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THE EFFECT OF LOCAL ANESTHETICS ON THE HYDROLYSIS OF FREE AND MEMBRANE-BOUND PHOSPHOLIPIDS CATALYZED BY VARIOUS PHOSPHOLIPASES

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SUMMARY

1. A number of local anesthetics related to procaine were shown to inhibit the hydrolytic activity of pure pancreatic phospholipase A towards membrane-bound phospholipids in mitochondria as well as in microsomes from rat liver and towards egg-yolk suspensions. With all substrates, the inhibitory activity decreased with increasing Ca^{2+} concentrations, and with egg yolk as a substrate we found good evidence of a true competitive effect of Ca^{2+} on the inhibition.

2. Under slightly variable conditions local anesthetics also inhibited phospholipase C from *Bacillus cereus* as well as endogenous mitochondrial phospholipase A and lipase activities.

3. Endogenous microsomal phospholipase A activity was stimulated rather than inhibited by the presence of nupercaine.

4. The inhibitory activity of a series of local anesthetics closely paralleled the anesthetic potency of the compounds.

5. Nupercaine, the most powerful inhibitor, was able to maintain respiratory control ratios of isolated rat-liver mitochondria at a constant high level for long periods of time. The effect of phospholipase A inhibition by local anesthetics on the stabilization of biological membranes is discussed and its possible involvement in the mechanism of local anesthesia is suggested.

6. The inhibitory effect of the anesthetics on the pancreatic phospholipase A is dependent on their uptake by the substrate particle: Sonicated liposomes prepared from egg-lecithin failed to take up nupercaine as was indicated by its ultraviolet absorbance spectrum, while their phospholipase A-catalyzed hydrolysis was not inhibited by nupercaine. We suggest that local anesthetics, particularly when applied in conjunction with pure phospholipases, may be useful tools for the detection of the phospholipid arrangement in biological membranes.

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INTRODUCTION

Local anesthetics of the procaine type have been shown to antagonize the binding of Ca^{2+} and other ions to phospholipids[1,2]. Kwant and Seeman[3] demonstrated that these anesthetics are also able to displace Ca^{2+} from erythrocyte membranes, while Scarpa and Azzi[4] found that the same compounds are competitive inhibitors of the binding of a number of mono- and divalent cations to submitochondrial particles. The displacement of Ca^{2+} from membranes in addition to the absolute requirement of various phospholipases for Ca^{2+} led us to investigate the effect of local anesthetics on a number of phospholipase activities.

Inhibition of a membrane-associated phospholipase A could theoretically lead to a decrease of the normally occurring level of lysophospholipids in such a membrane if transacylase activity (acyl-CoA:monoacyl-3-*sn*-glycerylphosphorylcholine acyltransferase) is available. Both phospholipase A[5–13] and transacylase activity[14–19] have been found in a number of biological membranes. In view of the lytic effect of lysophospholipids on biological[20] as well as artificial[21] membranes it would seem that a decrease of the amount of such compounds in a membrane would lead to decreased membrane permeability.

Although local anesthetics inhibit the increase in Na^+ and K^+ conductance which accompanies excitation of axon membranes[22] and although they were found to increase trans-membrane resistance of nervous membranes[23], Ohki[24] recently demonstrated that these compounds decrease membrane resistance of artificial bimolecular phospholipid membranes. This suggests that the mechanism through which these compounds induce anesthesia cannot be explained merely on the basis of their physical presence in the lipid phase of the membrane. An indirect involvement in membrane permeability, *e.g. via* prevention of lysophospholipid formation is, therefore, more likely to occur.

Partly on the basis of these considerations we thought it of interest to undertake the present study of the effect of local anesthetics on phospholipase activities.

While the experimental work described in this paper was in progress, we learned of the work of Seppälä *et al.*[25] who reported on the inhibition of phospholipase A-induced swelling of mitochondria by local anesthetics and related agents. Inhibition of swelling was accompanied by inhibition of phospholipid hydrolysis. These authors did not find inhibition of hydrolysis when the mitochondrial phospholipids were first solubilized by Triton X-100 treatment. They concluded that the inhibition of hydrolysis of membrane-bound phospholipid was to be ascribed to subtle modifications of the membrane structure, rendering the phospholipid less accessible for the enzyme.

In the present paper we confirm the inhibitory effect of local anesthetics on the action of some phospholipases and we provide evidence that this effect is due to the Ca^{2+}-displacing potency of these compounds. Furthermore the stabilizing influence of local anesthetics on mitochondrial membranes is demonstrated and discussed in view of their ability to block phospholipase A activity. In addition, some results are presented as to the condition of the substrate, required for the anesthetic to exert its inhibitory action. These data are discussed with special reference to the local anesthetics as auxiliary tools in membrane research. Parts of these results were presented at the 7th Meeting of the Federation of European Biochemical Societies[26].

METHODS

Sources of anesthetics and phospholipases

Samples of the local anesthetics procaine, lidocaine, cocaine, butacaine, tetracaine and nupercaine were gifts from Dr S. Luciani from the Department of Pharmacology, University of Padova, Italy. One 2-g sample of nupercaine was a gift from Ciba, A.G. (Basel, Switzerland). Pure pancreatic phospholipase A_2 (EC 3.1.1.4) was kindly donated by Dr de Haas and co-workers and pure phospholipase C (EC 3.1.4.3) from *Bacillus cereus* was generously supplied by Dr Zwaal and co-workers, both from this laboratory.

