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Effect of Local Anesthetics on Phospholipases from Mitochondria and Lysosomes. A Probe into the Role of the Calcium Ion in Phospholipid Hydrolysis†

Moseley Waite* and Patricia Sisson

ABSTRACT: Local anesthetics which contain a tertiary amine have marked effects on solubilized and membrane bound phospholipase A_2 of rat liver mitochondria. The compounds with greatest influence, dibucaine and butacaine, had opposite effects; at 10–50 μM dibucaine stimulated whereas butacaine inhibited. At higher concentrations (200–300 μM) dibucaine inhibited whereas butacaine stimulated which indicates that local anesthetics might have more than one mechanism of action. Studies on the mechanism of inhibition by dibucaine led to the following conclusions. (1) The substrate phosphatidylethanolamine does not bind dibucaine tightly enough to account for the inhibition. (2) The inhibition is uncompetitive, with respect to substrate. (3) The inhibition is greatest during the initial part of the reaction which suggests that the availability of the active site of the enzyme to the 2-acyl ester of phosphatidylethanolamine is reduced. This does not imply, however, that the physical interaction of the enzyme with the liposome is decreased when dibucaine is present. (4) Under the appropriate conditions, Ca^{2+} protects against inhibition by dibucaine, but Ca^{2+} concentrations higher than 1 mM are also inhibitory which suggests a common binding site for the two compounds on the enzyme. Lysosomal phospholipases A_1 and A_2 were also inhibited by either dibucaine or Ca^{2+} which further suggests the mechanism of inhibition by dibucaine is similar to that found with the mitochondrial enzyme. Dibucaine causes a severalfold increase in the hydrolysis of phosphatidylethanolamine of mitochondria in the absence of added Ca^{2+}. On the other hand, when the membrane-bound enzyme was fully stimulated by Ca^{2+}, dibucaine inhibited. These results are interpreted as being the result of two effects of dibucaine; one, dibucaine displaces Ca^{2+} from the membrane which makes it more available to the phospholipase, and two, when sufficient Ca^{2+} is available for maximal activity, dibucaine inhibits.

Nearly a decade ago Lehninger and his coworkers (1964) established that in mitochondria there is relationship between lipolytic activity and structure-linked functions such as respiratory control. More recently the phospholipase A_2 of mitochondria (EC 3.1.1.4) responsible for this lipolytic activity was described and its involvement in mitochondrial swelling was more clearly defined (Waite *et al.*, 1969b). This phospholipase is located primarily in the outer membrane (Nachbaur and Vignais, 1968; Waite, 1969) although some activity has been found in the inner membrane (Waite, 1969). Extraction of the mitochondria with ammoniacal acetone solubilized the enzyme which has now been partially purified and characterized (Waite and Sisson, 1971).

Recent work by Seppala *et al.* (1971) and Scherphof and Scarpa (1972) showed that local anesthetics such as dibucaine and butacaine inhibit both the phospholipase A_2 activity of the venom from *Crotalus adamanteus* and of pancreas, and the hydrolysis of mitochondrial phospholipid catalyzed by the mitochondrial phospholipase A_2. It was observed also that dibucaine competes with Ca^{2+} for binding sites on membranes (Scarpa and Azzi, 1968; Kwant and Seeman, 1969) and to phospholipids (Blaustein, 1967). The question arose: does dibucaine inhibit the phospholipase A_2 directly or is the effect primarily on the mitochondrial membrane and only secondarily

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on the mitochondrial phospholipase A_2? We decided to investigate further the mechanism of action of local anesthetics on phospholipase A_2 of mitochondria both in the intact mitochondria and in the lipid-free state. Also, we were able to compare the effect of dibucaine on the lysosomal phospholipases A_1 and A_2 which are inhibited by Ca^{2+} with the mitochondrial enzyme which required Ca^{2+}. In this communication we report the results of studies on the hydrolysis of phosphatidylethanolamine, the substrate most rapidly hydrolyzed by these enzymes.

Experimental Section

The mitochondrial phospholipase A_2 was solubilized by extraction of rat liver mitochondria with ammoniacal acetone (Waite and Sisson, 1971). Unless otherwise noted, the phospholipase referred to hereafter is this solubilized enzyme. The phospholipases A_1 and A_2 from the soluble fraction of lysosomes (Franson *et al.*, 1971) were generously donated by R. Franson of this laboratory. The reaction mixtures contained, unless otherwise noted, 150 mM Tris buffer (pH 8.0), 2mM $CaCl_2$, 50 μM (15,000 cpm) 2-[^{14}C]linoleoylphosphatidylethanolamine (added as an 0.25 mM ultrasonic suspension in H_2O), and 0.2–0.4 mg of soluble mitochondrial enzyme in a total volume of 1.0 ml. When an aqueous solution of anesthetic was included, the substrate was the last addition (except for the enzyme) so the substrate contacted the $CaCl_2$ and anesthetic at the same time. The mixture was incubated 10 min at 37°, the enzyme was added, and the incubation continued for 10 min. In preliminary experiments we found that the time of the first incubation had no effect on the enzymatic reaction but was kept at 10 min for uniformity. The conditions for assay of the lysosomal enzyme were the same except the buffer was 100 mM sodium acetate (pH 4.0) and 2 mM EDTA replaced the $CaCl_2$, and for the indicated amount of enzyme protein.

Assays of the membrane-bound mitochondrial enzyme are previously reported (Waite *et al.*, 1969a). Mitochondria labeled with [^{14}C]ethanolamine were resuspended in 125 mM KCl containing 10 mM Tris buffer (pH 7.4) and incubated at 37° at a concentration of 5.6 mg of protein/ml (15,000 cpm of radioactive phospholipid).

