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PHOSPHOLIPID MODEL MEMBRANES

III. ANTAGONISTIC EFFECTS OF Ca^{2+} AND LOCAL ANESTHETICS ON THE PERMEABILITY OF PHOSPHATIDYLSERINE VESICLES

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SUMMARY

Five local anesthetics (dibucaine, tetracaine, cocaine, lidocaine and procaine) were studied for their effect on the permeability of phospholipid model membranes. The membrane system was the unilamellar vesicles produced by sonication of phosphatidylserine in aqueous salt solution. The permeability property measured was the efflux of $^{22}Na^+$. The results indicate the following:

1. In the absence of Ca^{2+} the local anesthetics tend to increase the $^{22}Na^+$ efflux according to their relative nerve-blocking potency but at comparatively high concentrations.

2. In the presence of Ca^{2+} the local anesthetics completely inhibit the Ca^{2+}-induced high efflux rate of $^{22}Na^+$. This inhibition is observed at physiologically relevant concentrations and relative potencies comparable to the relative nerve-blocking potencies as reported in literature.

3. As Ca^{2+} concentration increases, the ability of each local anesthetic to inhibit the Ca^{2+}-induced increase of permeability is diminished, indicating antagonistic effects.

4. Tetracaine is more active at low pH (6.0) compared to pH's of 7.4 or 9.0.

5. The electrophoretic mobility of phosphatidylserine particles is diminished in the presence of each local anesthetic, indicating neutralization of the negative charges at the surface of the phospholipid particles. The ability of each anesthetic to reduce the mobility of phosphatidylserine is also related to the potency of each compound.

It was concluded that the similarity of the behavior of the system of artificial phosphatidylserine membranes studies here, to the properties of nerve membranes suggests a possible involvement of this phospholipid at the physiological site of action of local anesthetics and in general during the excitation process.

INTRODUCTION

It is generally recognized that local anesthetics exert their action on nerve membrane by inhibiting the increased Na^+ conductance associated with generation

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

of the action potential[1]. It appears that the mechanism of action involves external Ca^{2+} and that both local anesthetics and Ca^{2+} compete for the same "sites" on the nerve membrane[2,3]. The nature of these sites is still not known, in spite of the accumulating electrophysiological data on the effects of local anesthetics on nerve axons[4,5].

FEINSTEIN[6], on the basis of competitive binding studies with a two-phase model system, suggested that negative groups of phospholipid molecules might serve as the binding sites for Ca^{2+} and local anesthetics on membranes. Subsequently, BLAUSTEIN AND GOLDMAN[7,8] substantiated and extended these observations utilizing a variety of drugs and concluding similarly that acidic groups of phospholipids might be the physiologically active binding sites on nerve membranes.

Although the binding studies produced evidence on Ca^{2+}–local anesthetic antagonism for phospholipid negative groups, they provided no indication of the effect of this antagonism on the permeability of phospholipid membranes. Evidence on this point was forthcoming through the use of artificial membranes. FEINSTEIN[6] utilized the Millipore filter system introduced by TOBIAS *et al.*[9] and observed an increase of electrical resistance in the presence of tetracaine. Using an entirely different system, BANGHAM *et al.*[10] reported a decrease of the permeability of negatively charged liposome membranes in the presence of local anesthetics. Subsequently, PAPAHADJOPOULOS AND BANGHAM[11] and OHKI AND PAPAHADJOPOULOS[12] reported that tetracaine inhibited the Ca^{2+}-induced increase in cation permeability of vesicles (liposomes) composed of phosphatidylserine.

The technique of monolayer penetration has also been used to study the mechanism of interaction of anesthetics with membrane constituents[13–15]. SKOU[13] has observed that the rate of penetration of several local anesthetics into monolayers of lipids extracted from nerve gave excellent correlation of the potency of the same compounds in blocking the excitation of nerve axons.