Preparation of cell fractions

Mitochondrial and microsomal fractions from rat liver were prepared from 10% homogenates in 0.25 M sucrose–1 mM EDTA–5 mM Tris–HCl (pH 7.4) by sedimentation at $1.5 \cdot 10^5$ and $6 \cdot 10^6$ $g \cdot$min, respectively. The fractions were washed once or twice. Radioactively labeled fractions were isolated similarly from rats injected intraperitoneally with 10 or 20 μCi [1,2-$^{14}C_2$]ethanolamine 15–18 h prior to sacrifice. Specific activities of phosphatidylcholine and phosphatidylethanolamine were approximately similar under those conditions, both in mitochondria and microsomes. Fractions were suspended in the isolation medium at final concentrations of 20–40 mg of protein per ml and were either used immediately or frozen and stored at −20 °C. Phospholipid-depleted mitochondria were prepared according to the method of Fleischer *et al.*[27] using a mixture of water, acetone and ammonia at 0 °C and washed twice with sucrose–Tris.

Phospholipase A assays

(a) With labeled subcellular fractions as substrates. Aliquots of labeled mitochondrial or microsomal suspensions, containing 10–15 mg of protein, were added to 0.25 M sucrose–5 mM Tris–HCl (pH 7.4), containing the indicated amounts of $CaCl_2$ and local anesthetics, the final volume being 2.0 ml. Pancreatic phospholipase A in amounts of 0.5–1.0 μg was added in 25 μl water and the mixture was incubated for the indicated period of time at 37 °C in a shaking water bath. The reaction was stopped by addition of 0.1 ml 0.3 M EDTA followed by 4.5 ml of methanol. The lipids were extracted and separated by thin-layer chromatography and the labeled spots were assayed for radioactivity. The extent of hydrolysis was calculated as the percentage of total radioactivity of each phospholipid class, recovered in the relevant monoacyl derivative. Hydrolysis of cardiolipin (diphosphatidylglycerol) was measured by phosphorus analysis of the substrate remaining.

Endogenous phospholipase activities were measured in a similar way with omission of the pancreatic enzyme. Incubation times were necessarily prolonged in these experiments.

(b) With egg yolk as a substrate. One egg yolk was suspended in 150 ml distilled water. 5 ml of this suspension was pipetted into a thermostated vessel, 0.4 ml 32 mM sodium deoxycholate was added followed by the desired amounts of $CaCl_2$ and local anesthetic, both as concentrated solutions and pH was adjusted to pH 8.0 with 0.1 N NaOH. The reaction was initiated by the addition of 5 μg pancreatic phospholipase in 50 μl water. The fatty acids liberated were titrated automatically at pH 8.0 under a

stream of nitrogen with 0.04 M NaOH with a Radiometer pH-stat equipment. Initial rates of hydrolysis were calculated from the graphs as μmoles of NaOH consumed per 10 min.

Chromatographic procedures

Known aliquots of lipid extracts were applied to 0.25 mm layers of silicagel H (Merck, Darmstadt) on glass plates. Phospholipids were separated with chloroform–methanol–15% ammonia (65:35:5, by vol). For separation of neutral lipids we used light petroleum–diethyl ether–formic acid (60:40:1.5, by vol.). The lipids were visualized with iodine vapour and the relevant spots were scraped from the plates for radioactivity measurements or, after elution with chloroform–methanol (1:2, by vol.), for phosphorus analysis.

Radioactivity assay

After thin-layer chromatography the radioactive compounds were scraped directly from the plates into scintillation vials, containing 18 ml of the dioxane–water scintillation mixture described by Snyder[28]. A Packard Tricarb Liquid Scintillation spectrometer was used for radioactivity measurement, applying the external standard method for quenching corrections.

Respiratory control

Mitochondria used for these experiments were isolated in a medium containing 0.225 M mannitol, 0.075 M sucrose, 0.5 mM EDTA and 3 mM morpholinopropane sulphonate (pH 7.4). Mitochondria were resuspended in the same medium at a concentration of 35 mg protein per ml in the absence or presence of 400 μM nupercaine and stored at 0–4 °C. After the intervals indicated a 0.1 ml sample was withdrawn from each tube. This sample was added to 2.4 ml of a medium containing 0.25 M sucrose, 15 mM morpholinopropane sulphonate (pH 7.4), 10 mM K_2HPO_4 and 3 μM rotenone. 8 mM sodium succinate was added to induce State 4 of respiration and then 280 μM ADP to induce State 3. Oxygen consumption was measured polarographically with a Clark-type electrode in a chamber thermostated at 24 °C and equipped with a stirrer, and monitored with a potentiometric recorder. Respiratory control ratios were calculated according to Chance[29] as the ratio of respiration in the presence of added ADP and the rate obtained upon ADP expenditure.

