	
Dimyristoyl	24	88	9	90	66	9	86
Dimyristoyl/ Dipalmitoyl	32	40	4	90	57	13	77

in the liquid-crystalline phase [23, 24]. Liposomes of an equimolar mixture of dimyristoyl and dipalmitoyl phosphatidylcholine show one phase change at about 32 °C, in between the transition temperatures of the individual phospholipids [23]. One would expect dibucaine to affect the phospholipase-catalyzed degradation of such composite liposomes at 32 °C in the same manner as it influences the hydrolysis at 24 °C of liposomes prepared from dimyristoyl phosphatidylcholine alone. Table I shows that at 32 °C the activity of pancreatic as well as *Naja naja* phospholipase A towards the mixture is strongly inhibited by 1 mM dibucaine. Since both dimyristoyl and dipalmitoyl phosphatidylcholine are degraded to appreciable extents in the control incubations without dibucaine, as determined by gas chromatography (results not shown), the hydrolysis of both phosphatidylcholines must have been inhibited by the anesthetic. However, when the two phospholipids are incubated separately with phospholipase A the degradation of dipalmitoyl phosphatidylcholine by the snake venom enzyme is stimulated by dibucaine, whereas the hydrolysis of dimyristoyl phosphatidylcholine is inhibited. The degradation of both dimyristoyl and dipalmitoyl phosphatidylcholine as catalyzed by the pancreatic enzyme is inhibited by the anesthetic. In these experiments, the results of which are presented in Table II, the concentration of dibucaine in solution was kept at exactly identical values for the two substrates used as described in the legend to Table II. In an equimolar mixture of dilauroyl and distearoyl phosphatidylcholine, cocrystallization of the paraffin chains does not occur; the mixture shows two phase changes of the individual phospholipids [24]. At a temperature in between the transition temperature of the two constituents the bilayer is composed of a gel phase consisting of almost pure distearoyl phosphatidylcholine and a liquid-crystalline phase of dilauroyl phosphatidylcholine only. The results, presented in Table III, indicate that in the presence of dibucaine the hydrolysis by *Naja naja* phospholipase A of the liquid-crystalline species is inhibited,

TABLE II

DEGRADATION OF DIMYRISTOYL AND DIPALMITOYL PHOSPHATIDYLCHOLINE LIPOSOMES BY PHOSPHOLIPASE A AT 30 °C IN THE PRESENCE OF IDENTICAL DIBUCAINE CONCENTRATIONS IN SOLUTION

Dimyristoyl and dipalmitoyl phosphatidylcholine liposome suspensions in 0.1 M Tris · HCl (pH 7.0), containing 1.0 mM $CaCl_2$, were equilibrated with respect to dibucaine by means of equilibrium dialysis (see Materials and Methods). Dimyristoyl phosphatidylcholine was present in the one compartment of a dialysis cell and dipalmitoyl phosphatidylcholine in the other, both at a concentration of 0.75 mM. Initially the dibucaine concentration in both compartments was 1.0 mM. No anesthetic was present in the controls. After equilibration at 30 °C the liposome suspensions were incubated for 60 min at 30 °C with phospholipase (final concentrations: pancreatic phospholipase, 3 μg/ml; *Naja naja* phospholipase, 10 μg/ml). As could be expected, at equilibrium the total amount of anesthetic in the compartment containing the fluid dimyristoyl phosphatidylcholine exceeded the amount in the compartment with the solid dipalmitoyl phosphatidylcholine by approx. 100 nmol.