Although previous work with model membranes[10,13] has shown good correlation of potency of local anesthetics with their ability to penetrate lipid membranes, it does not take into account the well-known antagonism of local anesthetics with Ca^{2+} on nerve axon membranes[2,3] and on artificial two-phase systems containing phospholipids[6,7]. The present work describes a model membrane system which is responsive to both Ca^{2+} and local anesthetics. This system is based on the use of liquid–crystalline phospholipid vesicles[11,16] and was originated by BANGHAM *et al.*[17]. The ability of five different local anesthetic agents to inhibit the Ca^{2+}-induced increase in permeability of phosphatidylserine vesicles correlates very well with the relative potency of these agents. These results provide further evidence for the previously postulated[6,7] antagonism of Ca^{2+} and local anesthetics for phospholipid-negative sites on nerve axon membranes. Furthermore it identifies the possible phospholipid species that might be involved in the local anesthetic action. Finally, the similarity of of the properties of the present model system with those of natural membranes gives further credence to a recently proposed mechanism for phospholipid participation in excitable membranes[12].

MATERIALS AND METHODS

Phosphatidylserine

Phosphatidylserine in chromatographically pure form was prepared from beef brain by a combination of the extraction method of FOLCH[18] and column chromato-

graphy based on the method of ROUSER *et al.*[19]. The whole procedure has been described in detail elsewhere[20]. As isolated by this method, phosphatidylserine is in the protonated form and has to be converted to the salt form by titrating the carboxyl groups with an equivalent amount of the appropriate base. The utmost care was taken to avoid air oxidation during the preparation and during storage. The purified phospholipids were stored as a solution in chloroform in sealed ampules under N_2 at $-50°$. Each ampule contained approx. 10 μM of phosphate in 1 ml of solution and was newly opened for each experiment.

Phosphatidic acid

Phosphatidic acid was obtained by enzymatic hydrolysis of egg yolk lecithin according to a procedure described earlier[20].

Local anesthetics

Dibucaine (Nupercaine) was obtained from K and K Laboratories, in the HCl form (95–99% purity). Tetracaine and procaine from Mann Research Laboratories were also in the HCl form (over 95% purity). Cocaine was obtained from Merck and Co. as HCl (USP purity) and lidocaine (xylocaine) from Astra Pharm. Prod. (USP purity). They were all used without any further purification.

Other chemicals

L-Histidine·HCl (Sigma grade) and TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid) were both obtained from Sigma Chem. Co. Sephadex G-50 (coarse) was obtained from Pharmacia. $^{22}Na^+$ was obtained from New England Nuclear as a solution of NaCl (10 mC/mg minimal specific activity). All other chemicals were analytical reagent grade. Water was twice distilled, the second time in all-glass apparatus.

Formation of phospholipid vesicles

The procedure used was basically similar to a previously described method[16], modified to avoid air oxidation. The chloroform solution of phosphatidylserine (usually 10 μmoles estimated by phosphate determination) was transferred from a newly opened ampule to a glass tube which has been flushed with a stream of high purity N_2 (Linde, H.P. Dry, 99.996%). The chloroform was evaporated under vacuum while the tube was rotated by hand in a water bath at 22–24°. N_2 was then allowed to flow over the dried phospholipid, while 2 ml of a buffer solution containing $^{22}Na^+$ (10–20 μC per experiment) were pipetted into the tube. The buffer solution contained NaCl (100 mM), histidine (2 mM), TES (2 mM), adjusted to pH 7.4 by the addition of 0.1 M NaOH unless otherwise specified. This buffer combination can be successfully used over a wide pH range, 5.0–10.0. A small stream of N_2 (previously saturated with water by passing through a gas-washing bottle) was passed through the tube during the rest of the procedure. The phospholipid was dispersed into the aqueous solution by mechanical (Vortex) shaking for 10 min. The suspension was then sonicated for 30 min by placing the tube in a small bath-type sonicator (Heat systems, Model 5 × 5, 40 kHz). The temperature of the sonicating bath was maintained at approx. 22° by circulating cold water. After the sonication, the suspension was almost clear ($A_{700\,nm} = 0.01\ mM^{-1} \cdot cm^{-1}$) and the phospholipid was in the form of small unilamel-

lar vesicles[20,21]. The suspension was then left at room temperature in a N_2 atmosphere for 1 h before further processing.