Other methods

Ultraviolet absorbance spectra of nupercaine were recorded on a Hitachi–Perkin Elmer doublebeam spectrophotometer, Model 356, at room temperature in 1-cm light-path cuvettes. The same instrument, equipped with a thermostated cuvette, was used to measure the rate of swelling of mitochondria by recording the change in absorbance at 540 nm. Protein was determined by the biuret method described by Gornall *et al.*[30]. Lipid extractions were carried out according to Bligh and Dyer[31] or by the method of Folch *et al.*[32], when complete extraction of cardiolipin was required. Phosphorus content of phospholipids was measured as described by Chen *et al.*[33], after destruction of the lipid according to the procedure of Ames and Dubin[34]. Liposomes were prepared from a mixture of 96% egg lecithin and 4% phosphatidic acid as described by de Gier *et al.*[35]. These multilayered liposomes were submitted for 10 min

to ultrasonic vibration with a Branson Sonifier, Model 575 in a sealed nitrogen-containing chamber which was kept in ice-cold water.

RESULTS

Both swelling and phospholipid degradation induced by pure phospholipase A from porcine pancreas[36] can be inhibited when sufficient amounts of local anesthetics are added prior to the phospholipase, as can be seen in Fig. 1. A close relationship

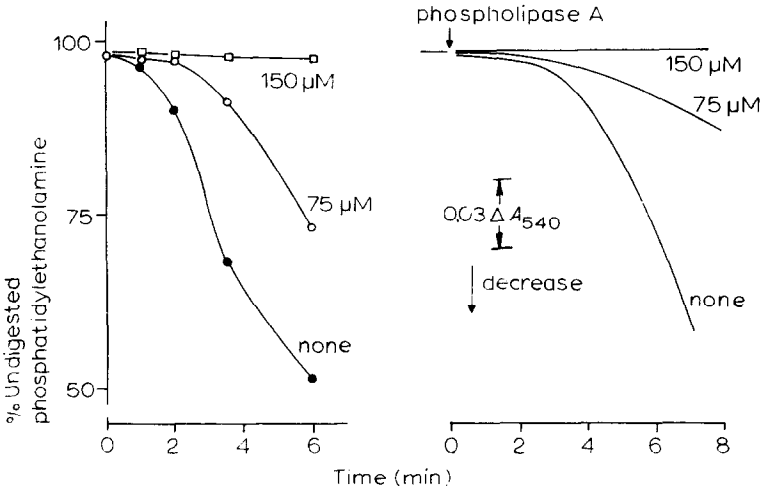

Fig. 1. Inhibition by nupercaine of phospholipase A-induced swelling of rat-liver mitochondria and hydrolysis of phospholipid. Mitochondria containing labeled phospholipids were incubated at a concentration of 1.8 mg of protein per ml at 24°C in a medium containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5.), 1 mM $CaCl_2$, 4 μM rotenone and 1.4 μg/ml antimycin A, either in presence or absence of the indicated amounts of nupercaine. Phospholipase A was added at a final concentration of 1 μg/ml and at the time intervals indicated a sample was withdrawn for assay of phospholipid hydrolysis. (See Methods.) Changes in absorbance were recorded continuously at 540 nm.

appeared to exist between the extents of inhibition of hydrolysis and of swelling, which justifies the use of mitochondrial-swelling measurements as an assay for the phospholipase-inhibiting potency of various local anesthetics, as is done in the following figure.

Although not apparent in the experiment described in Fig. 1 we repeatedly observed an actual decrease in the amount of monoacylphospholipids during incubation, particularly under conditions of complete inhibition of swelling. The presence of acyltransferase activity in mitochondria[15,16] probably accounts for this phenomenon.

Fig. 2 shows that the inhibitory activity of the series of local anesthetics used, decreased in the following order: nupercaine, tetracaine, butacaine, cocaine, lidocaine, procaine. For all these anesthetics a close parallelism was observed between the data of swelling and those of phospholipid digestion as is shown in Fig. 1. The anesthetic potency of these compounds[37,38] decreases in exactly the same order as their phospholipase-blocking capacity. When the inhibitory activities were measured with an egg-yolk suspension as a substrate we found the same sequence. This is shown in Table I. In this system higher concentrations of anesthetics were required to obtain