The reaction products were extracted and separated by thin-layer chromatography on silica gel G plates (Waite and van Deenen, 1967). When phosphatidylethanolamine with the label in the fatty acid was the substrate, the chloroform–petroleum ether (bp 30–60°)–acetic acid system (65:35:2, v/v) and the chloroform–methanol–H_2O system (65:35:4, v/v) were used sequentially to separate the products. Chloroform–methanol–H_2O–NH_4OH system (65:36:3, v/v) was used when [^{14}C]ethanolamine-labeled phosphatidylethanolamine in intact mitochondria was the substrate. The silica gel containing the radioactive compounds (located with I_2 vapor) was scraped into scintillation vials and counted.

The 2-[^{14}C]linoleoylphosphatidylethanolamine (specific activity 3×10^5 cpm/μmole) was prepared as described earlier (Waite and van Deenen, 1967) except rat liver microsomes were the source of the acyl-CoA–monoacyl phosphoglyceride acyl transferase rather than the whole liver homogenate.

[1,2-$^{14}C_2$]Ethanolamine (specific activity 9.6 μCi/μmole) was purchased from New England Nuclear Corp., Boston, Mass. Dibucaine and procamide were purchased from K & K Laboratories, Plainview, N. Y. Sigma, St. Louis, Mo., was the source of procaine and butacaine; Eastman Organic Chemicals, Rochester, N. Y., supplied the *N,N*-diethyldecylamine. Lidocaine was kindly given by Astra Pharmaceutical Products, Worcester, Mass.

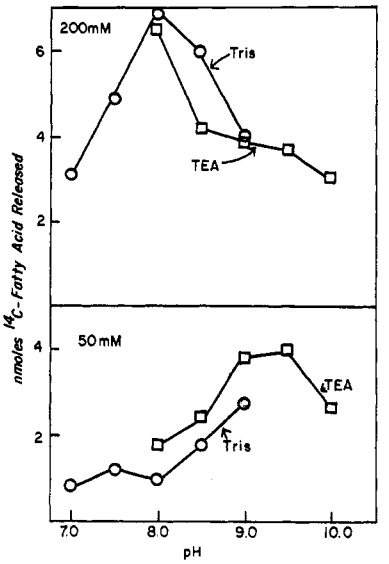

FIGURE 1: The effect of buffer concentration and pH on phospholipase A_2 activity of the soluble enzyme. The assay conditions are described in the Experimental Section, except for the variation in buffer concentration and pH.

Results

Effect of Local Anesthetics on the Soluble Phospholipase A_2 of Mitochondria. Preliminary experiments established conditions to best study the effect of anesthetics on phospholipase A_2 activity. Increased buffer concentration enhanced activity between pH 7.0 and 8.5 (Figure 1), but had less influence on activity at higher pH values. A Tris concentration of 0.15 M (pH 8.0) is optimal for the solubilized enzyme and 0.20 M was optimal for the enzyme in the mitochondria (not shown). The optimal conditions for the soluble enzyme were used for measurement of its activity unless otherwise noted. Scherphof and van Deenen (1965), and Bjørnstad (1966) reported optimal activity at pH 7.5–8.5; we reported optimal activity at pH 9.5 (Waite and Sisson, 1971). In those experiments we used low buffer concentrations which accounts for the difference in the reported optimal pH.

Five anesthetics, all of which contain a tertiary amine moiety (Figure 2), were tested at several concentrations as possible inhibitors (Figure 3). Lidocaine, procaine, and procainamide inhibit slightly. The other two, dibucaine and butacaine, have pronounced and apparently opposite effects under these conditions. Butacaine, at low concentrations inhibits; higher concentrations causes a twofold stimulation. Conversly, dibucaine stimulates activity at low concentrations and inhibits at higher concentrations. It is obvious from these experiments that the effects of anesthetics are complex and must depend upon a number of characteristics of the anesthetic molecule. The maximal inhibition by dibucaine was usually about 75%, but in a few experiments was more than 90%. The range of maximal inhibition observed was 60 to 92%. Generally, dibucaine stimulates and butacaine inhibits at an anesthetic concentration similar to that of the substrate (50 μM) although this varied somewhat between experiments. Further, procaine, and procainamide inhibit maximally at this low concentration.

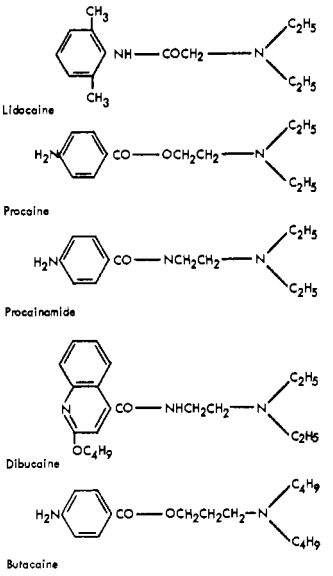

FIGURE 2: Structures of local anesthetics.

The effect of dibucaine is dependent upon the pH of the assay media (Figure 4). At pH 7.0 and 8.0 there is a pronounced inhibition by dibucaine, whereas pH 9.0, little, if any effect was observed. Although no precipitate was observed at this pH value, it is possible that dibucaine is insoluble at pH 9.0 and therefore would be ineffective as an inhibitor.

Inhibition of the reaction by dibucaine is greatest at short incubation times. As seen in Figure 5, and as shown before (Waite and Sisson, 1971), hydrolysis is linear for 4–6 min only. In the presence of dibucaine, however, there is several-fold inhibition during the first 10 min. The difference in activity noted between the control and inhibited enzyme is proportionately less at the longer (20–30 min) incubation periods.

