

Reaction of Local Anesthetics with Phospholipids

A possible chemical basis for anesthesia

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ABSTRACT Local anesthetics (LA) have been found to interact with phospholipids and lipids extracted from nerve and muscle. This reaction is demonstrated by: (a) Inhibition by LA of phospholipid (and tissue lipid) facilitated transport of calcium from a methanol: water phase into chloroform. This action is dependent upon the cationic form of the LA. (b) LA increase the electrical resistance of "membranes" prepared by impregnating Millipore filters with cephalin:cholesterol or tissue lipid extracts and bathed with NaCl or KCl solutions. (c) LA coagulate aqueous dispersions of cephalin, phosphatidyl serine, phosphatidyl ethanolamine, and inositide, an action shared by calcium. The order of potency in coagulating cephalin sols is tetracaine > calcium > butacaine > procaine. Na⁺ and K⁺ do not coagulate phospholipid dispersions at 0.1 M concentration and antagonize the effect of Ca²⁺. (d) LA produce a marked fall in the pH of cephalin sols equivalent to that produced by calcium. (e) Ca²⁺ and LA form 1:2 molar complexes with phospholipids probably by ion-ion and ion-induced polar type of binding at the phosphate groups of the lipid. It is suggested that such reactions with cell membrane phospholipids may underlie inhibitory effects of LA on cellular ion fluxes and provide a chemical basis for anesthetic action.

INTRODUCTION

It has become increasingly evident in recent years that the blockade of impulse conduction along muscle and nerve fibers by local anesthetics probably depends upon their ability to inhibit the increased sodium conductance associated with generation of the action potential. This is indicated by a decrease in rate of development and magnitude of the action potential (1-3), and by voltage clamp experiments which show that the rapid initial rise in sodium conductance is inhibited (4, 5). Interference with the increase in sodium conductance has been attributed to a specific competitive antagonism to Na⁺ (2, 3), or to a decrease in the availability of a hypothetical sodium "car-

rier" system (1, 6). It has been proposed that in the resting state the membrane Na^+ -carrier sites are occupied by calcium, and that depolarization leads to removal of this calcium so that the carrier becomes available to Na^+ (7-9). However, the action of local anesthetics is not restricted solely to a specific inhibition of the sodium influx during excitation as there are numerous examples of interference with the movement of other ions such as K^+ (10, 11), Ca^{2+} (12, 13), and Li^+ (14).

The site of local anesthetic action is generally accepted to be at the cell membrane (7, 15), but the mechanism is unknown. This is primarily due to the lack of knowledge as to the specific structural and functional modifications of the membrane which underlie the variations in its permeability. Structurally the membrane is a highly ordered unit, which appears to contain specific types of lipids (phospholipids, cerebroside, glycolipids, cholesterol) and proteins (16-19). The phospholipid content of the membrane is particularly interesting, because of the well known ion-binding properties of these compounds (20-26). This property suggests that they may serve as carriers for ions across the plasma membranes of cells.

The experiments reported in this paper were undertaken to determine what interactions, if any, might occur between local anesthetics and lipids of the type present in cellular membranes, and whether their cation-binding properties would be affected. The results indicate that local anesthetics (as well as calcium) are capable of reacting stoichiometrically with the phosphate group of at least some phospholipids. Complexes composed of 2 moles of phospholipid per mole of local anesthetic are formed, probably by an ion-ion and ion-induced polar type of binding. This leads to an inhibition of cation-binding by the phospholipids. The possibility that this reaction is a chemical basis for anesthetic action will be discussed.

METHODS

Transport of Calcium into Chloroform by Phospholipids

Lipid compounds were dissolved in chloroform:methanol (2:1) at a concentration of 1 mg/ml. This is equivalent to 1.25 $\mu\text{moles/ml}$, assuming an average molecular weight for the phospholipids of 800. Rabbit skeletal muscle microsomes (granular relaxing factor preparation, as prepared by Weber, Herz, and Reiss (27)) were extracted for 1 hour with 1.5 ml chloroform:methanol solution per mg of microsomal protein. Rabbit sciatic nerve was extracted with 20 ml of chloroform:methanol solution per gm wet weight of tissue. Uptake of calcium was measured by mixing 2.0 ml of a solution of phospholipids, or tissue lipids, in chloroform:methanol with 1.0 ml of Ringer's solution (NaCl 116 mM, KCl 2.5 mM, CaCl_2 1.0 mM) containing Ca^{45} . After vigorous mixing for 10 minutes centrifugation resulted in separation into two clear phases, one chloroform and the other water plus methanol. Aliquots of each phase were evaporated on stainless steel planchettes and the radioactivity determined

in a gas flow counter. The following lipids were used: tripalmitin (Mann Research Laboratories), cholesterol (Sigma), animal cephalin (Sigma and Nutritional Biochemicals Corp.), synthetic DL- α -cephalin (β - γ -dipalmitoyl-DL- α -cephalin, Sigma), lecithin (Sigma), synthetic L- α -lecithin (β - γ -dipalmitoyl-L- α -lecithin, Sigma), phosphatidyl-L-serine, fraction III of Folch (Sigma), phosphatidyl ethanolamine, fraction V of Folch (Sigma), phosphatidyl inositide, fraction I of Folch (Sigma), sphingomyelin (Mann Research Laboratories).