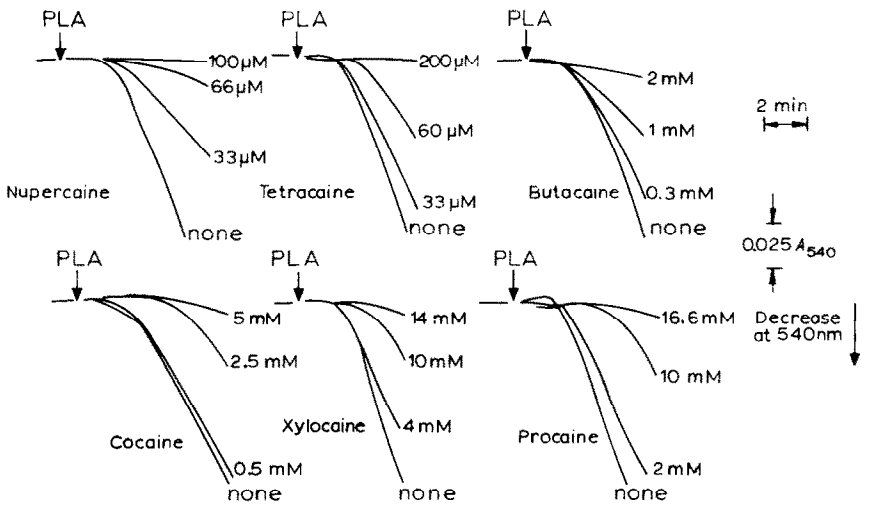

Fig. 2. Inhibition of phospholipase A-induced swelling of mitochondria by various local anesthetics. Mitochondria were incubated at 24 °C at a concentration of 1.3 mg of protein per ml in 2 ml of the medium described in Fig. 1. Local anesthetics were added as indicated and swelling was induced by addition of 0.5 μg of pancreatic phospholipase per ml and recorded as described. PLA = phospholipase A.

TABLE I

RELATIVE INHIBITORY ACTIVITY OF LOCAL ANESTHETICS ON THE DIGESTION OF EGG-YOLK PHOSPHOLIPIDS BY PANCREATIC PHOSPHOLIPASE A

Initial rates of hydrolysis of egg-yolk phospholipids by pancreatic phospholipase A_2 were measured by automatic titration of the fatty acids released as described in Methods. Ca^{2+} and local anesthetics were both added to final concentrations of 4 mM.

Anesthetic	% *Inhibition phospholipase* A_2
None (control)	0
Procaine	17
Lidocaine	25
Butacaine	39
Tetracaine	87
Nupercaine	98

the same extent of inhibition. This can be ascribed to the considerably larger amount of substrate and to the high Ca^{2+} concentration in this type of experiment. The effect of Ca^{2+} concentration on the inhibitory action of nupercaine is demonstrated in Fig. 3. Hydrolysis of mitochondrial and microsomal phosphatidylethanolamine and phosphatidylcholine, catalyzed by pancreatic phospholipase A, was measured in presence and absence of nupercaine with three different Ca^{2+} concentrations. The data on phosphatidylcholine which are not shown, are essentially similar. It is obvious that increase of Ca^{2+} concentration greatly reduces the nupercaine effect. The titration method with egg yolk as a substrate provided a suitable means to quantify these results. Initial rates of hydrolysis were measured with three different nupercaine concentrations and varying levels of Ca^{2+}. The results are presented in Fig. 4 as a double

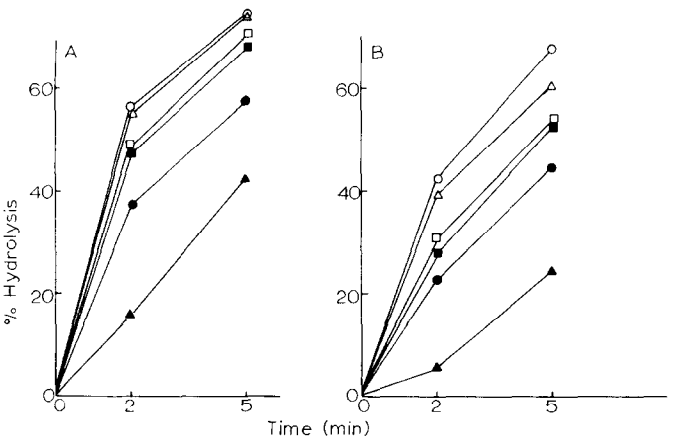

Fig. 3. Influence of Ca^{2+} concentration on inhibitory activity of nupercaine with mitochondria and microsomes as substrates. (A) Labeled mitochondria (13 mg of protein) were incubated for 2 or 5 min with 2.5 μg of pancreatic phospholipase A in presence or absence of 0.7 mM nupercaine and with various Ca^{2+} concentrations. (B) The same set of incubations under identical conditions with 9 mg of microsomal protein (labeled microsomes). The extent of hydrolysis of phosphatidylethanolamine was measured as described in the experimental part. Open symbols, no nupercaine added. Filled symbols, nupercaine added. Triangles, 1 mM $CaCl_2$; circles, 2 mM $CaCl_2$; squares, 5 mM $CaCl_2$.

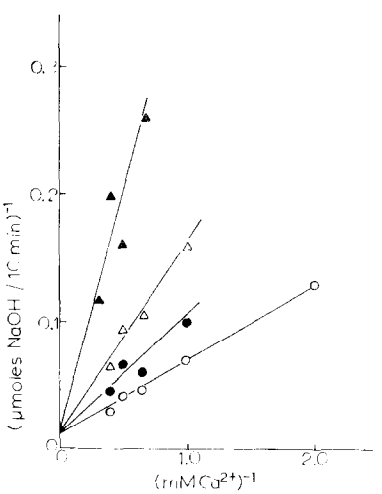

Fig. 4. Influence of Ca^{2+} concentration on inhibitory activity of nupercaine with egg yolk as a substrate. Pancreatic phospholipase A activity on an egg-yolk suspension was measured as described in the Methods section in presence of variable Ca^{2+} and nupercaine concentrations. ○—○, no nupercaine added; ●—●, 0.9 mM nupercaine; △—△, 1.8 mM nupercaine; ▲—▲, 3.6 mM nupercaine.

reciprocal plot. Although there is some scattering of the data, the figure is strongly indicative of a competition between the Ca^{2+} and the anesthetic.