Measurement of permeability

The procedure was a modification of the method described by BANGHAM *et al.*[17]. The suspension of vesicles was dialyzed by elution through a 2.7-g column of Sephadex G-50 (coarse) with the same buffer. The lipid peak, which was eluted with the void volume, was collected between 12 and 17 ml of eluting buffer. Aliquots of it (0.5 ml), containing 0.5–1.0 μmole of phosphate, were transferred into small pre-washed dialysis bags (1.0 cm width). After a preliminary 30-min dialysis against buffer, the bags were transferred into individual tubes filled with 10 ml of buffer containing the appropriate amount of the local anesthetic under study. At the end of a 60-min period, the bags were transferred into new tubes filled with 10 ml of buffer containing again different amounts of the anesthetics in addition to a fixed concentration of $CaCl_2$ as indicated. For most of the experiments with phosphatidylserine, the concentration of $CaCl_2$ was 2 mM. The controls were tubes containing just buffer, buffer with anesthetic only, or buffer with Ca^{2+} only. The amount of radioactive $^{22}Na^+$ contained in the dialysate and in the bag after the 1st- and 2nd-h intervals were determined in a liquid-scintillation counter. The results were tabulated as the amount of $^{22}Na^+$ diffusing through the vesicles and the bag into the dialysate at 1-h intervals and expressed as the percentage of the total $^{22}Na^+$ present inside the bags at the beginning of the time period. The total capture of $^{22}Na^+$ was calculated as 0.4 μequiv/μmole of phosphate. The rate of diffusion of $^{22}Na^+$ through the phosphatidylserine vesicles in the absence of either anesthetic or Ca^{2+} was 0.1 %/h or $0.62 \cdot 10^{-3}$ μequiv/μmole per h. From this, the ionic flux can be estimated assuming a 55-Å area per molecule and an average of two lamellae per vesicle[16]. The flux for Na^+ was calculated to be $2.0 \cdot 10^{-16}$ equiv/cm^2 per sec which is very low compared to biological membranes[22]. However, it is in accordance with the high electrical resistance obtained with bilayer (black film) phosphatidylserine membranes[23,24]. The value quoted for Na^+ flux through phosphatidylserine membranes is considerably lower than the value reported earlier[16]. It is apparent from very recent work (unpublished observations) that air oxidation could be responsible for the difference. The more successful the avoidance of exposure to air during purification, storage, sonication and evaporation, the more impermeable these artificial membranes appear to be.

Electrophoretic mobility

The mobility was determined by microelectrophoresis in a cylindrical cell[25]. Phosphatidylserine was evaporated to dryness and suspended at room temperature in buffered NaCl solution at a concentration of 3 mM by mechanical shaking for 10 min. Aliquots (0.5 ml) of this suspension were then equilibrated by dialysis for 1 h at room temperature against the same buffered salt solution containing various concentrations of local anesthetics. Finally, the dialyzed suspensions were diluted with the same buffered salt/anesthetic solution to a concentration of 0.15 mM and equilibrated at 30° for 30 min to 1 h just prior to the determination of the electrophoretic mobilities.

RESULTS AND DISCUSSION

Effect of local anesthetics on $^{22}Na^+$ efflux through phosphatidylserine vesicles

Five different anesthetics were studied at a concentration ranging from 0.1 to 20 mM. The total concentration of salt was kept 100 mM in NaCl and 4 mM in histidine–TES buffer (pH 7.4). The results are given in Fig. 1. In general, it appears that at low concentrations of anesthetic there is no appreciable effect on permeability. At high concentrations the efflux of $^{22}Na^+$ increases appreciably. Dibucaine and tetracaine produce a 10-fold increase (from 0.1 to 1.0%/h) at concentrations of 1 mM, while the other anesthetics produce a similar effect at concentrations between 5 and 20 mM.