Measurement of Electrical Resistance of Lipid-Impregnated Millipore Filters

Millipore filter discs (100 ± 20 A pore size, 0.15 mm thickness; Millipore Corp., Bedford, Massachusetts) were impregnated, as described originally by Tobias, Agin, and Pawlowski (28), with cephalin plus cholesterol or tissue lipids. The discs were soaked in benzene solutions of the lipids for several minutes and then dried in air. This process was repeated several times after which the dried filter disc was placed in a plastic chamber, so that it could be exposed on both sides to salt solutions. A 2.0 cm² area of membrane was exposed to the solutions on each side of the chamber. Resistance was measured with a Schering bridge circuit (29) by passing 5000 cps alternating current through platinum electrodes in the solutions on either side of the membrane. A Tektronix oscilloscope was used as a null detector. Absolute resistance values given in this paper are not considered of significance, rather it is the qualitative changes induced by salts or local anesthetics which are of primary interest. The total resistance for electrodes, solution, and *untreated* (no lipid) Millipore filters averaged 180 ohms. These data have been corrected only for differences in resistance between equimolar solutions of NaCl, KCl, or CaCl₂ with untreated Millipore filters in the chamber. These variations amounted to no more than 10 to 20 ohms. The addition of local anesthetics at 3 mM concentration had no effect on the resistance of untreated Millipore filters in the presence of 0.1 M salt solutions.

Coagulation of Aqueous Dispersions of Phospholipids

Aqueous dispersions of some phospholipids were prepared at a concentration of 1 mg/ml. One ml portions of the phospholipid dispersions were mixed with equal volumes of salt solutions (NaCl, KCl, and CaCl₂) at various concentrations. Varying concentrations of calcium chloride containing 0.2 M KCl, 0.2 M NaCl, or 0.06 M HCl were also prepared and mixed with equal volumes of the phospholipid dispersions. Solutions of the local anesthetics, procaine HCl, tetracaine HCl, and butacaine sulfate, were similarly prepared and mixed in equal proportions with the phospholipid dispersions. The turbidity of the mixed salt- or local anesthetic-phospholipid solutions was determined by the optical density readings in a spectrophotometer at 520 m μ . Phospholipid dispersions diluted with an equal volume of deionized water served as blanks. The turbidity of solutions containing calcium, once developed fully, decreased about 10 per cent per hour, whereas local anesthetics, especially at the higher concentrations, caused a rapid increase in turbidity followed by a large, rapid, fall as small dispersed particles coalesced into large particles and settled out or floated to the surface. When the final solution contained 0.5 mg/ml bovine serum albumin,

the coagulated phospholipid particles did not coalesce and the optical density readings declined slowly at about the same rate as with calcium.

RESULTS

Local Anesthetic Inhibition of Calcium-Binding by Phospholipids

Several investigators have observed that phospholipids bind inorganic cations and facilitate their transport from water into organic solvents in which they are normally not soluble. Transport of Na^+ and K^+ across an organic phase interposed between two aqueous phases was accomplished by cephalin (30–33). At low concentration (1 to 2 mM) Ca^{2+} blocked the Na^+ - K^+ carrier transport across a 1-pentanol:cephalin membrane (32). The relative affinities of various phospholipids for Na^+ and K^+ differ but the cephalin group appears to have a much greater affinity for Ca^{2+} than for any of the monovalent cations.

The results of experiments which demonstrate the ability of phospholipids, and lipid extracts of muscle and nerve, to transport calcium from water into chloroform are shown in Table I. Lecithin transported about 30 per cent of the total calcium present into the chloroform phase, whereas cephalin (which contained mainly phosphatidyl serine and phosphatidyl ethanolamine and traces of phosphatidyl inositide and sphingomyelin) and phosphatidyl serine transported 70 to 80 per cent. This is particularly striking as it occurred in the presence of a Na:Ca ratio of about 100 to 1. Relatively low concentrations of local anesthetics inhibited the calcium uptake into chloroform. Local anesthetics also blocked uptake of calcium by lipids extracted with chloroform:methanol (2:1) from rabbit skeletal muscle microsomes and rabbit sciatic nerve. Tetracaine was more potent than procaine in inhibiting calcium uptake by cephalin. At concentrations of 0.22 and 2.2 mM tetracaine HCl inhibited Ca^{2+} uptake by 23 and 79 per cent respectively, whereas 4.4 mM procaine HCl inhibited uptake by 44 per cent. The tetracaine experiments were performed at a lower cephalin concentration of 0.5 mg/ml.

These experiments suggest that local anesthetics may react with polar groups of the phospholipids which bind calcium at the chloroform/water:methanol interface and transport it into the chloroform phase. This could result from an ionic type of binding between procaine (or tetracaine) and polar groups of the phospholipid. A test of the pH dependence of the reaction (Fig. 1) supports this hypothesis, as calcium uptake by rabbit muscle lipid extracts was inhibited most strongly in acidic solutions and decreased as the pH approached the pK_a for the alkyl tertiary amine group of procaine (~ 9). More strongly alkaline solutions (pH > 8.5) were not used because of the possibility of calcium precipitation. Experiments with cephalin demonstrated

TABLE I
CALCIUM TRANSPORT INTO CHLOROFORM
FROM A WATER:METHANOL PHASE

Two ml aliquots of chloroform:methanol (2:1) containing 1 mg/ml of lipid, or extracted lipids from nerve or muscle, were shaken for 10 minutes with 1.0 ml aqueous solution containing NaCl 116 mM, KCl 2.5 mM, CaCl₂ 1.0 mM, and 1 μ c/ml Ca⁴⁵ at pH 6.0. Centrifugation produced separation into two clear phases (1) water + methanol and (2) chloroform. The concentrations of calcium were calculated from the radioactivity found in aliquots from each phase.

