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EFFECT OF LOCAL ANESTHETICS ON PHOSPHOLIPID BILAYERS

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SUMMARY

A study was made on the effect of four local anesthetics (nupercaine, tetracaine, cocaine and procaine) on d.c. resistance and the inhibition of Ca^{2+} binding of various phospholipid membranes. When the local anesthetics were applied on one side of the membrane, the electrical resistance of phospholipid membrane decreased with the increase of the concentrations of the local anesthetics. On the other hand, when the local anesthetics were applied on both sides of the membrane, the membrane showed high resistance at low concentrations of the local anesthetics. Also, the instability of the acidic phospholipid asymmetric membrane due to Ca^{2+} binding on one side of the membrane was inhibited by the presence of the local anesthetics in the solution. The potency of the lowering resistance of the membrane and the inhibition of instability of the asymmetric membrane with Ca^{2+} binding by the local anesthetics was similar to earlier works (nupercaine > tetracaine > cocaine > procaine).

INTRODUCTION

Phospholipid membranes have recently become a subject of intensive research as a model for biological membranes. Since most biological membranes are complex mixtures composed predominantly of lipids and proteins, artificial membranes may serve as a very simple model for the biological membranes. Nevertheless, some of the properties of such artificial membranes have been shown to have striking similarities to those of the biological membranes[1–3]. One of the studies of these properties has been the investigation of the effect of local anesthetics on isolated lipid model systems[4]. Some striking correlations have been found between the ability of the local anesthetics to block conduction of nerve impulses and their effects on these model systems, particularly on lipid monolayers at air–water interfaces[5–10], and on lipid membranes separating two aqueous phases[11–14].

The most widely accepted role of local anesthetics in a descriptive theory centers on their interaction with actual membrane constituents and their related ionic environment. Essentially there are three basic theories currently proposed. The theory that NACHMANSOHN[15] and his co-workers have proposed is that acetylcholine plays a role in nonsynaptic axonal conduction. THIMANN[16] proposed a competition between local anesthetics and acetylcholine at critical membrane sites. The other two

theories involve the role of Ca^{2+} in excitation[17] and so-called "channels" in the cell membrane[18], through which Na^+ and K^+ are assumed to pass. One of the basic studies has been the investigation of the interaction between the local anesthetics and lipid phases. Since Overton and Meyer (see ref. 19) proposed the following theory of narcosis; that is, the narcotic action is produced by dissolving drugs into the lipid phase of nervous tissues, several studies have been made on the correlation between physical properties of anesthetics in oil phases or lipoid phases and their narcotic potency[20–22].

In the studies of monomolecular films, SKOU[5] observed that local anesthetics were able to penetrate a monolayer of stearic acid, and that there was a certain similarity between their ability to penetrate the monolayer and toxic potencies. SHANES[8], SHANES AND GERSHFELD[9] and GERSHFELD[10] have proposed that local anesthetics block impulse conduction by virtue of increasing the lateral pressure of the lipid monolayer that constitutes the nerve membrane, with a resultant occlusion of the pores through which Na^+ and K^+ move.

In the studies of model systems which consist of lipid membranes separating two aqueous phases, FEINSTEIN[11] investigated the effect of local anesthetics on lipid-impregnated millipore filters. He found that the electrical conductance of the millipore filter was decreased in the presence of tetracaine when K^+ or Na^+ was the cation in the soluiton, and suggested the possibility of competition between local anesthetics and Ca^{2+} for phospholipid binding sites *in vitro*. BLAUSTEIN AND GOLDMAN[12,17] have extended the above work and proposed that negative polar groups of phospholipid molecules in nerve membranes might serve as physiologically active sites. BANGHAM *et al.*[13] and OHKI AND PAPAHADJOPOULOS[14] have studied the effect of local anesthetics on phospholipid vesicles whose structure is essentially that of bimolecular lipid membranes. BANGHAM *et al.*[13] reported that the local anesthetics resulted in the reduction of the diffusion rate of cations and in the reduction of ζ potentials for the egg phosphatidyl choline liquid crystals. However, no work has been done on interaction between the local anesthetics and membranes by using a planar type of phospholipid membrane developed recently[1,3] and whose structure is a two-dimensional bimolecular sheet.