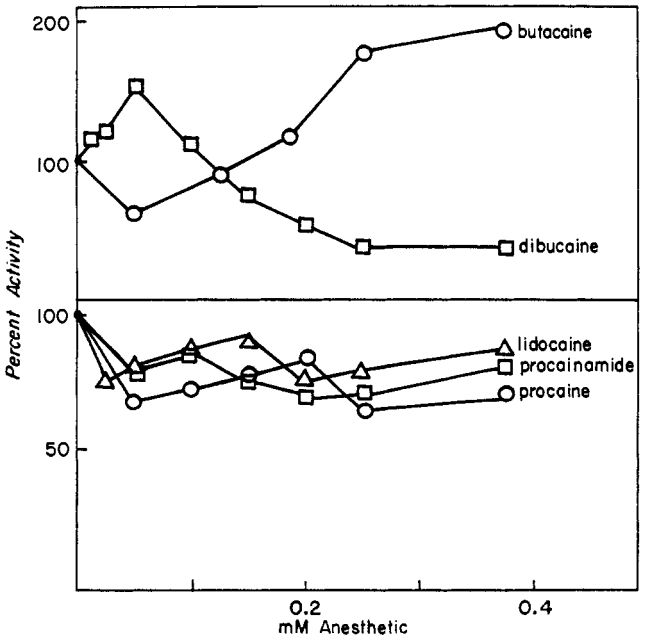

FIGURE 3: The effect of local anesthetics on the activity of the soluble phospholipase A_2. Incubations were run as described in the Experimental Section with the indicated concentrations of the various anesthetics.

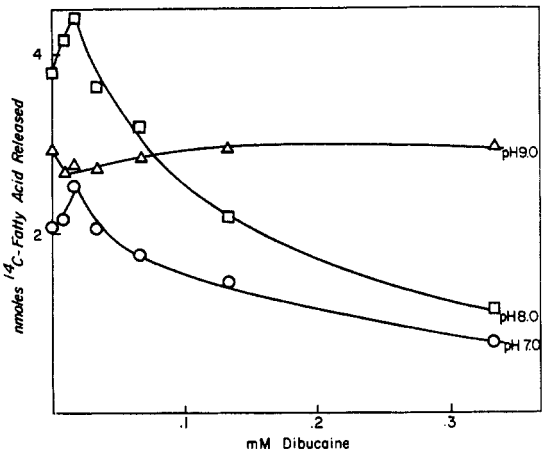

FIGURE 4: Inhibition of the soluble phospholipase A_2 by dibucaine at various pH values. The assay conditions are described in the Experimental Section except for the indicated variation on the pH of the Tris buffer (150 mM).

These results indicate that the main effect of dibucaine is to reduce the initial rate of hydrolysis, but apparently allows the reaction to continue for a longer period of time. Presumably the substrate available to the enzyme in the liposome is less rapidly used up when dibucaine is present. Butacaine also causes a decrease in the apparent initial rate of hydrolysis (Figure 6), although the effect is less marked than with dibucaine. Since the reaction proceeds longer with than without butacaine and since the reduction in initial rate is not as great as found with dibucaine, butacaine causes an overall increase in the accumulation of products. These data reinforce the conclusion (Figure 3) that local anesthetics have more than one role in the reaction and the effects should be studied separately. Accordingly, we have selected conditions which lead to inhibition with dibucaine for most of the following studies.

Inhibition of the reaction by dibucaine was not reversed by increasing the concentration of the substrate. Figure 7 shows that dibucaine inhibited the reaction at all concentrations of substrate that were used. Actually, with 0.33 mM dibucaine, there is greater inhibition at the higher concentrations of substrate. The data, when presented in a double-reciprocal plot

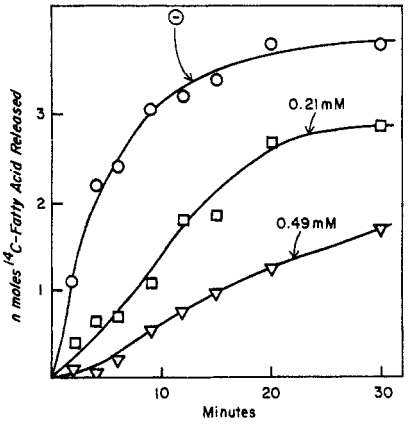

FIGURE 5: Inhibition by dibucanine of the soluble phospholipase A_2 at increasing incubation times. The conditions are described in the Experimental Section except for the time of incubation which was changed as indicated. The concentrations refer to dibucaine; ⊖ designates no dibucanine.

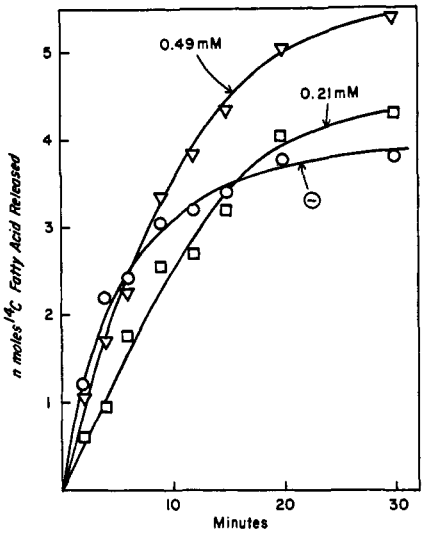

FIGURE 6: Butacaine inhibition of the soluble phospholipase A_2. The assay conditions are described in the Experimental Section except for the variation of time and the addition of the indicated concentrations of butacaine.