Lipid	Calcium in CHCl ₂	Inhibition of
	phase	calcium uptake into CHCl ₂
	<i>μmoles</i>	<i>per cent</i>
None	0	—
Cholesterol	0	—
Tripalmitin	0	—
Lecithin (animal)	0.327	—
Lecithin (animal) + procaine HCl 4.4 mM	0.080	76
Lecithin (synthetic)*	0.284	—
Lecithin (synthetic) + procaine HCl 4.4 mM	0.046	84
Cephalin (animal)	0.746	—
Cephalin (animal) + procaine HCl 4.4 mM	0.417	44
Cephalin (animal)†	0.485	—
Cephalin (animal) + tetracaine HCl 0.22 mM	0.375	23
Cephalin (animal)‡	0.451	—
Cephalin (animal) + tetracaine HCl 2.2 mM	0.095	79
Phosphatidyl serine	0.804	—
Phosphatidyl serine + procaine HCl 4.4 mM	0.539	33
Rabbit muscle microsomal extract§	0.047	—
Rabbit muscle microsomal extract + procaine HCl 4.4 mM	0.010	79
Rabbit muscle microsomal extract + procaine HCl 22 mM	0.000	100
Rabbit sciatic nerve extract	0.337	—
Rabbit sciatic nerve extract + procaine HCl 4.4 mM	0.222	34
Rabbit sciatic nerve extract + tetracaine HCl 4.4 mM	0.006	98

* β - γ -Dipalmitoyl-L- α -lecithin.

† Cephalin was present in these experiments at a concentration of 0.5 mg/ml.

§ Rabbit muscle microsomal fraction extracted with 1.5 ml chloroform:methanol (2:1) per mg of microsomal protein.

|| Nerve extracted with 20 ml chloroform:methanol (2:1) per gm.

the same sort of pH dependence for local anesthetic inhibition of calcium binding.

Electrical Resistance of Lipid-Impregnated Millipore Filters

Another approach to the investigation of local anesthetic effects on cation transport by phospholipids is provided by a study of the behavior of Millipore filters impregnated with cephalin plus cholesterol. The phospholipid-impregnated membrane was mounted between two chambers containing salt solutions (see Methods). The experiments demonstrate that membranes impregnated with cephalin plus cholesterol had a lower resistance in 0.1 M KCl

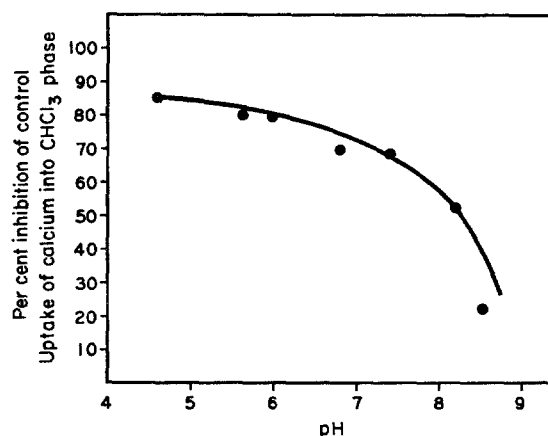


FIGURE 1. The effect of pH on the inhibition by procaine (4.4 mM) of rabbit muscle lipid-facilitated transport of calcium from a water:methanol phase into chloroform. Rabbit muscle microsome fraction extracted with 1.5 ml chloroform:methanol (2:1) per mg of microsomal protein.

than in 0.1 M NaCl solutions (range: 180 to 230 ohms for KCl, 310 to 400 ohms for NaCl. Values obtained on several different membranes). Calcium increased resistance considerably but not to as great an extent as that observed by Tobias *et al.* (28). This may be due to the fact that our membranes were impregnated with less cephalin and cholesterol than employed by the latter group. A considerable increase in resistance occurred when 0.1 M NaCl or KCl was replaced by 0.05 M NaCl, or KCl, plus 0.05 M CaCl₂ (Figs. 2 A and B). However, a substantial portion of the increased resistance in this case is due solely to lowering the concentration of the more permeable monovalent cations. This is shown by Fig. 2B where reduction of KCl from 0.1 M to 0.05 M increased resistance twofold. There is on the other hand an additional increase in resistance due to the effect of the Ca²⁺ itself in the presence of K⁺. These experiments indicate that the conductance of the membrane was

greatest in the presence of K^+ , lower with Na^+ , and least in the presence of Ca^{2+} .