Phosphatidylcholine species	Pancreatic phospholipase			*Naja naja* phospholipase		
	% Hydrolysis		Inhibition (%)	% Hydrolysis		Inhibition (%)
	Control	+Dibucaine		Control	+Dibucaine	
Dimyristoyl	73	9	87	50	12	76
Dipalmitoyl	31	14	55	27	78	−189

TABLE III

EFFECT OF DIBUCAINE ON THE PHOSPHOLIPASE-CATALYZED DEGRADATION OF DILAUROYL PHOSPHATIDYLCHOLINE/DISTEAROYL PHOSPHATIDYLCHOLINE LIPOSOMES

Liposomes were prepared from a mixture of dilauroyl and distearoyl phosphatidylcholine (molar ratio 1 : 1). Incubations were carried out at 20 °C under conditions as described in the legends of Figs. 3 and 4. The degree of hydrolysis of each phosphatidylcholine was determined by gas chromatographic analysis of the fatty acids from the lysophosphatidylcholine produced and from the remaining phosphatidylcholine.

Phospholipase	% Hydrolysis dilauroyl phosphatidylcholine		Inhibition (%)	% Hydrolysis distearoyl phosphatidylcholine		Inhibition (%)
	Control	+Dibucaine		Control	+Dibucaine	
Pancreatic	62	24	61	21	4	81
Naja naja	86	71	17	13	17	−31

whereas the degradation of the crystalline species is stimulated. Hydrolysis by the pancreatic enzyme of both the fluid as well as the solid species is inhibited by the anesthetic.

The non-identical response of the two phospholipases to dibucaine could suggest that the anesthetic interferes with one or both of the phospholipase activities at the enzyme level. On the other hand, it is well known that phospholipases may display different requirements with respect to the molecular packing of their substrates [21, 22, 25–27]. Consequently a change in the structure of the substrate brought about by foreign molecules may evoke dissimilar responses of different enzymes attacking the same substrate. Since the data of our earlier studies on this subject [13] as well as the results presented so far in this paper indeed favor a substrate-level localization of the dibucaine effects, we tried to establish further the possible relationship between the influences of the anesthetic upon phospholipase activities and its uptake by the substrate. The presence of a positively charged compound, like stearylamine, in the phosphatidylcholine bilayer will reduce the uptake of dibucaine, which carries a positively charged group as well. If the stimulation of the snake venom phospholipase and the inhibition of the pancreatic phospholipase at 10 °C is due to the interaction of dibucaine with the substrate, one would expect both effects to be reduced when stearylamine is present in the bilayer. If, on the other hand, an enzyme-level effect would be responsible for stimulation and inhibition, if anything, an enhancement of both would result when using positively charged liposomes due to a slight increase of the concentration of dibucaine in solution. The results, presented in Table IV, show that the incorporation of stearylamine into the liposomes reduces the inhibition by dibucaine of the pancreatic phospholipase A from 79 to 41 % and simultaneously reduces the stimulation of the snake venom enzyme from 175 to 52 %. Table IV also shows that stearylamine-containing liposomes do indeed bind lower amounts of dibucaine than those prepared from phosphatidylcholine alone, as is required for our hypothesis to be correct. The effect of relatively high $CaCl_2$ concentrations upon the phospholipase-inhibiting potency of dibucaine is essentially the same as the effect of the presence of stearylamine in the substrate bilayer. Dimyristoyl phosphatidylcholine liposomes were

TABLE IV

REDUCED EFFECT OF DIBUCAINE ON THE PHOSPHOLIPASE-CATALYZED DEGRADATION OF DIMYRISTOYL PHOSPHATIDYLCHOLINE/STEARYLAMINE LIPOSOMES IN COMPARISON WITH THE ANESTHETIC-AFFECTED HYDROLYSIS OF DIMYRISTOYL PHOSPHATIDYLCHOLINE ALONE

Liposomes were prepared from a mixture of dimyristoyl phosphatidylcholine and stearylamine in a molar ratio of 5 : 1. Incubations were carried out at 10 °C under conditions as described in the legends of Figs. 3 and 4. Lipid concentration, 0.75 mM with respect to phosphatidylcholine. The uptake of dibucaine by the liposomes at 10 °C was determined by means of equilibrium dialysis.