(Figure 8), show that dibucaine does not compete with the substrate, rather, the inhibition appears to be uncompetitive with respect to the substrate (Webb, 1963). This simple relationship does not hold at higher inhibitor concentrations, however (Figure 7).

There are at least two possible explanations for these results. If dibucaine is bound to the liposome, it could prevent Ca^{2+} from binding to the liposome or, on the other hand, if the inhibitor is bound to the enzyme it could prevent Ca^{2+} from interacting with the enzyme or enzyme–substrate complex. These two possibilities are suggested since local anesthetics have been shown to bind to phospholipids (Blaustein, 1967) and to displace Ca^{2+} from membranes (Scarpa and Azzi, 1968; Kwant and Seeman, 1969), even though little Ca^{2+} is bound to phosphatidylethanolamine (Blaustein, 1967). We tested first of these possibilities by incubating the sonicated substrate with dibucaine, sedimenting the liposomes to remove the nonbound anesthetic, and resuspending and resonicating the substrate. The concentrations of dibucaine

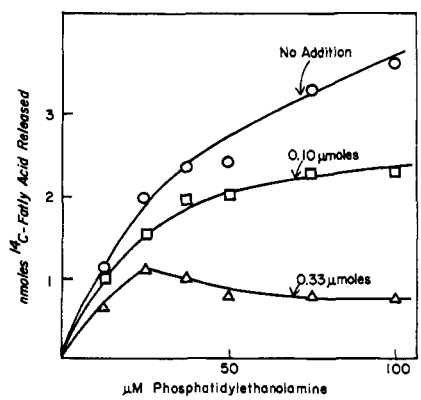

FIGURE 7: The lack of reversibility of dibucaine inhibition by high substrate concentrations. The assay conditions, except for the indicated variation in substrate concentration and the addition of the indicated amount of dibucaine, are described in the Experimental Section.

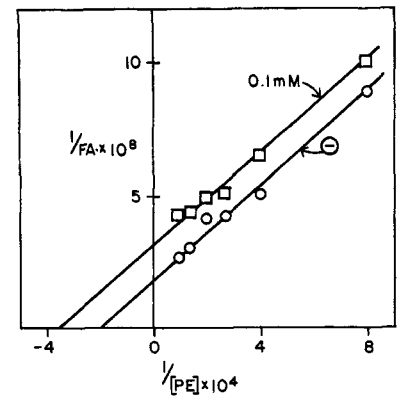

FIGURE 8: Double-reciprocal plots of the data from Figure 7 without and with 0.1 μmole of added dibucaine.

were chosen such that if all the dibucaine had been bound to the substrate, the final concentration of dibucaine would have been 0.16 mM. The substrate treated with dibucaine in this manner was hydrolyzed to the same extent as that not treated or that which was treated in the same manner except dibucaine was omitted (Table I). Dibucaine (0.16 mM) inhibits the hydrolysis of the three samples to the same extent when added to the assay mixture, however. Dibucaine, therefore, is not bound tightly to the substrate, although some binding cannot be excluded by these experiments.

We next examined the second possibility, namely, that dibucaine prevents Ca^{2+} from interacting with enzyme. This is complicated since dibucaine stimulates the activity at low concentrations and Ca^{2+} inhibits at high concentrations (Figure 9). High concentrations of Ca^{2+} presumably would

TABLE I[a]

Treatment of Substrate	Dibucaine in Assay	% of Control
A. Not Centrifuged	−	100
	+	38
B. Centrifuged	−	109
	+	48
C. Centrifuged with dibucaine	−	106
	+	38

[a] A study of the possible binding of dibucaine to liposomes of phosphatidylethanolamine. Six milliliters of a solution which contained 1.5 μmoles of [^{14}C]phosphatidylethanolamine were incubated for 15 min without (B) or with (C) 4.0 μmoles of dibucaine. The solutions were then centrifuged at 22,000g for 30 min. The supernatant liquid was decanted and 6.0 ml of H_2O was added to the sediment and the phosphatidylethanolamine was resuspended by sonification. About 50% of the substrate in both B and C was recovered following this treatment. A sample was taken from B prior to centrifugation to serve as control (A). These substrates (an equal concentration in all incubations) were then used in assays described in the Experimental Section. Dibucaine was present in the assay where designated at concentration of 0.16 mM. The hydrolysis of the control sample that was centrifuged was taken as 100% activity (8.5 nmoles of [^{14}C]fatty acid were released). All other values are compared to this control.

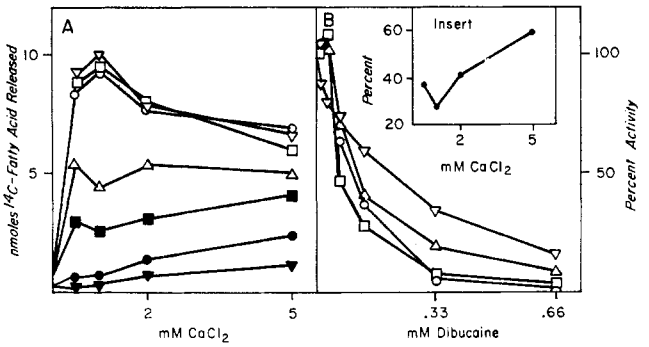

FIGURE 9: The competition between dibucaine and Ca^{2+}. The standard assay conditions described in the Experimental Section were employed except for the indicated variation of dibucaine and Ca^{2+} concentrations. Part A and part B are from the same experiment. Part B is plotted as the per cent of the activity found with each concentration of Ca^{2+} without dibucaine equal to 100%. In part A the designations are for dibucaine: none (○), 13 mM (□), 33 mM (▽), 66 mM (△), 132 mM (■), 330 μM (●), 660 μM (▼). The designation in B are for Ca^{2+} concentration: 0.5 mM (○), 1.0 mM (□), 2.0 mM (△), and 5.0 mM (▽). The per cent activity with 0.132 mM dibucaine is plotted *vs.* Ca^{2+} concentration in the insert.