The ability of each anesthetic to increase $^{22}Na^+$ efflux seems to be correlated to the relative potency of each drug, but the concentrations needed for an appreciable effect are much higher than those effective either on nerve membranes or on the system containing Ca^{2+} described below.

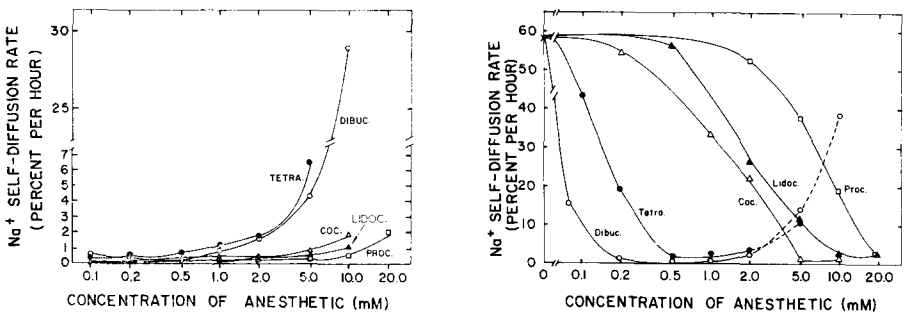

Fig. 1. Effect of local anesthetics on the $^{22}Na^+$ self-diffusion rate through phosphatidylserine vesicles. Buffer solution: 100 mM NaCl, 4 mM histidine–TES buffer (pH 7.4). Self-diffusion rate is expressed as percent $^{22}Na^+$ diffusing out of the vesicles per h. For total capture of $^{22}Na^+$ and diffusion rate in equiv/h, see MATERIALS AND METHODS. ○, dibucaine; ●, tetracaine; △, coacaine; ▲, lidocaine; □, procaine. All experiments performed at room temperature (22°).

Fig. 2. Effect of local anesthetics on the $^{22}Na^+$ self-diffusion rate through phosphatidylserine vesicles in the presence of 2 mM $CaCl_2$. For details see MATERIALS AND METHODS. The effect of 2 mM $CaCl_2$ in the absence of anesthetics is shown by ×. ○, dibucaine; ●, tetracaine; △, cocaine; ▲, lidocaine; □, procaine. The values for minimal blocking concentrations given in Table I are taken from the intercept of each curve with the horizontal line at the 10% diffusion rate.

Effect of local anesthetics in the presence of 2 mM Ca^{2+}

As previously described, Ca^{2+} added on one side of phosphatidylserine membranes produces instability which is reflected either in a lowering of d.c. resistance and breaking of membranes[23] or in an abrupt increase of the efflux of ions through vesicles composed of the same phospholipid[11,16]. If Ca^{2+} is added in the presence of tetracaine, the change in permeability is partially or completely inhibited[11,12]. Experiments with five different anesthetics, described in Fig. 2, indicate that the inhibition of the Ca^{2+} effect occurs at concentrations of each anesthetic similar to those needed for blocking the action potential of nerve membranes[2,3,5]. At low anesthetic concentrations, the efflux of $^{22}Na^+$ is very high due to the effect of Ca^{2+} (2 mM in all these experiments). As the concentration of anesthetic is increased, the $^{22}Na^+$ efflux is reduced to very low levels, comparable with the controls containing anesthetic alone.

Thus the Ca^{2+}-induced high efflux of $^{22}Na^{+}$ can be inhibited completely. Table I is a compilation of data taken from Fig. 2 and from the work of others[7,13] on nerve membranes, indicating minimum concentrations for blocking and the relative potency of the local anesthetics. There seems to be a fairly good correlation between the effects on real and artificial membranes.