The principal purpose of employing this model membrane system was to study its behavior towards local anesthetics, as it is known that a characteristic effect of these agents on the electrical properties of nerve is to bring about an increase in the transmembrane resistance (34). It is evident from Fig. 2 that tetracaine (3 mM) produced significant increases in resistance when either Na^+ or K^+ was the cation present in solution. Similar experiments with pro-

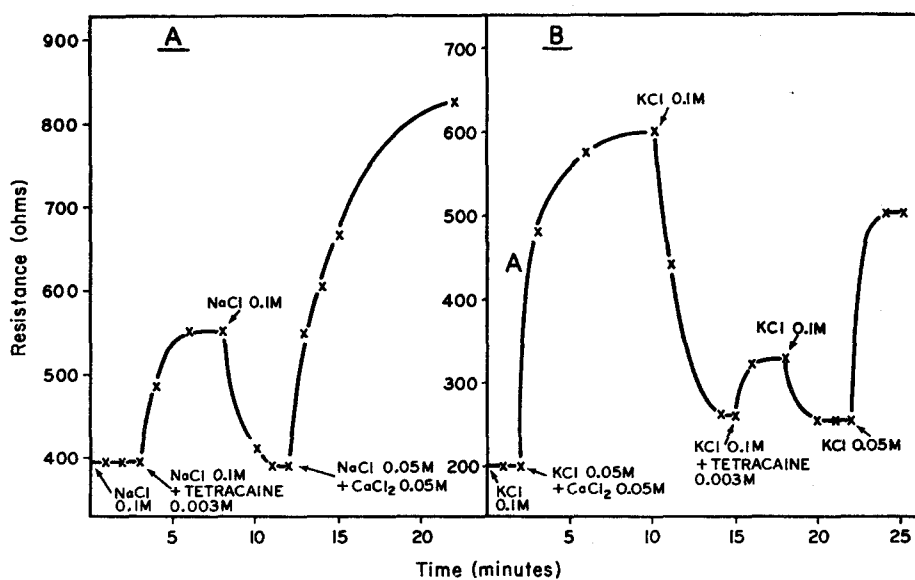


FIGURE 2. The effects of Na^+ , K^+ , Ca^{2+} , and tetracaine HCl on the resistance of Millipore filter membranes impregnated with cephalin plus cholesterol. Solutions were of the same composition on both sides of the membrane and were changed as indicated by the arrows.

caine gave the same qualitative effect but procaine was only about one-sixth as effective as tetracaine. Tetracaine was about twice as effective in raising resistance in the presence of Na^+ (average increase 40 per cent) compared to K^+ (average increase 20 per cent). These results indicate that tetracaine at relatively low concentration decreases the membrane conductance in the presence of Na^+ or K^+ with relatively greater effect in the presence of Na^+ .

Membranes prepared by impregnating Millipore filters with lipids extracted from rabbit muscle microsomes responded to tetracaine in the same manner as cephalin-cholesterol membranes. These membranes, however, differed in their response to calcium, which uniformly produced a decrease in resistance. In the presence of 0.1 M $CaCl_2$ tetracaine (3 mM) produced only a slight, but still detectable, increase in resistance. It should be noted that the

microsomes were extracted with chloroform:methanol (2:1) which also extracts some lipoproteins (35) in addition to phospholipids. The high conductance in the presence of calcium is not altogether surprising as the muscle microsomal fraction has a very great ability to transport Ca^{2+} (27). It is possible that the solvent extracted a Ca^{2+} -carrier substance involved in this transport process. Membranes prepared with lipid extracts of rabbit sciatic nerve were unsatisfactory for study as they exhibited very high initial resistances, which continuously decreased with time in any of the salt solutions used.

Reaction of Ca^{2+} and Local Anesthetics with Aqueous Dispersions of Phospholipids

Calcium coagulated aqueous dispersions of cephalin, phosphatidyl serine, phosphatidyl ethanolamine, and inositide but had little or no effect on lecithin or sphingomyelin. Robins and Thomas (36) report that precipitation of phosphatidyl ethanolamine sols began at a calcium concentration of 0.4 mM whereas phosphatidyl serine sols began to coagulate between 0.5 and 1 mM (37). Concentrations of NaCl and KCl (0.1 M), which did not produce turbidity in phospholipid sols, inhibited development of turbidity with calcium. The calcium concentration at which half-maximum turbidity developed increased from 6.5 mM to 11 to 12 mM in the presence of 0.03 M HCl or 0.1 M NaCl, and to 16.7 mM with 0.1 M KCl. The order of effectiveness in inhibiting turbidity was $\text{H}^+ > \text{K}^+ > \text{Na}^+$ and indicates the relative affinity for binding by the phospholipids.

Tetracaine, butacaine, and procaine also coagulated phospholipid sols (Fig. 3). Half-maximal turbidity of cephalin sols was produced at tetracaine 4 to 5 mM, butacaine 30 mM, and procaine 120 mM. The order of potency corresponds to the relative order of anesthetic potency *in vivo*. Tetracaine coagulated cephalin sols at slightly lower concentrations than calcium. Thus, the order of ability to coagulate cephalin sols is tetracaine $>$ Ca^{+2} $>$ butacaine $>$ procaine.

Tetracaine was more effective at coagulating solutions of cephalin, phosphatidyl serine, and phosphatidyl ethanolamine than phosphatidyl inositide. The tetracaine concentrations at which half-maximum turbidity developed were ~ 5 mM for cephalin and ~ 15 mM for phosphatidyl inositide. Phosphatidyl serine and phosphatidyl ethanolamine sols are almost indistinguishable in behavior compared with cephalin (Fig. 3).