TABLE II[a]

Addition of Ca^{2+}	Dibucaine	% of Control
A. With assay	−	100
	+	39
B. With substrate prior to assay	−	104
	+	39

[a] The lack of protection against dibucaine inhibition by incubation of the substrate with Ca^{2+} prior to the addition of dibucaine. The assays were run in A part as described in Experimental Section. In the B part of the experiment 2.0 mM $CaCl_2$ was incubated with the substrate for 5, 10, 15, and 25 min. Dibucaine (0.33 mM) was then added to the reaction mixture, the mixture was incubated 10 min more and then the enzyme was added. The hydrolytic reaction was run 10 min. The control hydrolysis (A), taken as 100%, was 4.0 nmoles of [^{14}C]fatty acid released.

protect against inhibition by dibucaine if the two were competing for the same site on the enzyme. With or without low concentrations of dibucaine (13–26 μM) maximal activity was found with 1.0 mM Ca^{2+}; the highest concentration of $CaCl_2$ (5.0 mM) inhibited. However, at the concentrations of dibucaine in the range of 66–330 μM, activity increases with increasing concentrations of Ca^{2+} which suggests that Ca^{2+} does protect against dibucaine inhibition under these conditions. This is better seen in the B part of Figure 9 where the data are replotted as the per cent of the activity found with each concentration of Ca^{2+} in the absence of dibucaine. In the presence of 130 μM dibucaine, 5 mM Ca^{2+} doubles the relative activity found at 1.0 mM Ca^{2+}; 2 mM Ca^{2+} gives moderate protection. Calcium did not protect effectively against inhibition by 0.66 mM dibucaine, which indicates that the enzyme (or enzyme–substrate complex) has a much greater affinity for dibucaine than Ca^{2+}. It is of interest to note that 5.0 mM Ca^{2+} not only provides maximum protection against dibucaine inhibition but also abolishes the slight stimulation of activity at low concentrations of dibucaine (part B). The concentration of Ca^{2+} which allows maximum activity, 1.0 mM, gave least protection against the inhibition by dibucaine (Insert, Figure 9B).

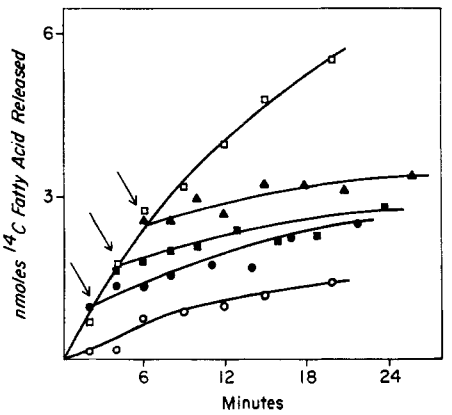

FIGURE 10: Inhibition of the soluble phospholipase A_2 activity by dibucaine after initiation of the reaction. The reaction was run with the concentrations of reactants indicated in the Experimental Section except the total volume was 8.0 ml. At the indicated times, 1.0 ml of the reaction mixture was pipetted into 3.0 ml of $CHCl_3$–methanol (1:2) to stop the reaction. The open squares designate the results obtained in the absence of dibucaine whereas the open circles designate the results obtained when the original incubation mixture contained 0.66 mM dibucaine. Dibucaine (0.66 mM final concentration) was added to reaction mixtures after 2- (●), 4- (■), and 6-min (▼) incubation. Aliquots (1.0 ml) were removed from these assay mixtures at the indicated times.

The foregoing results suggest that we could use the competition between Ca^{2+} and dibucaine to learn more of the role of Ca^{2+} in the reaction. If Ca^{2+} controlled binding of the enzyme to the liposome, we might expect that once the enzyme–substrate complex was formed, dibucaine could no longer inhibit. Such a possibility was tested by adding dibucaine to the complete reaction mixture since under these conditions the enzyme–Ca^{2+}–substrate complex is formed. Dibucaine inhibited (Figure 10) even after the reaction was initiated, however. Further, the order of addition to the reaction mixture made no apparent difference since incubation of the substrate with Ca^{2+} did not protect against inhibition by dibucaine (Table II). Similar results were obtained when the enzyme and Ca^{2+} were mixed prior to the addition of dibucaine. There are two possible explanations for the results; one, the active complex dissociates completely, or two, dibucaine binds to the complex (or the enzyme within the complex) and competes with Ca^{2+}, perhaps by dissociating Ca^{2+} from the complex. We tend to favor the latter interpretation since the enzyme appears to bind lipid tightly since vigorous treatment with organic solvent is required to remove the enzyme from the mitochondrial membrane (Waite and Sisson, 1971). Therefore, it is tentatively concluded that Ca^{2+} is not involved in binding of the liposome to the enzyme, but rather is bound to the enzyme and is involved in the hydrolytic process.