Fig. 5 demonstrates the dependence of inhibitory activity on the nupercaine concentration. It is clear that for both types of membrane and for both phosphatidylcholine and phosphatidylethanolamine as well as the cardiolipin in the mitochondria

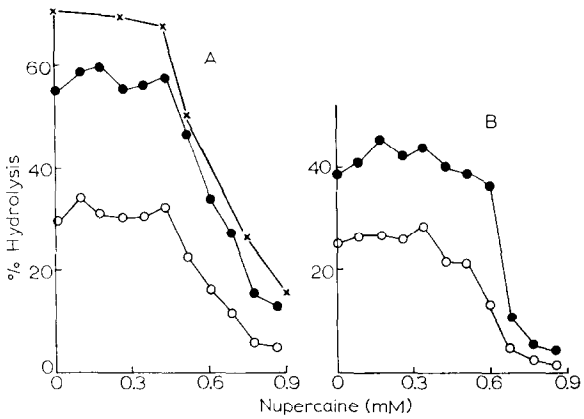

Fig. 5. Effect of nupercaine concentration on phospholipase A inhibition. (A) Labelled mitochondria (13 mg of protein) were incubated for 2 min as described in the experimental part with 2.5 μg of pancreatic phospholipase A in presence of 1 mM $CaCl_2$ and variable amounts of nupercaine. (B) Same experiment with 9 mg of microsomal protein. ○—○, hydrolysis of phosphatidylcholine; ●—●, hydrolysis of phosphatidylethanolamine; ×—×, hydrolysis of cardiolipin.

there is no inhibition of phospholipase activity at relatively low levels of anesthetic. If anything, the results rather seem to point to a slight stimulation of the hydrolytic activity up to a certain nupercaine concentration. Beyond that concentration there is a rapid decline in the extent of phospholipid hydrolysis. The concentration at which the nupercaine starts to inhibit was found to be dependent on the total amount of membrane present in the incubation mixture. For mitochondria this was at approx. 60 nmoles per mg protein as was measured spectrophotometrically at 325 nm.

The experiments so far described dealt with the pure pancreatic phospholipase A. Table II demonstrates that also the phospholipase A associated with the mitochondrial fraction proper[6,7] can be inhibited by nupercaine. The extent of inhibition in this case is not as great as when we measured the pancreatic enzyme. This can be ascribed to the relatively high Ca^{2+} concentration (6.7 mM) in this experiment. With lower levels of Ca^{2+} (1 mM) the mitochondrial phospholipase can be inhibited nearly completely. This is in striking contrast with the phospholipase which is found in the

TABLE II

EFFECT OF NUPERCAINE ON MITOCHONDRIAL PHOSPHOLIPASE A

Labeled mitochondria equivalent to 12 mg of protein were incubated for 30 or 90 min in presence of 6.7 mM $CaCl_2$ and the indicated amounts of nupercaine. Further details on the incubation procedure and the phospholipase A assay can be found in the Methods section.

Incubation time (min)	*Nupercaine concentration (μM)*	*% Hydrolysis*	
		Phosphatidyl-choline	*Phosphatidyl-ethanolamine*
30	0	6.9	17.8
30	50	6.0	20.8
30	100	6.2	17.2
30	200	4.9	12.2
90	0	20.4	62.5
90	50	19.6	56.6
90	100	15.6	49.6
90	200	13.1	44.8

microsomal fraction[5,6] as can be seen in Table III. The percentage of lysophospholipid formed is considerably increased by the addition of nupercaine. At conditions giving rise to almost 90% inhibition of the pancreatic phospholipase (see Fig. 5B) there is a nearly 100% increase in the formation of lysophosphatidylethanolamine by the endogenous microsomal enzyme. The microsomal phospholipase distinguishes itself from the pancreatic and mitochondrial enzymes in that it is specific for the 1-position of the substrate[7] and, probably more important, it does not require Ca^{2+}, but is rather inhibited by Ca^{2+} (ref. 39).

TABLE III

EFFECT OF NUPERCAINE ON MICROSOMAL PHOSPHOLIPASE A

Labeled microsomes equivalent to 0.5 mg of protein were incubated for 120 min in presence in 1 mM $CaCl_2$ and the indicated amounts of nupercaine. Experimental details are given in Methods.

Nupercaine concentration (mM)	% *Hydrolysis*	
	Phosphatidylcholine	*Phosphatidylethanolamine*
0	2.1	8.8
0.25	3.5	10.6
0.50	4.1	12.8
0.75	3.2	16.7
1.00	3.0	15.4
1.25	3.6	16.2

The inhibition of the mitochondrial phospholipase A by nupercaine can be usefully applied to the storage of isolated mitochondria. It is well known that isolated mitochondria, upon storage in sucrose media at 4 °C gradually loose their energy-linked functions as is indicated by a decrease in respiratory control ratio[40]. Fig. 6 demonstrates that nupercaine is capable of maintaining respiratory control for over three days at levels close to those in freshly isolated mitochondria. At 4 °C there is a small but significant increase in the amount of lysophospholipids (a few percent per day), which is nearly completely abolished when nupercaine is present (results not shown).