Although relatively high concentrations of local anesthetics are required for coagulation, much lower concentrations have a considerable effect upon Ca^{2+} uptake at a CHCl_3 -water interface. This is probably because there may be a direct relationship between the number of phospholipid-local anesthetic complexes formed and the degree of turbidity. In the case of inhibition of Ca^{2+} binding it is a matter of competition between calcium and the drugs and

depends mainly upon the relative concentrations of calcium and local anesthetic, and the relative affinity constants for the calcium-cephalin and local anesthetic-cephalin complexes.

Stoichiometry of Calcium and Local Anesthetic Reaction with Cephalin

Calcium, tetracaine, and butacaine were added to cephalin sols as indicated in Table II. The concentrations were chosen to produce an excess over that required to produce complete coagulation of the phospholipid sol. The coagulated phospholipid was separated by centrifugation and the residual calcium, or local anesthetic, present in the supernatant was analyzed by flame photometry (calcium) or ultraviolet absorption (tetracaine and butacaine). It

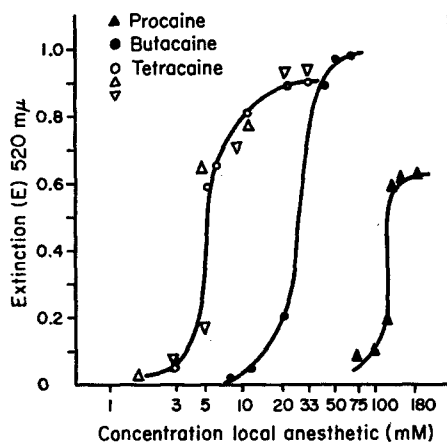


FIGURE 3. The effect of local anesthetics on the turbidity of aqueous dispersions of phospholipids. Tetracaine HCl was added to cephalin (○), phosphatidyl ethanolamine (Δ), and phosphatidyl serine (▽) dispersions. Butacaine sulfate (●) and procaine HCl (▲) were added to cephalin dispersions. Phospholipids and bovine serum albumin were present at a concentration of 0.5 mg/ml each. Turbidity was measured at 520 mμ in a spectrophotometer.

was observed that 1 mole of calcium or local anesthetic disappeared from the supernatant for every 2 moles of cephalin which precipitated. The phospholipid apparently precipitates as a (cephalin)₂-Ca or (cephalin)₂-local anesthetic complex. In some experiments the precipitated phospholipid, after several washes with deionized water, was shaken for several hours with 2 N HCl, and the acid extract analyzed for tetracaine or butacaine. About 70 per cent of the drug could be recovered from the precipitate in this way. Some was lost in the preliminary water washes. Only a very small fraction of the drug extracted with HCl could have been present in any water trapped in the phospholipid precipitate as the precipitate occupied slightly less than 0.05 ml volume out of a total solution volume of 2.0 ml. If the precipitate contained as much as 50 per cent water, which is highly unlikely due to its hydrophobic character, about 5 to 10 per cent of the drug extracted by acid could have been present without necessarily being bound in any way to the phospholipid. However, the preliminary washes with deionized water should have removed almost all unbound drug present in any trapped water. The

tetracaine and butacaine recovered from the precipitate must therefore have come down as a complex with the cephalin. The complex could be split by providing a high H^+ concentration to depress the ionization of the cephalin phosphate groups ($pK_a \simeq 1$).

Titration of Aqueous Dispersions of Cephalin with Salts and Tetracaine HCl

Aqueous dispersions of cephalin were prepared at a concentration of 2 mg/ml (~ 2.5 mM) and adjusted when necessary to a pH of 6.00 ± 0.01 with small

TABLE II
STOICHIOMETRY OF REACTION BETWEEN CEPHALIN
AND CALCIUM OR LOCAL ANESTHETICS

	Cephalin added	Amount calcium or local anesthetic added	Decrease in calcium or local anesthetic in supernatant after total precipitation of cephalin	Ratio of cephalin precipitated to calcium or local anesthetic precipitated
	μmoles	μmoles	μmoles	
Calcium	25	50	11	2.3
	25	50	12.5*	2.0
Tetracaine	25	33.4	13.7†	1.8
	25	33.4	12.3	2.0
Butacaine	25	100	12†	2.1
	25	100	12	2.1

Volume of solution was 2.0 ml in all experiments.

* Determined with Beckman DU flame photometer at 554 $m\mu$.

† Determined with ultraviolet spectral analysis on Bausch and Lomb 505 recording spectrophotometer.

amounts of dilute HCl or NaOH. Portions (30 ml) of the cephalin sol were titrated, under nitrogen, with 0.05 M solutions of NaCl, $CaCl_2$, and tetracaine HCl, the pH of the solution having been adjusted in each case to 6.00 ± 0.01 . The changes in pH were measured with a Radiometer model 25 pH meter equipped with a scale expander and glass and calomel electrodes. NaCl produced a fall in pH which approached a maximum of about 0.3 after 5 to 6 ml had been added. Both $CaCl_2$ and tetracaine produced a pH fall of 1.0 under the same conditions (Fig. 4). Note that the initial rate of fall in pH with increase in Ca^{2+} or tetracaine is markedly greater than with Na^+ indicating the relative affinities for interaction with the phospholipid. It has been observed that uranyl $^{2+}$, curare $^{2+}$ as well as Ca^{2+} ions had similar effects on the pH of cephalin dispersions, and the correspondence between this reaction and the ability of the same substances to inhibit the carrier transport between Na^+ and K^+ across a non-aqueous layer was pointed out (32).