Substrate	Dibucaine taken up (nmol/μmol phosphatidylcholine)	Pancreatic phospholipase			*Naja naja* phospholipase		
		% Hydrolysis		Inhibition (%)	% Hydrolysis		Stimulation (%)
		Control	+Dibucaine		Control	+Dibucaine	
Dimyristoyl phosphatidylcholine	62	34	7	79	28	77	175
Dimyristoyl phosphatidylcholine/stearylamine	21	27	16	41	27	41	52

incubated at 10 °C with pancreatic phospholipase A in the presence of 1 mM dibucaine and varying amounts of $CaCl_2$. The results, presented in Table V, demonstrate that the inhibitory effect of dibucaine is slightly reduced by $CaCl_2$. Since the uptake of dibucaine by dimyristoyl phosphatidylcholine liposomes at 10 °C is decreased to similar extents, the antagonism by Ca^{2+} of the phospholipase inhibition can be ascribed completely to a lower anesthetic concentration in the substrate membrane, at least under the conditions employed in the present investigation.

TABLE V

INFLUENCE OF $CaCl_2$ ON THE BINDING OF DIBUCAINE TO DIMYRISTOYL PHOSPHATIDYLCHOLINE LIPOSOMES AND ON THE ANESTHETIC-AFFECTED DEGRADATION OF THE LIPOSOMES BY PANCREATIC PHOSPHOLIPASE A

Dimyristoyl phosphatidylcholine liposomes were incubated with pancreatic phospholipase A at 10 °C under the conditions as described in the legends of Figs. 3 and 4, except that the $CaCl_2$ concentration was varied. The uptake of dibucaine by the liposomes at 10 °C was studied by means of equilibrium dialysis.

$CaCl_2$ concentration (mM)	Dibucaine taken up (nmol/μmol phosphatidylcholine)	% Hydrolysis		Inhibition (%)
		Control	+Dibucaine	
0	63	–	–	–
1	60	26	7	73
5	56	26	8	69
10	53	26	9	65
20	48	26	10	62

DISCUSSION

Binding of local anesthetics to lipids has been observed by many investigators (for reviews see refs. 1 and 2). In most studies, however, lipid systems were used containing at least one negatively charged component, such as phosphatidylserine or cardiolipin. Interaction between lipid and anesthetic could, therefore, at least in part be ascribed to electrostatic attractive forces, the anesthetic carrying a positively charged tertiary amino group. With a molecule like phosphatidylcholine, which has no net charge, such an interaction would seem less likely to occur. In the present study, however, the uptake of the local anesthetic dibucaine by phosphatidylcholine liposomes was clearly demonstrated, providing indirect evidence that hydrophobic bonding predominates in this interaction. Moreover, the dibucaine-induced shift in the phase transition temperature of the phosphatidylcholine suggests that the hydrophobic attractive forces between the alkyl chains of the lipids are affected by the anesthetic.

From thermodynamic considerations on freezing point depression it can be predicted that, in case of dipalmitoyl phosphatidylcholine, a 4.4 % concentration of anesthetic in the bilayer (mol anesthetic/mol phosphatidylcholine $\times 100$ %) will cause a 1 degree downward shift in the phase transition temperature of the lipid. Hill proved this relationship to be valid for the alcohols *n*-octanol and *n*-nonanol [18]. However, our results indicate that the effect of dibucaine does not fit the theoretical relationship governing freezing point depression, $\Delta T = (RT^2/Q)\,(c_1 - c_2)$ [18], in which c_1 and c_2 are the concentrations of the solute in the liquid and in the solid, respectively, and Q the transition enthalpy. Having measured the uptake of dibucaine by dimyristoyl phosphatidylcholine above as well as below the phase transition temperature (Fig. 2) we calculate a ΔT of 2.1 °C, using 0.14 and 0.06 as values for c_1 and c_2, respectively, and 6.64 kcal/mol as a value for Q [33]. Alternatively, one might postulate that below the phase transition temperature the binding of dibucaine by the phosphatidylcholine is of a different nature in comparison with that above the transition temperature and possibly irrelevant with respect to the freezing point depression. In that case ($c_2 = 0$) one calculates a value for ΔT of 3.7 °C. However, we measured a ΔT of 5.0 °C (Fig. 1). Similar deviations from the ideal behaviour were observed by Cater et al. [31] for drugs like morphine and desipramine. As pointed out by these authors additional complications could be introduced owing to specific lipid-drug interactions. They even showed stearyl alcohol to increase rather than decrease the phase transition temperature of dipalmitoyl phosphatidylcholine [31].