Recently, Zwaal *et al.*[41] in our laboratory, succeeded in obtaining a pure phospho-

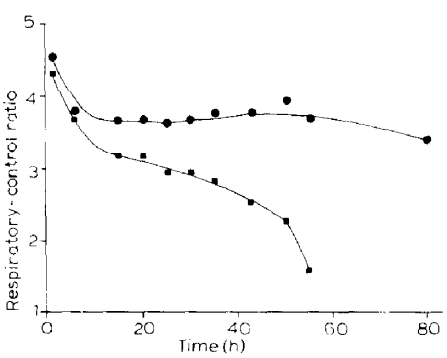

Fig. 6. Influence of nupercaine on respiratory-control ratios during cold storage of mitochondria. Mitochondria were incubated at 0–4 °C at a concentration of 35 mg protein per ml in absence or presence of 0.4 mM nupercaine. At the time intervals indicated respiratory control ratios were measured as described in Methods. ■—■, without nupercaine; ●—●, with nupercaine.

lipase C (EC 3.1.4.3) from *Bacillus cereus*. We thought it of interest to know whether this enzyme, which is stimulated by Ca^{2+}, is inhibited by the local anesthetics. The accumulation of 1,2-diglycerides after phospholipase treatment is evident from Fig. 7, as is the appearence of monoglycerides, undoubtedly due to lipase activity. In Lane A (control) the spot which approximately co-chromatographs with 1,2-diglyceride in Lane B probably is cholesterol rather than diglyceride, as judged from the reddish discoloration during charring. Nupercaine, present during the incubation with phos-

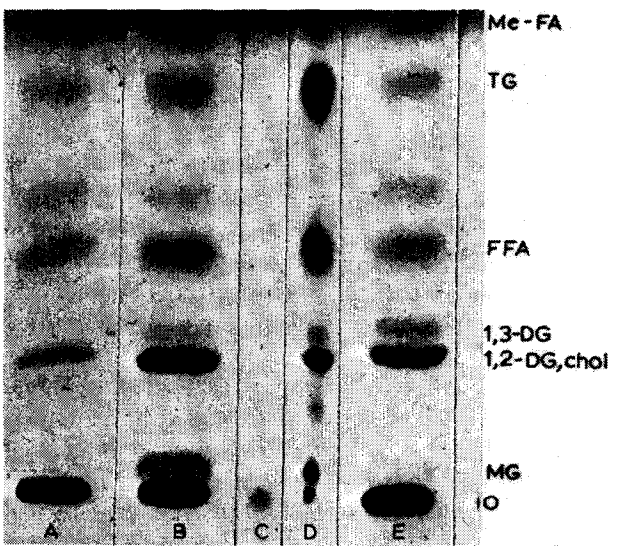

Fig. 7. Effect of nupercaine on phospholipase C-catalyzed hydrolysis of mitochondrial phospholipids. 13 mg of mitochondrial protein were incubated for 2 min at 37 °C with 0.5 μg of phospholipase C in a final volume of 2.0 ml sucrose–Tris (pH 7.4). Ca^{2+} was added to a final concentration of 2 mM. The reaction was stopped by addition of 50 μl of 0.3 M EDTA and the mixture was immediately extracted according to Bligh and Dyer[31]. Identical aliquots of the lipid extracts of each incubation mixture were chromatographed as described. After development and evaporation of the solvent the chromatograms were sprayed with 20% sulfuric acid and charred. A, control incubation without enzyme; B, incubation with enzyme; C, nupercaine; D, standard mixture of glycerides and fatty acid; E, as B with addition of 0.8 mM nupercaine; O, origin; MG, monoglycerides; DG, diglycerides; FFA, free fatty acids; TG, triglycerides; Me-Fa, methylated fatty acids; chol, cholesterol.

pholipase C, was not able to prevent the accumulation of considerable amounts of diglycerides. Monoglyceride production however, was nearly completely blocked. Quantitative experiments with labeled mitochondria showed that at 2 mM Ca^{2+} the extent of inhibition by nupercaine is larger than is suggested by the chromatogram, but not as pronounced as under the same conditions with phospholipase A. At low Ca^{2+} concentrations, however, nupercaine was able to block phospholipase C completely, as is demonstrated in Table IV. The concentration at which nupercaine starts to inhibit is comparable to that for phospholipase A.

Preliminary experiments suggest that response to nupercaine varies with the nature of the substrate. Although the hydrolysis of egg lecithin is readily blocked by nupercaine when the lecithin is present as a yolk suspension (Table I and Fig. 4) no inhibition was observed when sonicated liposomes of purified egg lecithin were used

TABLE IV

INHIBITION OF PHOSPHOLIPASE C BY NUPERCAINE AT LOW Ca^{2+} CONCENTRATION

Mitochondria labeled with [^{14}C]ethanolamine were incubated for 3 min at 37°C at a concentration of 3.4 mg of protein per ml sucrose–Tris pH 7.4 with 0.25 units of phospholipase C (approx. 0.25 μg). $CaCl_2$ was added to a final concentration of 15 μM and nupercaine as indicated. Hydrolysis was assayed from the amounts of radioactivity in the undigested phospholipids.