TABLE I

COMPARISON OF RELATIVE POTENCIES OF LOCAL ANESTHETICS ON NERVE AND PHOSPHATIDYLSERINE MEMBRANES

Anesthetic	*Data of* SKOU*		*Data of* BLAUSTEIN AND GOLDMAN**	*From Fig. 2****	
	Minimum blocking concn. (mM)	*Relative blocking potency*	*Relative blocking potency*	*Minimum blocking concn. (mM)*	*Relative potency*
Procaine	4.6	1	1	13.5	1
Lidocaine	—	—	3.8	5.5	2.5
Cocaine	2.6	1.8	—	3.5	3.9
Tetracaine	0.01	460	36	0.3	45
Dibucaine	0.005	920	53	0.1	135

* Taken from ref. 13, obtained with desheathed frog sciatic nerve at pH 7.0.

** Taken from ref. 7, obtained with frog sciatic nerve trunk at pH 7.2.

*** The concentrations of each local anesthetic producing the same degree of inhibition to Ca^{2+} as shown in Fig. 2. In this case the arbitrary value of 10% per h was taken for comparison. The values shown in this column as minimum blocking concentrations were obtained from the intercept of each curve with the horizontal line at 10% $^{22}Na^{+}$ self-diffusion rate. Concentration of $CaCl_2$ during the experiment, 2 mM.

It should be pointed out that, of all the different purified phospholipids studied as artificial membranes, only phosphatidylserine gives good correlation of the properties of natural (axon) membranes in terms of both the concentration of Ca^{2+} and the concentration of local anesthetics. The neutral phospholipid phosphatidylcholine does not have appreciable affinity for Ca^{2+} (see refs. 16 and 26). Of the other acidic phospholipids, phosphatidylinositol and phosphatidylglycerol show changes in permeability only at higher Ca^{2+} concentrations[16,27] and were not tested with anesthetics. Finally, phosphatidic acid at pH 7.4, which is close to the pK_2 for the phosphate group[26], is very sensitive to even a low (0.5 mM) Ca^{2+} concentration[16,27]. However, when phosphatidic acid was studied under conditions similar to those described here for phosphatidylserine (except at 1 mM $CaCl_2$), it was found that local anesthetics could not completely inhibit the Ca^{2+}-induced permeability. There was only partial inhibition at relatively high anesthetic concentrations (1.0 mM for dibucaine and 20 mM for cocaine) compared with the phosphatidylserine system. This could be explained if the affinity constant of the phosphatidic acid–Ca^{2+} complex is higher than that of the phosphatidic acid–anesthetic complex and thus the competition would favor the phosphatidic acid–Ca^{2+} complex.

Effect of raising $CaCl_2$ concentration

All the experiments with phosphatidylserine described above were performed with 2 mM $CaCl_2$. When $CaCl_2$ concentration was increased to 5 or 10 mM, the effec-

tiveness of the anesthetics decreased. These experiments are described in Fig. 3. Curve I shows the effect of Ca^{2+} on the permeability of phosphatidylserine vesicles to $^{22}Na^+$ in the absence of anesthetics. Here, 2 mM $CaCl_2$ produces an abrupt increase in $^{22}Na^+$ efflux. When cocaine (Curve II) is present at 5.0 mM concentration, 2 mM Ca^{2+} produces only a small increase in $^{22}Na^+$ efflux, and now 5 mM $CaCl_2$ is needed to produce high permeability to Na^+. The same antagonism can be seen in Curves III and IV with 0.5 mM dibucaine and 1.0 mM tetracaine, respectively. While both drugs are effective in inhibiting the Ca^{2+} effect at 2 and 5.0 mM $CaCl_2$ concentration, they become ineffective when $CaCl_2$ concentration is increased to 10 mM. Similar results have been obtained with nerve membranes and procaine at similar concentrations of Ca^{2+} (see ref. 2).