Phosphatidylcholine bilayers in the liquid-crystalline state were shown to be more easily penetrated by dibucaine in comparison with bilayers in the gel state. In this respect the anesthetic resembles the spin label TEMPO (2,2,6,6,-tetramethylpiperidine-1-oxyl), which is expelled from lipid bilayers in the gel state, but readily taken up when the lipids are in the liquid-crystalline state [23]. The fluidity of membranes could appear to be a factor of major importance in determining whether anesthetics are excluded or taken up. It is well established that divalent cations may greatly influence the fluidity of lipid bilayers, particularly of those composed of negatively charged phospholipids [32, 33]. Consequently, the penetration of membranes by anesthetics might in part be controlled by divalent cations. The fluidizing effect of anesthetics together with the solidifying effect of Ca^{2+} upon negatively

charged phospholipids could at least in part account for the observed antagonism of local anesthetics and Ca^{2+} for lipid bilayers [30, 34].

A correlation could be established between the effect of the anesthetic upon the two phospholipase activities studied and its concentration in the substrate membrane (Tables IV and V). This relationship strongly favors our view, as presented previously, that the effect of dibucaine upon phospholipase activities results from its interaction with the substrate rather than with the enzyme. Two different effects of dibucaine upon the substrate can be distinguished. Firstly, a structural, fluidizing effect, as discussed above, and secondly, an electrostatic effect; intercalation of a positively charged anesthetic within the bilayer evokes a positive charge on the liposomal surface. Since the phosphatidylcholines studied are degraded at high rates near their phase transition temperatures (refs 21 and 22, this paper), the fluidizing effect of dibucaine, resulting in a lowering of the transition temperature, must be inhibitory due to widening of the gap between incubation temperature and transition temperature, if the incubation is carried out above the transition temperature of the pure lipid. This is what we found for both phospholipases (Figs. 3 and 4). However, at temperatures below the transition temperature dibucaine will rather decrease the gap between incubation temperature and transition temperature. On that basis stimulation of the phospholipase activities could be expected. This was indeed observed for the snake venom phospholipase (Fig. 3). However, the activity of the pancreatic enzyme towards dimyristoyl phosphatidylcholine is inhibited near 10 °C and hardly affected near 15 °C (Fig. 4). This can possibly be ascribed to a greater sensitivity of this particular phospholipase, in comparison with the other enzyme, for the presence of a positive charge on the substrate surface. The inhibition of the pancreatic enzyme near 10 °C may then be due to the electrostatic effect of dibucaine, predominating the stimulatory fluidizing effect, while near 15 °C the two effects are balanced. In this respect it should be emphasized that the anesthetic concentration in the lipid phase and, consequently, the surface charge density, are much higher at temperatures above the phase transition temperature of the phosphatidylcholine than below (Fig. 2). Therefore, in addition to the structural influences of the anesthetic, the electrostatic effect may contribute considerably to the observed phospholipase inhibition at temperatures above the phase transition temperature of the substrate. The sensitivity of the pancreatic enzyme for a positive charge on the substrate surface is suggested by the results presented in Table IV on the control incubations. The pancreatic enzyme is inhibited owing to the presence of the positively charged stearylamine in the substrate, whereas the snake venom enzyme is hardly affected. Other studies are in progress in order to further substantiate these observations.

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