The present study was therefore concerned with the effect of the local anesthetics on the electrical conductance of various phospholipid bilayers (planar type) and on the instability of asymmetric phospholipid bilayers, due to Ca^{2+} binding on one surface of the membrane. On the basis of these results, some discussion is made on nerve excitation relating to the competition of Ca^{2+} and local anesthetics on the phospholipid membrane.

MATERIALS AND METHODS

Phosphatidyl choline (egg), phosphatidyl serine (bovine brain) and phosphatidic acid (modified from egg phosphatidyl choline) were prepared in Dr. Papahadjopoulos' laboratory. Method of preparation, purity and composition were as described earlier[23]. Also, these phospholipids in chromatographically pure form, purchased from Applied Science Laboratories (State College, Pa.), were used. All three phospholipids were stored in chloroform solution in ampules sealed under nitrogen gas (phospholipid 10 μM in 1 ml chloroform solution). In order to prepare a membrane-forming solution, the chloroform was driven out by blowing nitrogen gas, or evap-

orated in a vacuum chamber. The membrane-forming solutions were prepared by dissolving 10 mg of phospholipid in 0.5 ml of *n*-decane (over 99% purity; Fluka, Switzerland). Water was triple distilled, including a process of distillation from $KMnO_4$. Local anesthetic drugs (all U.S.P. grade) were purchased from Mann Research Laboratories (tetracaine·HCl, procaine·HCl; over 95% purity); K and K Laboratories (nupercaine·HCl, 95–99% purity); and Merck Co. (cocaine·HCl; U.S.P. purity). Tris–HCl was from Sigma (reagent grade). All other chemicals were A.R.

The experimental apparatus was similar to that described in the previous papers[24,25]. Capacitance of the membrane was measured with a Universal Bridge B221A ($2\pi \times$ frequency approx. 10^4) from the Wayne Kerr Laboratory (Chessington, Surrey, England). The low-frequency values for the capacitance of the lipid bilayers were measured judging from the capacitance measured at various frequencies. Electrical current was measured by using Electrometer 610C of Keithley Instrument Inc. (Cleveland, Ohio) and a recorder (Bausch & Lomb, Rochester). Ag–AgCl electrodes were used for reversal electrodes. All experimental measurements were made at $25° \pm 1°$.

EXPERIMENTAL RESULTS

The experiments can be categorized into three different types. For the first series of experiments, the electrical conductances of various types of phospholipid bilayers prepared in 0.1 M NaCl solution were measured adding the local anesthetics (nupercaine, tetracaine, cocaine, procaine) on one side of the solution. For the second series of experiments, the measurements were made on the electrical conductances of various types of phospholipid bilayers prepared in 0.1 M NaCl solution containing various concentrations of the local anesthetics. For the third series of experiments, the instability of the membranes was observed by adding Ca^{2+} on one side of the solution of the membrane which was composed of 0.1 M NaCl and various concentrations of the local anesthetics. All NaCl solutions contained 0.2 mM Tris–HCl as a buffer for the solution and 0.05 mM EDTA in order to remove small amounts of contaminant bi- and multivalent metals, which are present as contaminants in the monovalent salts and are also extracted along with phospholipids from natural sources. Metal determination performed by Spang Microanalytical Laboratory indicated the following: For a sample of bovine phosphatidyl serine purchased from Applied Science Laboratories there were 204 parts per million Ca^{2+} and 4030 parts per million Mg^{2+}. For a sample of bovine brain phosphatidyl serine prepared in our laboratory (D. Papahadjopoulos) there were 100 parts per million Ca^{2+} and 40 parts per million Mg^{2+}.