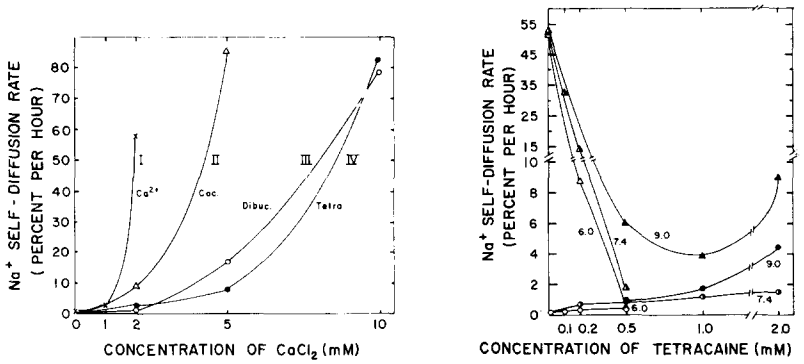

Fig. 3. Effect of increasing $CaCl_2$ concentrations of the potency of several local anesthetics. Curve I ($\times$—$\times$) represents the effect of $CaCl_2$ on phosphatidylserine vesicles without anesthetic. Curves II, III and IV show the effect of different $CaCl_2$ concentrations in the presence of: △, 5 mM cocaine; ○, 1.0 mM tetracaine; ●, 0.5 mM dibucaine. It is apparent that 2 mM $CaCl_2$ is antagonized by the anesthetics, while higher $CaCl_2$ concentrations compete successfully.

Fig. 4. Antagonism of tetracaine and Ca^{2+} at three different pH values. The effect of different concentrations of tetracaine alone on the diffusion rate of $^{22}Na^+$ through phosphatidylserine vesicles is represented by circles: ●, at pH 9.0; ◑, at pH 7.4; and ○, at pH 6.0. The effect of tetracaine in the presence of 2 mM of $CaCl_2$ is represented by triangles: ▲, at pH 9.0; ◭, at pH 7.4; and △, at pH 6.0.

Effect of varying pH

The question as to whether the ionized (positively charged) or nonionized form of a local anesthetic is the active species has been a point of controversy[1]. More recent investigations tend to favor the ionized species as the active one[1,4,5]. However, pH changes in the bathing solution of an axon membrane can affect the ionization of the anesthetics as well as groups of similar pK values in the membrane. With the model membrane system described here, no detectable change occurs in the ionization of the carboxyl and amino groups at the surface of phosphatidylserine at pH 6–9 (see ref. 26). It therefore seemed worthwhile to study the effect of local anesthetics on the phosphatidylserine membranes at different pH's within this range. The results of such an experiment are given in Fig. 4. It is clear that tetracaine is more effective in inhibiting the Ca^{2+}-induced permeability change at pH 6.0 compared with pH 7.4 or 9.0. At pH 9.0 tetracaine does not produce complete inhibition even at high concentrations. This result tends to support the earlier evidence that the positively charged form of these anesthetics is the active species in membranes. This evidence also favors

the concept of the competition between Ca^{2+} and local anesthetics (both strongly binding cations) for the anionic sites of the membranes (biological or artificial). It is also noteworthy (although not clearly understood) that tetracaine is more effective (in the absence of Ca^{2+}, lower curves) in inducing an increase in $^{22}Na^{+}$ efflux at high pH (9.0) compared with pH 7.4 or 6.0.

Influence of local anesthetics on the surface charge of phosphatidylserine vesicles

The binding of positively charged molecules of local anesthetics to the surface of negatively charged phosphatidylserine vesicles would be expected to reduce the surface charge density by a factor depending on the amount of anesthetic bound. Such a reduction occurs when Ca^{2+} binds to phosphatidylserine vesicles[26]. The method of microelectrophoresis has been used to measure a change of the electrokinetic potential after metal binding on phospholipid particles[28] or biological cells[29]. It has also been used recently to investigate the reactivity of amino groups at the surface of phospholipid particles[30]. Although the numerical value of electrophoretic mobility is not easily translated into absolute values for surface charge density[31], it provides a useful indication of changes in this parameter due to neutralization of charges within a distance of a few Å from the hydrodynamic slip plane[32].