Effect of Dibucaine on the Phospholipases of Lysosomes. The generality of inhibition of dibucaine was studied with the lysosomal phospholipases A_1 and A_2. The lysosomal enzymes are particularly suited for comparison since they are known not to require Ca^{2+} for activity; rather, Ca^{2+} inhibits (Franson *et al.*, 1971). As shown in Figure 11, 0.2 mM dibucaine is

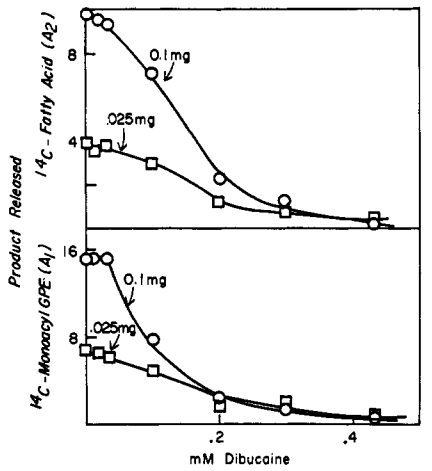

FIGURE 11: Inhibition of the lysosomal phospholipases A_1 and A_2 by dibucaine. The assay conditions are described in the Experimental Section except for the indicated amounts of the soluble proteins derived from lysosomes.

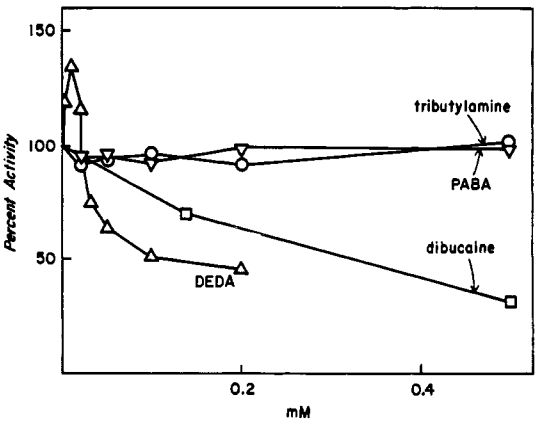

FIGURE 12: The effect on the soluble phospholipase A_2 of the addition of compounds with structural features similar to those of local anesthetics. The conditions described in the Experimental Section were used except for the addition of dibucaine, tributylamine, diethyldodecylamine (DEDA), and *p*-aminobenzoic acid (PABA). All were neutralized before addition to the reaction mixture. DEDA was added as an ultrasonic suspension prepared in water.

nearly sufficient to completely inhibit both phospholipases A_1 and A_2 at two concentrations of enzyme. These results demonstrate that dibucaine is a more potent inhibitor than Ca^{2+}; nearly 50 times as much Ca^{2+} (10 mM) is required to cause maximal inhibition (Franson *et al.*, 1971). At no concentration of dibucaine tested was there any stimulation of the lysosomal phospholipase, unlike the mitochondrial enzyme. The lysosomal phospholipase A_1 appears to be more sensitive to dibucaine than the lysosomal phospholipase A_2. The A_1 is inhibited 50% in the presence of 0.1 mM dibucaine whereas the A_2 is only inhibited about 25%. As is the case with the mitochondrial enzyme, the inhibition is not overcome by increasing the concentration of substrate (not presented here). Dibucaine inhibition appears to involve the same site of action as that of Ca^{2+}.

Structural Features Required for Inhibition. To determine which functional group(s) of anesthetics is critical for the inhibition, compounds similar to the various anesthetics were tested on the mitochondrial phospholipase A_2. The results show (Figure 12) that the ring group with a nitrogen (*p*-aminobenzoic acid) is not involved in the action of the anesthetics. Of the two amines shown, only the hydrophobic diethyldodecylamine was effective; tributylamine, which is water soluble, was not. Other amines tested, diethyl, dibutyl, and butyl, are not included in the figure since they also had no effect. These results implicate the tertiary amine as the active constituent. However, the molecule must be lipid soluble to be fully effective which explains why diethyldodecylamine is more potent than dibucaine. Similar results were obtained at pH 9.5 with trimethylhexadecylamine (Waite and Sisson, 1971).

Effect of Dibucaine on the Membrane-Bound Phospholipase A_2. Since dibucaine is an inhibitor of the solubilized mitochondrial phospholipase A_2, interest arose as to the effect dibucaine has on the activity of the enzyme associated with the mitochondrial membranes. Scherphof and Scarpa (1972) demonstrated that mitochondria stored at 0° in dibucaine maintained their respiratory control and that the hydrolysis of membrane phosphatidylethanolamine was reduced. Dibucaine therefore could inhibit the membrane bound phospholipase A_2 as well. Mitochondria labeled with [^{14}C]ethanolamine in phosphatidylethanolamine were incubated with dibucaine in the absence of or in the presence of 1.0 or 5.0 mM added Ca^{2+}. The results of this experiment (Figure 13) are somewhat surprising. Without the addition of Ca^{2+}, dibucaine stimulates hydrolysis of the membranous phosphatidylethanolamine and presumably causes swelling. Only with 5 mM Ca^{2+} added did dibucaine inhibit the phospholipase A_2. This level of Ca^{2+} is required for maximal phospholipase A_2 activity. The fact that high concentrations are required for inhibition (1.5 mM), relative to those used with the solubilized mitochondrial enzyme, can be explained by assuming that most dibucaine is not bound in the membrane specifically by the phospholipase A_2.