In the first series of experiments phosphatidyl choline, phosphatidyl serine and phosphatidic acid membranes were prepared in 0.1 M NaCl solution at pH 7.3 and d.c. resistances of the membranes were measured with the addition of various amounts of the local anesthetics (0.01, 0.1, 1.0 and 10 mM) on one side of the solutions separated by the membrane. The electrical resistances for phosphatidyl choline, phosphatidyl serine and phosphatidic acid membranes are shown in Figs. 1a–1c with the concentration of local anesthetics, respectively. As a general tendency it was found that as the amount of the local anesthetics was increased, the conductance of the membrane was increased; or the addition of the local anesthetics resulted in lowering resistance

of the membrane. All points are means of at least the five highest values of resistances of several membranes in order to avoid the results from leaky membranes. The degree of lowering the resistance of the membrane due to the local anesthetics was the highest with nupercaine among four local anesthetics for all three phospholipid membranes.

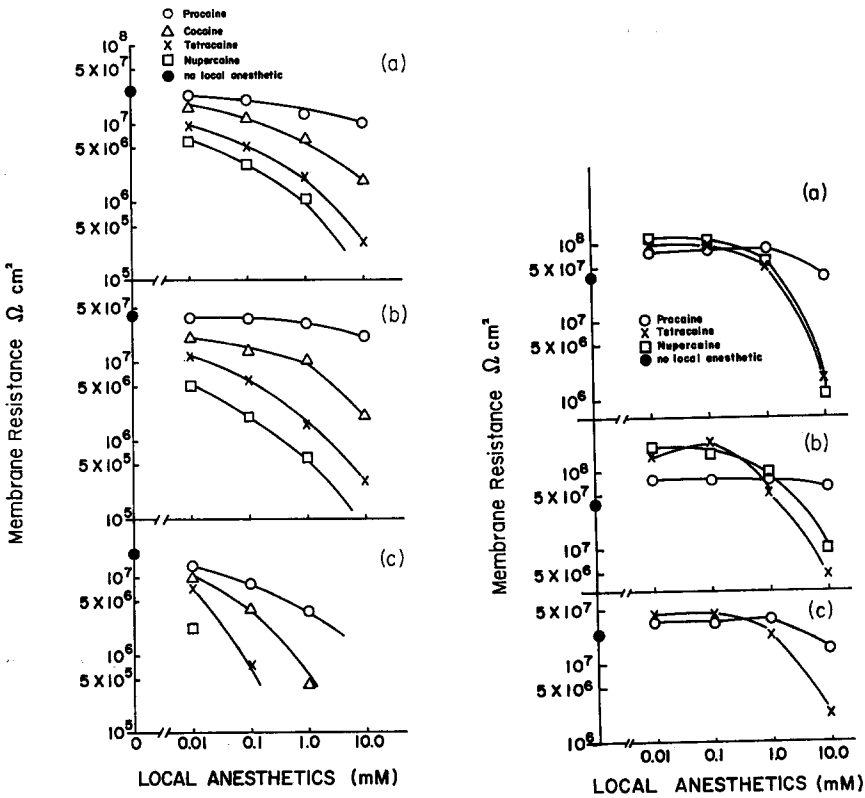


Fig. 1. Resistances of (a) phosphatidyl choline membrane, (b) phosphatidyl serine membrane and (c) phosphatidic acid membrane in 0.1 M NaCl solution at pH 7.3 with various concentrations of the local anesthetics on one side of the solutions.

Fig. 2. Resistances of (a) phosphatidyl serine membrane at pH 7.3 and (b) phosphatidyl serine membrane at pH 6.0 and (c) phosphatidyl choline membrane at pH 7.3 in 0.1 M NaCl solution containing various concentrations of the local anesthetics.