DISCUSSION

Mechanism of Local Anesthetic Reaction with Phospholipids

The evidence that local anesthetics react stoichiometrically with the phosphate groups of phospholipids may be summarized as follows: (a) The addition of salts to phospholipid sols produced a fall in pH (32, 37-40), with polyvalent cations inducing greater pH changes than monovalent cations. The pH fall has been attributed to ionic exchange between the hydrogen ion, associated with the phosphate group of the lipid, and the cation, although in the case of Na^+ and K^+ it is thought that one mole of cation may bind to the carboxyl

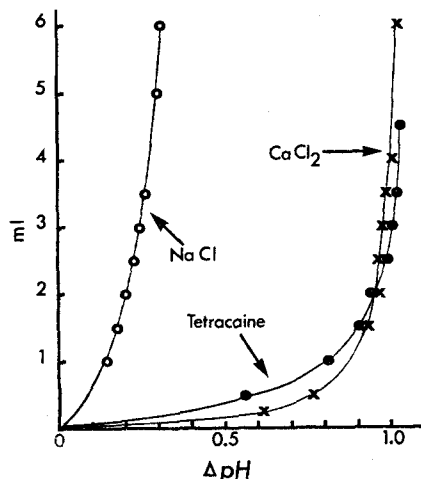


FIGURE 4. Titration of 30 ml aliquots of a cephalin dispersion in water by 0.05 M solutions of NaCl, CaCl_2 , and tetracaine HCl. All solutions were adjusted to $\text{pH } 6.00 \pm 0.01$ before titration. ΔpH indicates the fall in pH observed as the salts were added. Temperature 22°C , atmosphere N_2 .

group of serine (37, 39), while a second mole may bind to the phosphate group (37). The marked release of H^+ by calcium suggested that calcium was bound to the strongly acidic phosphate group. Tetracaine produced a fall in pH of cephalin sols equivalent to that due to Ca^{2+} which indicates that this compound also reacts with the phosphate group. Tetracaine reacted in the same way with phosphatidyl ethanolamine and inositide so that the reactive site in this case must also be the phosphate group as there is no carboxyl group present. (b) The formation of complexes between calcium and cephalin with a composition of 2 moles cephalin/mole calcium has previously been reported (38-41) and is confirmed in this paper. Tetracaine and butacaine form complexes of exactly the same type; *i.e.*, tetracaine $(\text{cephalin})_2$ and butacaine $(\text{cephalin})_2$. The local anesthetics (as well as calcium) probably bind two molecules of phospholipid together by forming a linkage through the two phosphate groups. (c) Gershfeld (42) observed that procaine reacted with monomolecular films of *n*-octadecylphosphate at an air/water surface. His

analysis of force:area curves for the films indicated that procaine penetrated into the films and oriented itself horizontally to the surface between the polar groups of the perpendicularly oriented *n*-octadecylphosphate molecules. Binding at the phosphate groups was also indicated by the fact that procaine did not penetrate uncharged monolayers of *n*-octadecyl alcohol or methyl stearate. The horizontal orientation of procaine at the air/water surface was attributed to the possession by procaine of two hydrophilic amino groups (tertiary aliphatic $pK_a = 9.05$ and aromatic amino $pK_a = 2.2$) at opposite ends of the molecule.

The reactions between each of the phospholipids and local anesthetics studied have certain similarities, but significant differences have also been noted which depend upon the chemical properties of the phospholipid. Although local anesthetics react with lecithin and the cephalins to inhibit uptake of calcium, neither these drugs nor calcium produced significant coagulation of lecithin sols. The same is true for sphingomyelin, which like lecithin also contains the quarternary ammonium group of choline. Dervichian's experiments (39) offer an explanation for this result. He titrated phosphatidyl serine and lecithin with salts of Na^+ , K^+ , Ca^{2+} , and Cu^{2+} . Phosphatidyl serine behaved as shown in Fig. 4 of this paper (and as also observed by Rosano *et al.*, reference 32; and Abramson *et al.*, reference 37); that is, Ca^{2+} was much more potent than Na^+ or K^+ in displacing H^+ from the lipid. In striking contrast was the response of lecithin (39, see Fig. 7) to Na^+ and Ca^{2+} both of which produced equally weak effects on pH and no coagulation, whereas Cu^{2+} had a strong H^+ liberating effect and also coagulated the lecithin sols. The Cu^{2+} apparently formed a $Cu-(lecithin)_2$ complex. The difference in reactivity of lecithin compared to the cephalin group probably resides in the different chemical properties of these compounds. At pH's in the physiological range lecithin does not exhibit acidic properties. It exists as a zwitterion over the pH range 3–11 due to the internal neutralization of the phosphate group by the strongly basic quarternary nitrogen of choline. Phosphatidyl serine on the other hand has three dissociable groups: amino ($pK_a \sim 10$), carboxyl ($pK_a \sim 3.8$ to 4.6), and phosphate (37, 43). The isoelectric point of phosphatidyl serine in 0.1 M NaCl is 1.2 (37). At this pH the carboxyl group is unionized so that the positively charged amino nitrogen of serine is balanced by the negatively charged phosphate group. The pK_a of the phosphate group must therefore be less than 1.2. It is interesting to note that the pK_a of the phosphate group in phosphatidic acid is 4.1 (39), so that a very considerable increase in acidic strength is produced by esterification with serine. Phosphatidyl ethanolamine like lecithin has two dissociable groups, but in this case the strong acidic phosphate group is balanced by a weak basic group (ethanolamine) so that the isoelectric point is 3.1 (44). The pK_a 's of the phosphate and amine group have been reported to be 1.1 and 8.9 respectively (45). Thus at