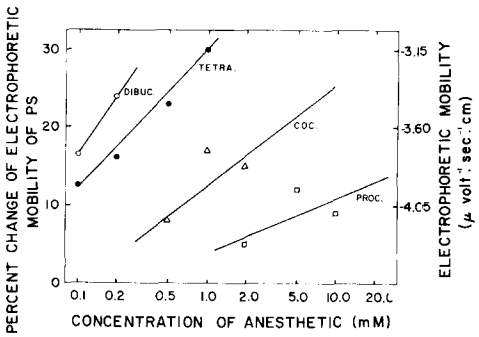

Fig. 5. Electrophoretic mobility of phosphatidylserine vesicles in the presence of local anesthetics. The mobility of phosphatidylserine in buffer alone was found to be $-4.5\ \mu \cdot sec^{-1} \cdot V^{-1} \cdot cm$. The mobility in the presence of the anesthetics was found to be lower and the numerical difference of the two mobilities at each concentration of anesthetic was expressed as a percentage of the initial mobility. The scale of the actual mobilities is also given on the left of the figure. ○, dibucaine; ●, tetracaine; △, cocaine; □, procaine.

The results obtained with local anesthetics and phosphatidylserine particles are summarized in Fig. 5. The electrophoretic mobility of the phosphatidylserine particles in 0.1 M NaCl at pH 7.4 in the absence of local anesthetics or Ca^{2+} is $-4.5\ \mu \cdot sec^{-1} \cdot V^{-1} \cdot cm$. In the presence of 2.0 mM $CaCl_2$ it is reduced to -1.3, a change of 71 %. In Fig. 5 the reduction of electrophoretic mobility (expressed as percent change) is plotted against the concentration of different amounts of anesthetic. The experiment is similar to those mentioned earlier with measurements of permeability except that the phosphatidylserine particles were not sonicated. This step was omitted because it reduces the size of the phospholipid particles to submicroscopic levels. It is apparent from Fig. 5 that the local anesthetics do not produce as great a reduction of the electrophoretic mobility of phosphatidylserine particles as does Ca^{2+}. However, the concentration of each anesthetic needed for a 15 % change in mobility is very similar

to the concentration of the same anesthetic producing appreciable inhibition of the Ca^{2+} effect, as shown in Fig. 2.

CONCLUDING REMARKS

The permeability studies described in this paper were performed with unilamellar phospholipid vesicles (approx. 500 Å diameter) obtained by ultrasonic radiation as described in detail in MATERIALS AND METHODS. Similar results have also been obtained with the larger multilamellar vesicles (liposomes) obtained by simple mechanical dispersion[20,21].

It appears from the microelectrophoresis experiments that the potency of local anesthetics is related to their ability to bind to lipid bilayer membranes. This result is in good agreement with the earlier studies of SKOU[13] on the penetration of lipid monolayers by the same drugs. These studies indicated that the same number of molecules of each drug must bind to the surface and penetrate the membrane before an effect can be recorded. As SKOU has remarked, the apparent correlation between penetration and nerve-blocking potency need not necessarily imply that physical changes produced by penetration in the membrane are decisive for the blockage[13]. The evidence presented in Figs. 1 and 2 seems to support the above supposition. Thus, it appears that at low concentrations local anesthetics have no appreciable effect on the permeability of phosphatidylserine membranes, although their effect on electrophoretic mobility indicates considerable binding. However, the same low concentrations are effective in producing full inhibition of the Ca^{2+}-induced increase of permeability of the same membranes. Higher concentrations of each local anesthetic induce an increase in Na^+ efflux even in the absence of Ca^{2+}. Essentially similar results were obtained recently (S. OHKI, personal communication) with bilayer membranes composed of phosphatidylserine.

These results at first sight appear to contradict earlier data presented by BANGHAM *et al.*[10] where low concentrations of local anesthetics were shown to decrease the permeability of multilamellar phospholipid particles. However, the phospholipid system used by BANGHAM *et al.* was egg lecithin mixed with 15 % dicetyl phosphate which tends to produce membranes relatively permeable to K^+ and Na^+. The phospholipid used here (beef brain phosphatidylserine) produces very impermeable membranes in spite of the fact that they are negatively charged[16,27]. Thus, the neutralization by local anesthetics of the phosphate groups of dicetyl phosphate (whose presence produces increased permeability to K^+ or Na^+) would be expected to reduce the permeability to these ions. It should be pointed out that local anesthetics produce a decrease of the permeability of the resting axon membrane[1], an effect essentially similar to the one described by BANGHAM *et al.*[10] with artificial membranes and also similar to the effect on phosphatidylserine–Ca^{2+} membranes described here.