The order of degree of lowering resistance among four local anesthetics is as follows: nupercaine > tetracaine > cocaine > procaine. This order corresponds to that of the potency of local anesthetics obtained by earlier workers[5,12] (see Table I).

For example, the ratio of the lowering resistance of the phosphatidyl serine membrane with respect to the four local anesthetics (1 mM) in the solution of pH 7.3 are: nupercaine : tetracaine : cocaine : procaine=60:20:3:1. This ratio corresponds fairly well to that of the relative blocking potency in the biological membrane obtained by BLAUSTEIN AND GOLDMAN[17]. The addition of 10 mM nupercaine on one side of the solution caused lowering of the resistance greatly and resulted in the breaking of any membrane. Essentially similar results were obtained recently from the study on the

TABLE I

RELATIVE BLOCKING POTENCY OF LOCAL ANESTHETICS ON NATURAL MEMBRANES AND VARIOUS PHYSICOCHEMICAL EFFECTS OF LOCAL ANESTHETICS ON ARTIFICIAL PHOSPHOLIPID MEMBRANES

	Refs. 5–7		*Ref. 12*	*Ref. 13*	*Relative power*	*Order of*
	Minimum blocking concn. (mM)*	*Relative blocking potency*	*Relative** blocking potency*	*Concn. for 5 mV reduction in ζ potential*	*of lowering resistance of the membrane*** (Fig. 1b)*	*inhibition of instability of phosphatidyl serine asymmetric membrane (Fig. 4)*
Procaine	4.6	1	1	5.01	1	Procaine
						>
Cocaine	2.6	1.8	3.8	0.89	3	Cocaine
						>
Tetracaine	0.01	460	36	0.10	20	Tetracaine
						>
Nupercaine	0.005	920	53	0.02	60	Nupercaine

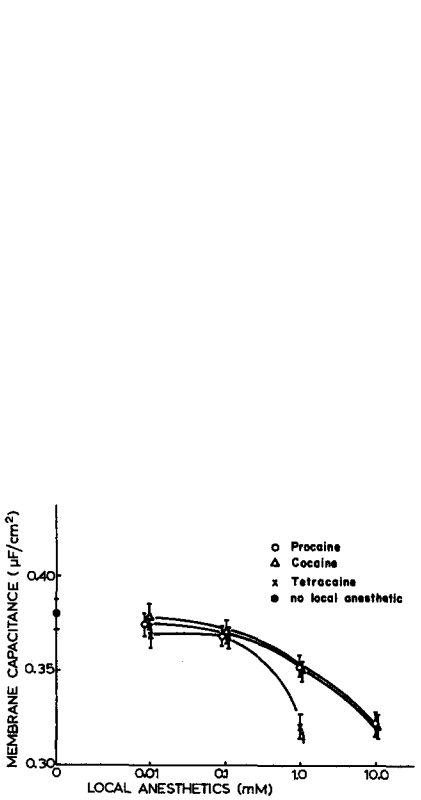
* Obtained with desheathed frog sciatic nerve at pH 7.0.
** Obtained with frog sciatic nerve at pH 7.2.
*** pH 7.3, 1 mM local anesthetics.

efflux of ions through phospholipid vesicles (D. PAPAHADJOPOULOS, private communication).