physiological pH phosphatidyl serine and phosphatidyl ethanolamine would behave as anions in contrast to lecithin. The marked fall in pH and coagulation of the acidic phospholipid sols produced by calcium and local anesthetics

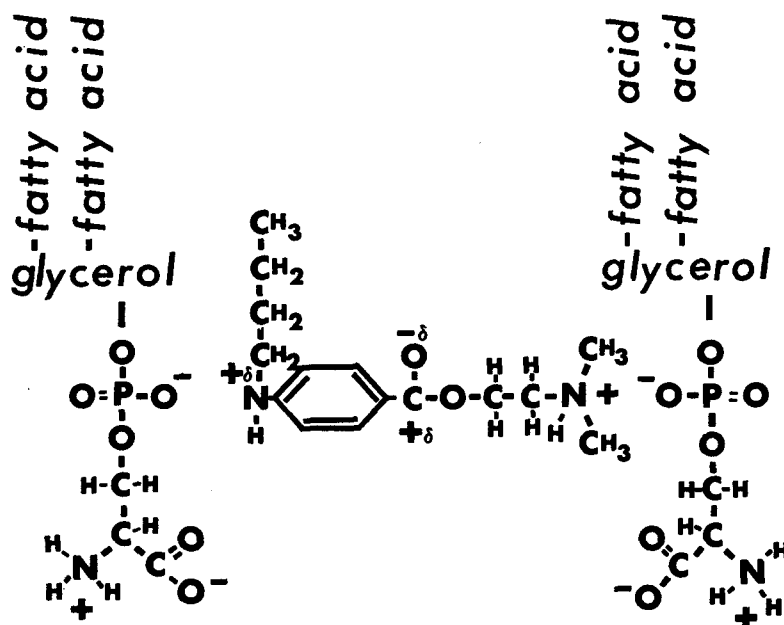
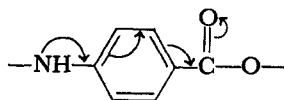


FIGURE 5. Proposed model for the mechanism of complex formation between acidic phospholipids and local anesthetics. The polar ($+\delta$) aromatic nitrogen and the positively charged tertiary alkyl nitrogen groups of tetracaine are shown oriented towards the negatively charged ionized phosphate groups of two phosphatidyl serine molecules. Polarization of the local anesthetics, such as tetracaine derived from *p*-aminobenzoic acid, results from electron displacement in the conjugated double bond system:



The considerable electron delocalization stabilizes the mesomeric structure. The polarizing effect of aromatic amino group would be reinforced by two factors: (a) the electron-repelling inductive effect of the *n*-butyl group attached to the aromatic nitrogen, and (b) the close approach to the negatively charged phosphate group of the phospholipid.

is undoubtedly related to the formation of complexes by reaction with the phosphate groups of two adjacent phospholipid molecules.

From the evidence cited above one may postulate that local anesthetics orient themselves in the membrane vis-à-vis phospholipids in the manner illustrated in Fig. 5 for tetracaine and phosphatidyl serine. At physiological pH the alkyl tertiary amino group of the local anesthetics would bear a formal

positive charge. The aromatic amino group would be essentially uncharged, but it would have polar properties which could be expected to become stabilized as it approached an acidic region such as is found at the surface of a cephalin micelle.¹ Thus, the bonds between the local anesthetic and the phosphate group of the acidic phospholipids would be ion-ion and ion-induced polar in nature. While controversy still exists over the question of the anesthetic activity of charged local anesthetic molecules (7, 47), several examples of anesthetic action dependent upon the cationic form have been reported (15, 48, 49).

Relationship of Reaction between Phospholipids and Local Anesthetics and the Mechanism of Anesthesia

The fact that local anesthetics react with phospholipids in the same manner as does Ca^{2+} suggests that the membrane "stabilizing" properties of both may be due to this specific reaction. The stabilizing properties would be explicable whether one accepts a "pore" theory or carrier theory for membrane permeability. Thus if the phospholipids act as Na^+ and K^+ carriers during excitation the local anesthetics could be potent inhibitors of this function by virtue of their much greater affinity for the phospholipids.² If Na^+ permeability increases during excitation by the opening of functional "pores" within the membrane the local anesthetics could oppose this effect by penetration between the polar groups of the phospholipids, thereby increasing lateral pressure within the membrane (42, 47, 50) or by occluding the pores by reaction with the polar groups lining their surfaces. The local anesthetics also appear to decrease markedly the hydration of cephalin micelles, as was previously noted for Ca^{2+} (18). This might interfere with ion fluxes related to water movement across the membrane (51, 52).