Nupercaine concentration (mM)	% *Hydrolysis*	
	Phosphatidylcholine	*Phosphatidylethanolamine*
0	35.0	32.5
0.13	37.1	34.7
0.27	14.0	20.3
0.40	1.5	4.0
0.54	−4.8	0.7
0.80	−2.6	−4.8

as a substrate for pancreatic phospholipase, either in presence or absence of sodium deoxycholate. Fig. 8 indicates that this is possibly due to a lack of uptake of the nupercaine in the lipid phase of the membrane. The ultraviolet spectrum of nupercaine is dramatically altered by the presence of mitochondrial or microsomal membranes, presumably due to an interaction of the apolar part of the molecule with the hydrophobic part of the membrane. This change is greatly limited when sonicated liposomes of the type described by Huang[42] prepared from egg lecithin are added to the nupercaine solution in an amount which is equivalent to or even exceeds the amount of phospholipid present in the added aliquots of mitochondria or microsomes. The spectral change was independent of the Ca^{2+} concentration, but is greatly reduced when

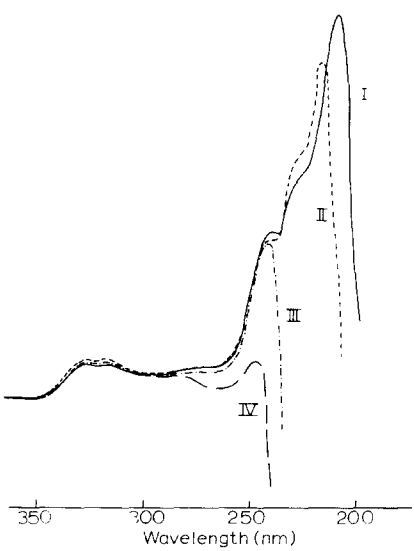

Fig. 8. Influence of various additions on the ultraviolet absorbance spectrum of nupercaine. I, 20 μM nupercaine in 0.25 M sucrose; 5 mM Tris·HCl (pH 7.4); II, as I, in presence of 0.4 mg/ml sonicated lecithin–phosphatidic acid liposomes; III, as I, in presence of mitochondria (0.5 mg of protein/ml); IV, as I, in presence of microsomes (0.5 mg of protein/ml).

lipid-depleted rather than intact mitochondria are added to the nupercaine (Fig. 9). This suggests that the spectral change is mainly due to the lipid constituents of the membrane.

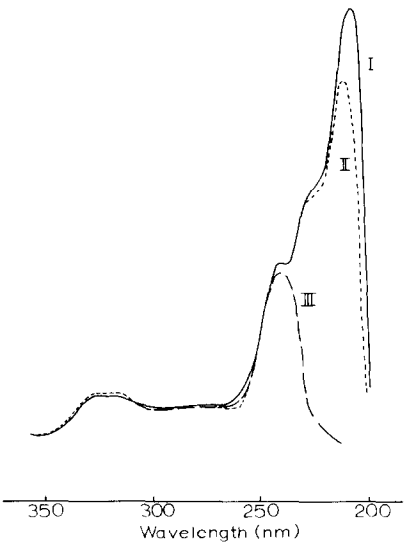

Fig. 9. Effect of lipid-depleted mitochondria on the ultraviolet absorbance spectrum of nupercaine. I, 20 μM nupercaine in 0.25 M sucrose; 5 mM Tris–HCl (pH 7.4); II, as I, in presence of acetone-extracted mitochondria[27] at a concentration of 0.4 mg of protein per ml; III, as I, in presence of intact mitochondria at a concentration of 0.5 mg of protein per ml.

DISCUSSION

Our experiments demonstrate that the inhibition by local anesthetics of phospholipases possibly is achieved through a displacement of Ca^{2+} from the membrane. Ca^{2+} is required by the pancreatic as well as the mitochondrial phospholipase, both of which can be blocked by local anesthetics. De Haas *et al.*[43] provided good evidence that during hydrolysis of short-chain lecithins by pancreatic phospholipase A Ca^{2+} randomly binds to either the enzyme alone or to the enzyme–substrate complex, whereas direct binding of Ca^{2+} to the substrate was not shown to occur. It is not certain that the same sequence of events takes place in case the substrate is part of a complex system such as a biological membrane or a yolk suspension. However, our results suggest that the presence in the membrane of the positively charged anesthetics ultimately inhibits the formation of the enzyme–Ca^{2+}–substrate complex and thus hydrolysis. Although preliminary experiments have suggested that nupercaine also inhibits hydrolysis of micellar dioctanoyllecithin, further experiments have to be done in order to ascertain a true competitive effect with Ca^{2+} when this substrate is used.