In the second series of experiments, the electrical conductances of phosphatidyl choline and phosphatidyl serine membranes prepared in 0.1 M NaCl solutions containing various concentrations of the local anesthetics (0.01, 0.1, 1.0 and 10 mM) were measured at pH 6.0 and 7.3 respectively. Also, the capacitance for phosphatidyl serine membrane was measured at pH 7.3. The results are shown in Figs. 2a–2c for phosphatidyl choline and phosphatidyl serine membranes in the solutions of pH 6.0 and 7.3. From Figs. 2a–2c the high membrane resistance increased slightly as the concentration of the local anesthetics was increased up to a certain concentration in the solution. When the concentration of the local anesthetics was increased over a certain concentration (for example, 1.0 mM tetracaine for phosphatidyl serine membrane (see Fig. 2a)), the electrical conductance was increased gradually and finally the membrane could not keep its form and broke into the solution. It is noticed that the results were contrary to those obtained in the first series of experiments in the solution containing low concentration of the local anesthetics on one side of the membrane. In a similar solution, a.c. capacitance (frequency=1592 cycles/sec, $\omega = 2\pi \times$ frequency$= 1 \cdot 10^4$) of the phosphatidyl serine membrane was measured varying the concentration (0.01, 0.1, 1.0 and 10.0 mM) of the local anesthetics (tetracaine, cocaine and procaine). The capacitance decreased gradually as the concentration of the local anesthetics was increased (see Fig. 3). The capacitance values were checked at low frequency ($\omega = 5 \cdot 10^2$) and at high frequency ($\omega = 1 \cdot 10^5$), respectively. The value at low frequency was identical to that at the frequency used for the capacitance measurement. However, the value at high frequency was lower than those for two other frequencies. Therefore, it may be concluded that the low-frequency values of the membrane were measured in this experiment.

In the third series of experiments, phosphatidyl choline, phosphatidyl serine and phosphatidic acid membranes were prepared in 0.1 M NaCl solution containing various concentrations (0.01, 0.1, 1.0 and 10 mM) of the local anesthetics at pH 6.0 and 7.3. After the membrane was formed, some amount of $CaCl_2$ was added on one side of the solutions separated by the membrane, until the membrane reached the breaking point. In order to compensate for the increase in the volume of the solution on one side, an amount of NaCl equal to the $CaCl_2$ was added to the other side of the solution. It has been found[14,26] that in acidic phospholipid membranes (phosphatidyl serine membrane), the addition of small amounts of Ca^{2+} on one side of the 0.1 M NaCl solution caused the breaking of the membrane. On the other hand, the addition of any amount of Ca^{2+} on one side of the solution separated by neutral phospholipid (phospatidyl choline membrane) does not result in the breaking of the membrane, because there is no strong binding of Ca^{2+} with neutral phospholipids[14,28].

We believe this instability of the membrane is due to the asymmetricity of Ca^{2+} binding with polar groups of acidic phospholipids at the membrane surface[14,27].



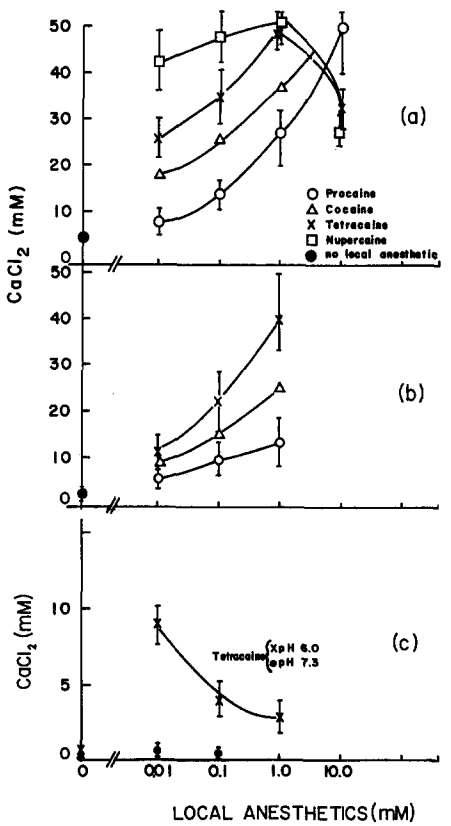


Fig. 3. Capacitance of phosphatidyl serine membrane in 0.1 M NaCl solution containing various concentrations of the local anesthetics at pH 7.3.

Fig. 4. The amount of $CaCl_2$ necessary to break the membrane on one side of 0.1 M NaCl solution containing various concentrations of the local anesthetics. (a) phosphatidyl serine membrane at pH 6.0, (b) phosphatidyl serine membrane at pH 7.3, (c) phosphatidic acid membrane at pH 6.0 and 7.3, respectively.