Discussion

The effects of local anesthetics on phospholipases are dependent upon the time of incubation and concentration and structure of the anesthetic. The basic requirement appears to be a lipophilic molecule with a tertiary amine. Structural comparison of the compounds tested indicates that dibucaine, which inhibits at high concentrations, differs in the ring struc-

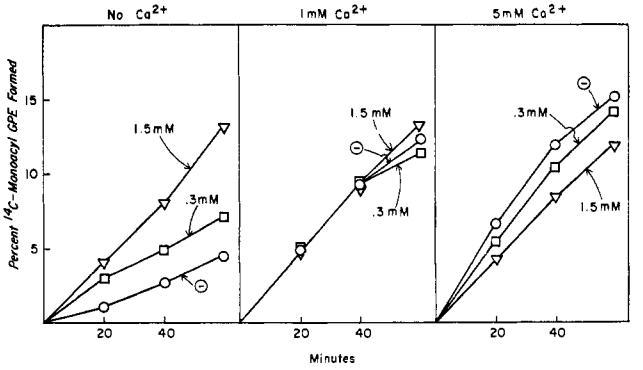

FIGURE 13: The effect of dibucaine and Ca^{2+} on the hydrolysis of membranous phosphatidylethanolamine by the membrane bound phospholipase A_2. The assay conditions are described in the Experimental Section. The concentrations on the figure refer to the concentration of dibucaine.

ture from the other anesthetics. It has a fused ring moiety which contains nitrogen and a butyl ether attached to the ring which adds to the lipophilic nature of the end of the molecule distant to the tertiary amine. The involvement of an amide or ester linkage between the aliphatic amine and the ring structure is not important since procaine and procainamide have similar effects. Butacaine, which stimulates at high concentrations, has butyl chains on the tertiary amine which makes the amine moiety lipophilic, relative to dibucaine. Indeed, the difference between the apparent effects with high concentrations of dibucaine and butacaine might be due to the relative lipophilic nature of the opposite ends of the molecule. Lidocaine, procaine, and procainamide, the smaller, less lipophilic compounds have less effect on phospholipase activity. Scherphof and Scarpa (1972) found procaine and lidocaine to be poor inhibitors of the pancreatic phospholipase, similar to the findings in this report. Diethyldodecylamine gave the same effect as dibucaine although lower concentrations of diethyldodecylamine were required. This compound is sparsely soluble in H_2O and is more lipophilic than dibucaine which might account for its greater potency. Trimethylhexadecylamine had a similar but not as pronounced effect as diethyldodecylamine at pH 8.0–9.5. (Waite and Sisson, 1971).

It is not possible at this stage of experimentation to clearly define the mechanism of inhibition by dibucaine although some features can be described. Seppala *et al.* (1971) concluded that the inhibitory action of a number of amines on the enzyme from the venom of *C. adamanteus* is not due to the displacement of Ca^{2+} from the enzyme. Scherphof and Scarpa (1972), on the other hand, found competition between dibucaine and Ca^{2+} in their studies with the pancreatic and mitochondrial (membrane-bound) phospholipase A_2. We also find competition between Ca^{2+} and dibucaine with the soluble mitochondrial phospholipase A and inhibition of both lysosomal phospholipases similar to the effect of Ca^{2+}. We conclude that dibucaine action is related to that of Ca^{2+} and that studies which incorporate dibucaine can yield information on the poorly defined role of Ca^{2+}. de Haas *et al.* (1971) and Wells (1972) concluded that Ca^{2+} binds to phospholipase from pancreas and the venom of *C. adamanteus* independently of the substrate. If the mitochondrial enzyme employs Ca^{2+} in the same manner as these enzymes, and if dibucaine binds to the same site(s) as Ca^{2+}, some predictions can be made. (1) Dibucaine, like Ca^{2+} should not bind to the substrate tightly. This was found to be the case when a concentration of dibucaine was used sufficient to cause 60% inhibition. (2) Inhibition by dibucaine should not be influenced by substrate concentration if the interaction of dibucaine and the enzyme is independent of substrate. The inhibition by 0.1 mM dibucaine is uncompetitive with substrate. The type of inhibition is more complicated at high concentrations of inhibitor which indicates other possibilities do exist. Possibly, at high concentrations of dibucaine there is interaction of dibucaine and phosphatidylethanolamine. (3) Enzymatic activity should not be protected by addition of substrate to the enzyme prior to the addition of dibucaine. We find that dibucaine stops when added as the reaction proceeds (Figure 10).

The inhibition of the soluble phospholipase A_2 from mitochondrial was most pronounced in the first 10 min of the reaction which suggests a change in the affinity of the enzyme for the substrates. Our tentative conclusion is, however, that dibucaine does not influence the physical binding of the substrate to the enzyme. This might be different, however, from the actual interaction of the 2-acyl ester and water, the other substrate, with the active site of the enzyme. It is our tentative hypothesis that Ca^{2+} exerts its effect at this stage of the hydrolytic reaction.

Scherphof and Scarpa (1972) concluded that dibucaine must be absorbed to the liposomes before inhibition will occur, which is different from our observations (no significant binding of dibucaine to purified rat liver phosphatidylethanolamine). Their conclusion is based on the observation that dibucaine did not inhibit hydrolysis of pure egg lecithin by the pancreatic enzyme. Under those conditions, dibucaine did not bind to the lipid, as shown by spectral studies. The differences between their report and ours could be due to differences in the mechanisms of action of the enzymes, the substrate used, or the method of studying the dibucaine–substrate interaction. However, the demonstration that substrate binding to the enzyme is independent of Ca^{2+} (de Hass *et al.*, 1971) plus the observation that there is competition between dibucaine and Ca^{2+} would suggest that the effect of dibucaine is on the pancreatic enzyme rather than the substrate as concluded by Scherphof and Scarpa. Clearly, more detailed work is required on the mechanism of inhibition.