The significance of the slight stimulation of phospholipase A activity at low nupercaine concentrations, if at all real, remains as yet a matter of speculation. It could be a reflection of an entirely different action of nupercaine, such as modifying the substrate density and thus facilitating the accessibility of the substrates[44,45]. A similar phenomenon might explain the stimulating effect of nupercaine on the micro-

somal phospholipase. However, the reported Ca^{2+} inhibition of this enzyme[39] would suggest that the Ca^{2+}-displacing capacity of the nupercaine could also contribute to the stimulation, in a way similar to the inhibition of the Ca^{2+}-requiring enzymes. The different response to nupercaine, at mM Ca^{2+} levels, of phospholipase C, as compared to the phospholipase A is likely to be related to the modest Ca^{2+} requirement of the former enzyme. With mitochondria as a substrate, 15 μM Ca^{2+} was sufficient to obtain maximal activity, whereas for phospholipase A the optimal Ca^{2+} concentration was between 1 and 2 mM. On the other hand, Bangham and Dawson[46] reported some years ago that certain long-chain cations could, to a certain extent, replace Ca^{2+} as activator of phospholipase C from *Clostridium perfringens*. Nupercaine somewhat resembles these long-chain cations and a stimulatory effect as described by Bangham and Dawson[46] might counteract the Ca^{2+}-displacing activity of nupercaine which leads to inhibition. An absolute Ca^{2+} requirement for the mitochondrial lipase is difficult to assess in our system, since we measured activity with diglycerides as a substrate, which are locally generated by the Ca^{2+}-requiring phospholipase C. Waite and van Deenen[39] found no effect of EDTA on triglyceride hydrolysis by a mitochondrial lipase, whereas the microsomal lipase in their hands was clearly stimulated by EDTA. Both enzymes yielded diglycerides and fatty acids as their products. Guder *et al.*[47] found no lipase activity at all in mitochondria when using tributyrin or triolein as substrates, but high activity at pH 5.0 in purified lysosomes and at pH 8.5 in microsomes, while Ca^{2+} was slightly inhibitory. It remains to be established which of these enzymes if any, is identical to the one that produces monoglycerides in our system.

The inhibition of phospholipase activity by local anesthetics apparently has a stabilizing effect on the membrane, as was shown by the inhibition of mitochondrial swelling and, even more so, by the preservation of oxidative phosphorylation in terms of respiratory-control levels. The accumulation of free fatty acids in mitochondria[48] which can be ascribed to the presence in these particles of phospholipase A^{5-7} is often held responsible for the uncoupling of oxidative phosphorylation during storage. On the other hand, exogenous fatty acids were shown to stimulate mitochondrial phospholipase A activity[49]. In addition to this, albumin, which can complex fatty acids, protects considerably less effectively against loss of respiratory control than does nupercaine[50]. These observations suggest that rather than the accumulation of fatty acids it is the very conversion of diacyl- to monoacylphospholipids and, possibly, the accumulation of the latter, which is responsible for the deleterious effect of phospholipase action on membranes.

The residual phospholipase A activity, observed during ageing at 4 °C in presence of EDTA can probably be explained on the basis of the observation by Azzi and Chance[51] that nM amounts of Ca^{2+} are retained in mitochondria under these conditions.

Lysophospholipids can cause lysis of red cells as well as of artificial bilayered phospholipid membranes[20] and can decrease the electrical resistance of the latter[21], both phenomena being indicative of increased membrane permeability. Furthermore, phospholipase A^{5-13} as well as acyltransferase[14-19] activities have been shown to be associated with a variety of membrane structures. The concerted action of these two types of enzyme conceivably governs the concentration of lysophospholipids in a given membrane, and thus its permeability properties (*cf.* Van Deenen *et al.*[52]). By blocking the deacylation with a local anesthetic, without interfering with the acylating

mechanism, the delicate balance between mono- and diacylphospholipids in the membrane would be disturbed, giving rise to a decrease in permeability. Such a decrease of membrane permeability towards Na^+ and K^+ has been shown to occur both in resting and in excited nerve upon administration of local anesthetics[53]. We feel that the demonstration of phospholipase A inhibition by local anesthetics combined with some well-established data from the literature, justify to postulate an involvement of phospholipase A inhibition in the pharmacological action of local anesthetics. This view is supported by the close relationship between anesthetic potency and inhibitory activity towards phospholipase A of a series of six local anesthetics. Not only is the order in which these properties decrease the same, but also in a quantitative sense are they related.

The shift which takes place in the ultraviolet spectrum of nupercaine when it is mixed with egg-lecithin liposomes is not nearly as dramatic as when equivalent amounts of mitochondrial or microsomal membranes are added. This is tentatively taken to indicate that the anesthetic is not taken up by the lipid vesicles to the same extent or in the same way as it is by the subcellular membranes. This presumed lack of uptake could easily explain why the nupercaine does not inhibit phospholipase A degradation of this type of vesicles. That the spectral shift in case of mitochondria or microsomes can be attributed to the lipid part of the membranes is suggested by the lack of influence of acetone-extracted mitochondria on the nupercaine spectrum, although it is quite possible that it is the protein which aids to make the lipid phase accessible to the nupercaine. On the other hand, it could be speculated that, unlike in the sonicated vesicles, the lipid constituents of mitochondrial and microsomal membranes are not in the closely packed configuration of a continuous bimolecular layer.

Summarizing these results, we may state that an approach using the combined application of local anesthetics and pure phospholipases holds promising possibilities for future work on the resolution of membrane structure, since it may provide valuable information as to the manner in which individual phospholipid molecules make up the integral lipid phase of biological membranes.

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