However, in the presence of the local anesthetics in the solution, this instability phenomenon due to Ca^{2+} was suppressed. For example, in order to break the phosphatidyl serine membrane in 0.1 M NaCl solution at pH 7.3, it was necessary to add 2 mM Ca^{2+} on one side of the membrane[14], while in 0.1 M NaCl solution containing 1.0 mM tetracaine, the membrane did not break even after the addition of roughly 50 mM of Ca^{2+}. It is likely that the local anesthetics compete with Ca^{2+} in the interaction with phospholipids and antagonize the interaction of Ca^{2+} with the acidic phospholipids. The results are shown in Figs. 4a–4c for phosphatidyl serine and phosphatidic acid membranes. For phosphatidic choline membranes, the above antagonistic effect between Ca^{2+} and local anesthetics was not observed.

The degree of the suppression of instability was stronger as the concentration of a local anesthetic in the solution was increased to a certain value. The order of potency of suppression among the local anesthetics for the phosphatidyl serine membranes was the same order as that of the first series of experiments: nupercaine > tetracaine > cocaine > procaine. The inhibition of the asymmetric membrane instability also depended upon the pH value of the solution. The potency to inhibit the instability of the asymmetrical membrane by the local anesthetic was greater in the solution of pH 6.0 than in the solution of pH 7.3 (see Figs. 4a–4c).

DISCUSSION

The results of the first series of experiments suggest that the local anesthetics may interact strongly with the polar groups of the phospholipids at the membrane surface as well as with the hydrophobic interior of the membrane. If the structure of one side of the membrane is different from that of the other, by applying local anesthetics on one side, the membrane would become asymmetric with respect to the structure or surface of the membrane. Bilayer membranes with asymmetric distribution with respect to surface charges are energetically unstable[27] and tend to reach their equilibrium states by changing structures or by changing the ionic distributions around the membrane phase. During these processes the permeability of the membrane to ions would be changed, or even disruption of the membrane would occur. This phenomenon of reduction of electrical resistance in asymmetric membranes is similar to that observed with Ca^{2+} on one side of the phosphatidyl serine membranes[14,25]. However, there is a difference between asymmetric membranes made with Ca^{2+} and the local anesthetics; that is, the local anesthetics on one side of the membrane showed similar effects in reduction of the electrical resistance for both neutral phospholipid (phosphatidyl choline) and acidic phospholipid (phosphatidyl serine, phosphatidic acid) membranes, while Ca^{2+} on one side of the acidic phospholipid membranes resulted in small reduction of electrical resistance of the membrane, but did not result in any reduction for the neutral phospholipid membrane (phosphatidyl choline)[14,26]. The following two mechanisms can be considered. That is, local anesthetics would interact with the polar groups as well as the hydrocarbon phase of the phospholipid membranes. The former mechanism may be dipole–dipole interaction as well as electrostatic interaction between polar groups of the local anesthetics and phospholipids. This causes the local anesthetics to exhibit an effect similar to that of Ca^{2+}, which interact with the polar groups on the surface of the membrane[14,29]. The latter interaction would be hydrophobic interaction between non-polar groups of the local

anesthetics and phospholipids, which suggests that some part of the local anesthetic can penetrate into the hydrocarbon phase of the membrane as shown in studies of SKOU[5] of lipid monolayers with the local anesthetic.

On the other hand, when the local anesthetics interact with the membrane from both sides of its surface, binding with the polar groups at the surfaces and penetration into the hydrocarbon phase can occur equally at both sides of the membrane surfaces. As a result, the membrane structure would be symmetric with respect to a plane in the middle of the membrane. Such a symmetric binding of the local anesthetic with the membrane at both sides of the surface was found to produce stable membranes with lower conductance for ions. The results of the second series of experiments (Figs. 2a–2c) correspond to the above interpretation. The results correspond to that obtained by FEINSTEIN[11]. However, in the solution of highly concentrated local anesthetics, the mechanism for the increased permeability would be more complicated. Further intensive experiments are required for the explanation. The variation of the a.c. capacitance of the phosphatidyl serine membrane with the concentration of the local anesthetics is also difficult to understand because many unknown factors[29] are involved for measurements of the membrane capacitance.