Our attempts to relate the inhibition of the solubilized mitochondrial phospholipase to the preservation of mitochondrial respiratory control has led to anomalous results. Dibucaine stimulated phospholipase A_2 activity when intact mitochondria were incubated in the absence of added Ca^{2+}. Presumably, this caused swelling and uncoupling of mitochondrial oxidative phosphorylation. When Ca^{2+} is present in sufficient quantities for optimal activity (5 mM), dibucaine caused inhibition. Under somewhat different conditions, Scarpa and Linsay (1972) and Scherphof and Scarpa (1972) found protection against uncoupling and inhibition of the hydrolysis of phospholipid by dibucaine when no Ca^{2+} was added. Their mitochondria were isolated in the presence of EGTA, however. Dibucaine could cause the membrane Ca^{2+} to become more "fluid" within the membrane which would activate the membrane-bound phospholipid. Indeed, Scarpa and Azzi (1968) found that dibucaine competed with Ca^{2+} for binding to mitochondria. The amount of Ca^{2+} bound to mitochondria, 25 nmoles/mg of protein (Scarpa and Azzi, 1968) is not sufficient to cause maximal phospholipase activity (Waite *et al.*, 1969a). This indicates that either dibucaine has a stimulatory effect on the membrane bound phospholipase similar to that of added fatty acid (Waite *et al.*, 1969a) or that the Ca^{2+} is not truly solubilized but remains in some association with the membrane and can activate the phospholipase. The latter possibility is supported by the observation that the enzyme from *C. adamanteus* is not stimulated under these conditions (Seppala *et al.*, 1971). The absolute requirement by the phospholipase A_2 for Ca^{2+} must be met in this case by the endogenous Ca^{2+} of the mitochondria since EDTA blocked all activity.

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Fluorometric Investigations of the Interaction of Polyene Antibiotics with Sterols†

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ABSTRACT: Changes in specific fluorescent properties, partial quantum efficiency (PQ), and corrected fluorescence (CO) can be used to monitor the interaction of various polyenes with sterols. The changes in PQ and CO, caused by addition of sterols, are different for each polyene antibiotic. Addition of cholesterol decreases the PQ and CO of filipin in aqueous solution 36 and 62%, respectively, but cholesterol increases the PQ and CO of pimaricin more than 80-fold. Addition of cholesterol to nystatin or amphotericin B has little effect on the fluorescence emission. The filipin complex as well as pimaricin strongly binds sterols that contain both a 3β-hydroxyl group and a long alkyl side chain that is attached to the D ring of sterols. The filipin complex and pimaricin interact weakly or not at all with cholesterol palmitate and 3-keto or 3α-hydroxy sterols. The methanol degradation product of filipin (a tetraene epoxide) does not interact with cholesterol. The stoichiometry of the filipin:cholesterol interaction is, within experimental error, 1:1. The interaction of filipin with cholesterol is independent of pH from pH 4.5 to 8, but is dependent on the method of isolation and preparation of the filipin complex. The filipin complex isolated from organic solvents does not interact with sterols—using fluorescence as a criteria of interaction—but it will interact with sterols after prolonged standing in aqueous solution or heating to 50° for 2 hr. These studies strongly indicate that filipins can exist in conformational states or bonded conditions such as dimers that do not bind sterols in a specific manner and that such forms undergo changes in aqueous solutions to a form or forms that can interact specifically with cholesterol and other 3β-hydroxy sterols.

Many polyene antibiotics can produce lethal permeability alterations in microorganisms containing sterols in their membranes (Whitfield *et al.*, 1955; Perritt *et al.*, 1960; Gottlieb *et al.*, 1960; Lampen and Arnow, 1961; Johnson *et al.*, 1962; Ghosh, 1963; Weber and Kinsky, 1965; Child *et al.*, 1969). These polyenes can also lyse erythrocytes (Kinsky *et al.*, 1962, 1967; Kinsky, 1963), and alter the structure and permeability of liposomes (Weissmann and Sessa, 1967; Sessa and Weissmann, 1968), and model membranes (Demel *et al.*, 1965; Van Zutphen *et al.*, 1966, 1971; Demel, 1968; Kinsky, 1970). Effects *in vitro* include stimulation of glucose utilization in mammalian fat cells (Kuo, 1968), decreased DNA synthesis and cellular respiration in Ehrlich ascites and Novikoff hepatoma tumor cells (Mondovi *et al.*, 1971), increased phospholipid turnover in beef thyroid slices (Larsen and Wolff, 1967), and rupture of beef spermatozoa (Morton and Lardy, 1967). Some polyenes, especially filipin, decrease serum cholesterol levels in dogs and chicks (Schaffner and Gordon, 1968; Fisher *et al.*, 1969), reduce canine prostatic hyperplasia (Gordon and Schaffner, 1968), mimic vitamin D mediated calcium transport in chick ileum (Adams *et al.*, 1970; Wong *et al.*, 1970), act as larvicides and chemosterilants in some insects (Sweeley *et al.*, 1970; Schroeder and Bieber, 1971), and are toxic to snails (Seneca and Bergendahl, 1955). The mechanism of polyene action that is suggested by these studies is the binding to sterols, free or membrane bound, by polyene macrolides. Spectrophotometric evidence for complex formation has been presented (Lampen *et al.*, 1960; Gottlieb *et al.*, 1961; Norman *et al.*, 1971, 1972) but interpretation of such data is difficult because reduction of the absorption peaks by sterols does not correlate with the ability of the sterol to alter the fungicidal activity of filipin (Gottlieb *et al.*, 1961). No spectral shifts were noted. Decreases in absorbance of filipin could indicate lowered solubility in aqueous solution (Kinsky, 1967), and, as shown herein, can be due to small differences in experi-

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