The mechanism of the increase in permeability of phosphatidylserine membranes at relatively high concentrations of local anesthetics, as shown in Fig. 1, could be rationalized in terms of an asymmetric distribution of these compounds in relation to the two sides of each membrane. This rationalization is based on evidence presented earlier[12,23] that asymmetric distribution of Ca^{2+} or fixed negative charges between the two surfaces of phosphatidylserine bilayer membranes reduces the electrical resistance and produces instability. This argument is also consistent with the finding

(S. OHKI, personal communication) that when local anesthetics are present on both sides of phosphatidylserine bilayer membranes they are considerably less effective in reducing the electrical resistance compared with when they are added on one side only.

It is clear from the above discussion that, although the potency of local anesthetics is determined by their ability to penetrate lipid membranes, their mechanism of action need not necessarily be directly connected with such nonspecific interaction. On the contrary, it appears from the results presented here that the mechanism of action could be correlated with the ability of the positively charged species of these "amphipathic" compounds to compete with Ca^{2+} for the anionic sites of negatively charged phospholipid molecules. Results obtained thus far indicate that of all phospholipids studied phosphatidylserine seems to be the physiologically active species for this interaction. The competitive binding was shown first by FEINSTEIN[6] in a water–chloroform biphasic extraction system and later by BLAUSTEIN AND GOLDMAN[7]. The mechanism is also supported further by the results of HAUSER AND DAWSON[33] who showed that local anesthetics can displace Ca^{2+} from monolayers of phosphatidylinositol at the air–water interphase. The binding of Ca^{2+} onto phosphatidylserine monolayers and membranes has also been observed before[16,26].

The mechanism by which Ca^{2+} induces the increased efflux of Na^+ through the vesicles, shown in Figs. 2 and 3, is still not well understood. However, this phenomenon has been described in detail and discussed in previous publications[11,12,16,20,23,27]. Based on the evidence obtained both from vesicles and black films, the suggestion was made that phosphatidylserine membranes become unstable only when Ca^{2+} is added to one side of the membrane[12,13]. The instability due to the asymmetric distribution of Ca^{2+} is manifested initially as a drop in the d.c. resistance and finally as a rupture of the membrane, when Ca^{2+} concentration reaches a certain concentration[23]. The observed instability has been attributed to the difference in surface energy between the two surfaces of the membrane[23] which would be expected to produce "inversion" of molecules or clusters of molecules of phosphatidylserine from one side of the membrane to the other. The binding of local anesthetics to phosphatidylserine membranes would of course be expected to reduce the affinity of the membranes for Ca^{2+} and thus inhibit the Ca^{2+} effect on the membrane permeability, as observed earlier in this paper.

It would appear that, in agreement with previous suggestions[6,7], the mechanism of action of local anesthetics involves: (1) Binding and penetration into a lipid membrane through the lipophilic part of the molecule. (2) Interaction of the positively charged amine groups with negatively charged phospholipid head groups (mostly phosphate and carboxyls). (3) The above interaction results in displacement of Ca^{2+} which is no longer available for the production of an action potential during excitation.

The experimental system used in the present study provided interesting information on the correlation between the nerve-blocking potency of local anesthetics and the inhibition of Ca^{2+}-induced permeability changes across phospholipid membranes. The same phospholipid system has been proposed recently as a model relating to the mechanism of nerve excitability and involving an unstable state due to an asymmetric distribution of Ca^{2+} (refs. 12 and 23). The present results can also be taken as further support for this mechanism. However, it must be emphasized here that such correlations between artificial and biological membranes can only be taken as suggestive evidence, in recognition of the limits of an extrapolative approach.

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