According to the results of the third series of experiments, the local anesthetics inhibit (1) the binding of Ca^{2+} with phospholipid polar groups, and (2) the formation of unstable asymmetric membranes due to Ca^{2+} binding. This phenomenon was clearly observed for the phosphatidyl serine membrane at both pH 6.0 and 7.3. The order of potency of the inhibition by the local anesthetics for the phosphatidyl serine membrane was the same order as that found in the first series of experiments: that is, nupercaine > tetracaine > cocaine > procaine. The mechanism of inhibition would be the following: as mentioned before, the local anesthetics interact strongly with polar groups of phospholipids and also penetrate between phospholipid molecules. The binding of the local anesthetics with the polar groups of phospholipids will be determined by the competition of the strength of the binding of Ca^{2+} and the local anesthetics with the phospholipid polar groups. The penetration by the local anesthetics among the lipid molecules would cause a separation between the polar groups and inhibit the satisfactory binding of Ca^{2+} with the polar groups among the phospholipids.

For the phosphatidic acid membrane, the effect of local anesthetic inhibition (tetracaine) was not significant in the solution of pH 7.3 ,but significant enough in the solution of pH 6.0. If the strength of the interaction of the local anesthetics with membranes is comparable to that of Ca^{2+}, the effect of antagonism between the local anesthetics and Ca^{2+} in binding with the polar groups of the lipids is expected to be significant, as observed with phosphatidyl serine membranes in our experiments. It is concluded that, as far as the three phospholipid membranes (phosphatidyl choline, phosphatidyl serine and phosphatidic acid) are concerned, the lipid which has a significant relationship to the antagonism of the local anesthetics and Ca^{2+} may be phosphatidyl serine molecules.

From the results for both phosphatidyl serine and phosphatidic acid membranes (Figs. 4a–4c) the inhibition of the local anesthetic with Ca^{2+} is more effective at pH 6.0 than at higher pH 7.3. Especially, for the phosphatidyl serine membrane, there, is no appreciable change of the ionization of the polar group of the membrane over the pH range 5–8 (one net negative charge per molecule[29]). The difference in the antag-

onism of the local anesthetics with Ca^{2+} at pH 6.0 and at pH 7.3 may be due to the difference in ionization of the local anesthetics. In solution at pH 6.0, the local anesthetics are more positively ionized than at 7.3. These results may correspond to the concept[4,13] that the narcotic action of the local anesthetics is more active for cation form than for the neutral form of the local anesthetics.

It should be mentioned that the result of the first series of experiments seems to be rather contradictory to that obtained in nervous membranes[28]. That is, the former showed the decrease of the membrane resistance when the local anesthetics were added on one side of the membrane, while the latter showed that the addition of the local anesthetics to the nervous membrane resulted in an increase in the transmembrane resistance of the nerve[30]. In order to discuss the results of the biological membrane compared with the results of the artificial membrane, it is necessary to investigate with the same type of experiments on lipid *plus* protein membranes (artificial membranes). However, it is understood that what we have obtained in our experiments is the degree of affinity (interaction) of the local anesthetics with various phospholipids which are components of biological membranes.

As we have proposed previously[14], if the instability of asymmetric membranes due to the Ca^{2+} binding on the outer surface of the membrane plays a role in the excitability of the biological membranes, the inhibition of membrane excitability[17,30,32] would be explained as the mechanism by which the local anesthetics inhibit the binding of Ca^{2+} with phosphatidyl serine membranes.

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