Yet one must point out that doubt has been expressed that Ca^{2+} and local anesthetics have a common locus of action. This view is based on examples of qualitative differences between the action of local anesthetics and Ca^{2+} on some types of nerve tissue. Both local anesthetics and high Ca^{2+} consistently increased the threshold to excitation in squid giant axon (4), and frog spinal ganglion cells (54), but Ca^{2+} increased both rate and height of the spike potential. Local anesthetics had the opposite effect, decreasing spike rate and height

¹ The electrostatic potential at an interface relative to the adjacent conducting phase alters the pH at the surface. At a surface between water and a micelle composed of anionic cephalin molecules the surface pH would be lower than the bulk pH because of the attraction of H^+ into the vicinity of the surface. Experimental evidence for differences between surface and bulk pH values of 3 to 4 units has been reported (46).

² Since the submission of this paper for publication Goldman (53) has presented a detailed theory of the molecular basis for the excitation properties of nerve. The important feature of this theory, which is pertinent to the experiments described above, is that the phosphate groups of certain phospholipids act as ion exchange sites for the transfer of Na^+ and K^+ across the surface of the membrane.

and the associated Na^+ and K^+ currents, as measured by the voltage-clamping method (4). These effects have been interpreted as indicating different sites of action for Ca^{2+} and the local anesthetics. However, they may be interpreted somewhat differently, Ca^{2+} having a dual role, stabilization of the resting membrane by increasing threshold, and an active role in supporting generation of the spike by decreasing the degree of inactivation in the resting state. Local anesthetics may act at the same site as Ca^{2+} to raise the threshold of the membrane, but lack the ability to substitute for Ca^{2+} in supporting the spike potential. The anesthetics may in fact displace or compete with Ca^{2+} at the site involved in spike generation. Shanes (58) suggested that the local anesthetics were not displaced from this site during the spike. It should be noted though, that in many instances Ca^{2+} and local anesthetics do not exhibit the differences in action noted above. At nodes of Ranvier of toad fibers excess Ca^{2+} reduces the amplitude of the spike (55), and in nodose fibers (56) and muscle (57) the rate of rise of the spike is decreased.

After-discharges and oscillations in squid axon (58, p. 192) and depolarization of frog nerve (59) due to Ca^{2+} lack can be blocked by local anesthetics. In these cases the local anesthetics may react with the same anionic sites in the membrane which normally bind Ca^{2+} . The analogy between these effects in tissues and the common reaction of Ca^{2+} and local anesthetic with phospholipids is obvious.

Ehrenpreis has extracted a phospholipoprotein from the electrical organ of electric eel (60, 61), which combines with Ca^{2+} and a number of neurotropic agents (60–67). Among these compounds are chlorpromazine, tetracaine, diphenhydramine, eserine, and atropine. A compound with similar properties was isolated from sciatic nerve (62). Ehrenpreis (64) suggests that the ability of these compounds to inhibit nerve conduction is related to reaction with this cellular component. Divalent ions (Ca^{2+}) and the anesthetic drugs are viewed as reacting with phosphate groups of this phospholipoprotein which line membrane pores, thereby impeding influx of Na^+ through the pores. Whether or not the anesthetics combine with the phospholipid portion of the lipoprotein has not been reported, but it behaves very much like the acidic phospholipids towards Ca^{2+} and local anesthetics.

Caffeine rigor in frog striated muscle is accompanied by an increase in calcium efflux from the muscle cells. Inhibition of the rigor by local anesthetics results in a concomitant abolition of the increased calcium efflux (12). The stimulation of striated muscle by caffeine is thought to be the result of a release of calcium from binding sites in the cell and it was therefore of interest to determine whether this alkaloid would alter the calcium-binding properties of phospholipids. The results were entirely negative. Caffeine neither inhibited calcium binding by cephalin or extracted frog muscle lipids, nor did pretreatment of frog muscle with caffeine result in any decrease in the extractability of

calcium-binding lipids (unpublished experiments). Thus the mechanism for caffeine's effect upon calcium mobility in frog muscle, and its inhibition by local anesthetics remains obscure.

Do Local Anesthetics and the Gaseous General Anesthetics Have a Common Mechanism of Action?

The effects of the local anesthetics described in this paper are dependent upon interactions with phospholipids through electrostatic forces. It is significant therefore that Pauling's recent discussion of his "hydrate microcrystal" theory of anesthesia (65) considers the tendency of gaseous anesthetics to form stable clathrate crystals with water to be dependent upon electric polarizability of the gas atoms. Pauling discounts interaction with lipids as a mechanism for action of gaseous anesthetics. However, a very striking correlation has been demonstrated between the potency of gaseous (66) as well as local anesthetics (46, 66) and their ability to penetrate into monomolecular films of polar lipids and thereby produce equivalent increases in film pressure. It would not be surprising if the tendency of gaseous anesthetics to react with the phospholipid film were also related to polarizability of the gas atoms. Considering the evidence, that the lipid portion of the nerve membrane is more vital for the maintenance of function than is the protein (67), the reaction of gaseous and local anesthetics with monomolecular lipid films attains greater significance. Recent experiments in brain also indicate a common mode of action for gaseous and local anesthetics (68).

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