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Trinitrobenzenesulfonic Acid and Fluorodinitrobenzene: Probes to Study Local Anesthetic Effects in Cell Membranes

P. Bradford and G.V. Marinetti

Department of Biochemistry, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642

Summary. The interaction of local anesthetics with intact erythrocytes was studied by monitoring the extent of reaction of phospholipids with trinitrobenzenesulfonic acid and fluorodinitrobenzene. Incubating erythrocytes with local anesthetics increases the amount of phosphatidylethanolamine and phosphatidylserine available for reaction with trinitrobenzenesulfonic acid and fluorodinitrobenzene. The order of potency of the local anesthetics corresponded to that reported for blocking nerve conduction: dibucaine > tetracaine > butacaine > lidocaine > procaine. Treatment of intact erythrocytes with 1 mM tetracaine at 37 °C allows 4–5% more of the phosphatidylethanolamine to react with trinitrobenzenesulfonic acid as compared to control cells. Treatment with tetracaine has no effect at 0 °C, a temperature at which there is only limited partitioning of the anesthetic into the bilayer. Kinetic analysis of the reaction with trinitrobenzene sulfonic acid showed that the increased number of reactive phosphatidylethanolamine molecules are located mainly on the outer half of the erythrocyte membrane. Tetracaine also increases the number of phosphatidylserine and phosphatidylethanolamine molecules in the erythrocyte membrane which are available to react with the penetrating probe fluorodinitrobenzene. The reaction with PE is increased from 67 to 77% and the reaction of PS is increased from 44 to 57%. Thus tetracaine affects both halves of the lipid bilayer.

Key words: Local anesthetics, amino-phospholipids, red cell membrane, chemical probes, TNBS, FDNB

That anesthetic potencies of a series of compounds correlated with their olive oil/water partition coefficients was indirect evidence for a lipid site of action. This was formulated into the Meyer-Overton rule of anesthesia, that chemically indifferent substances produce narcosis if they have dissolved to approximately the same molar concentration in the cell lipids (Meyer & Gottlieb, 1926). This parallelism between the strength of anesthetic action and solubility in fatty bodies was soon recognized for local anesthetics, as well (Gottlieb, 1923). Following both from the work of Hodgkin, Huxley, and Katz (1949), which established the ionic basis of impulse conduction in nerves, and from the observation that the rate of rise of the recorded action potential of nerves was reduced by anesthetics (Weidmann, 1955; Thesleff, 1956), the effect of local anesthetics could be deduced to be an inhibition of sodium conductance. Anesthetics may bind to the protein component of the Na^+ channel at one or more domains or may exert an indirect effect on the Na^+ channel by altering lipid-protein interactions. Since the Na^+ channel is a membrane component, a role for phospholipids in the functioning of the channel becomes apparent. Indirect effects of local anesthetics have been suggested to lead to nerve blockage. These include the displacement by anesthetics of functionally required membrane Ca^{2+} (Low, Lloyd, Stein & Rogers, 1979) and a conversion of the lipid surrounding the Na^+ channel from the crystalline gel state to a fluid liquid crystalline state (Lee, 1976). It is our hypothesis that specific lipid-protein and Ca^{++}-phospholipid interactions play a role in the functioning of the Na^+ channel and that local anesthetics can disrupt these interactions. To study this possibility, we have employed chemical probes to determine if local anesthetics perturb phospholipid interactions in the red cell membrane.

Chemical probes, such as 2,4,6-trinitrobenzenesulfonate (TNBS)[1], and 1-fluoro-2,4-dinitrobenzene (FDNB) have been used to determine the topology

[1] *Abbreviations:* TNBS = 2,4,6-trinitrobenzenesulfonate; FDNB = 1-fluoro-2,4-dinitrobenzene; PE = phosphatidylethanolamine; PS = phosphatidylserine; BSA = bovine serum albumin; NMR = nuclear magnetic resonance; TLC = thin layer chromatography.

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of PS and PE in a variety of membranes (Gordesky & Marinetti, 1973; Crain, Marinetti & O'Brien, 1978). Since TNBS is a nonpenetrating probe at 0 °C, it has been used to determine the sidedness of aminophospholipids in sealed vesicles and cell membranes. TNBS and FDNB have the potential to assess the effect of drugs such as anesthetics on protein-lipid and lipid-lipid interactions. The increased availability of PE of erythrocytes to TNBS after hexanol treatment has been reported by Haest and Deuticke (1975). Although the initial enhancement of reactivity was noted and attributed to the temperature-sensitive binding of hexanol to cell membrane, the authors concluded that a lack of energy supply induces a reorientation in the membrane and that drugs like hexanol enhance this metabolic effect. In this paper, we report results on the effects of local anesthetics on cell membranes, as monitored by the availability of membrane phospholipids to react with TNBS and FDNB.

Materials and Methods

TNBS and FDNB were obtained from Pierce Chemical Co. Local anesthetics were obtained from Sigma Chemical Co. Human blood was obtained from the Red Cross Blood Bank and stored at 4 °C until used. Merck-Darmstadt TLC plates (silica gel 60, 0.25 mm thickness, 20 × 20 cm) were purchased from VWR Scientific. Chloroform and methanol (ACS analytical grade) were purchased from Fisher Scientific and were redistilled prior to use. Phospholipid standards were obtained from Avanti and from Supelco.

Incubation of Erythrocytes with Anesthetics and Reaction with TNBS

Human blood was obtained from volunteer donors or the Red Cross Blood Bank. Freshly drawn blood was used immediately. Red Cross blood was used within 1–2 weeks. One half ml aliquots of packed erythrocytes were suspended in 19.5 ml Hank's buffer, pH 7.4, containing different amounts of local anesthetic. Control samples lacked the anesthetic. After a 30-min incubation at 37 °C, the cells were centrifuged at 1500 × *g* and washed with 19.5 ml ice-cold buffer. Nineteen and one half ml of Hank's buffer, pH 7.4, or 140 mM NaCl – 40 mM $NaHCO_3$ buffer, pH 8.5, containing 2 mM TNBS or 2 mM FDNB were added, and after incubation for different time periods at either 0, 21, or 37 °C the cells were centrifuged rapidly and lysed immediately in 20 ml of 10 mM Tris buffer, pH 7.4, containing 1 mM EDTA. Cell lysis due to the anesthetic was quantitated spectrophotometrically at 540 nm by measuring the amount of hemoglobin left in the supernatent after the cells and ghosts were centrifuged. The ghosts were obtained by centrifugation at 35,000 × *g* for 20 min. The extraction of lipids and the TLC analysis of PE, PS, and their dinitrophenyl or trinitrophenyl derivatives were carried out as described previously (Crain et al., 1978). Lipids were extracted with chloroform-methanol by the method of Folch, Lees and Stanley (1957). Phospholipids and their dinitrophenyl and trinitrophenyl derivatives were separated on TLC plates by development in chloroform/methanol/16.6 N NH_4OH (65:25:4). Yellow dinitrophenyl and trinitrophenyl derivatives of PE and PS were scraped from the plates, eluted in methanol, and quantitated spectrophotometrically (Gordesky & Marinetti, 1973). Lipid spots were made visible by exposure to iodine vapors. Aminolipids were detected by the ninhydrin reagent. Identification was made by comparison to authentic phospholipids and their derivatives developed under identical conditions. These phospholipids included PE, PS, TNP-PE, TNP-PS, DNP-PE and DNP-PS. After perchloric acid digestion of unreacted phospholipid, inorganic phosphate was measured by a modification of the method of Harris and Popat (1954). Under the experimental conditions of labeling red cells with TNBS, less than 1% hemolysis occurs.

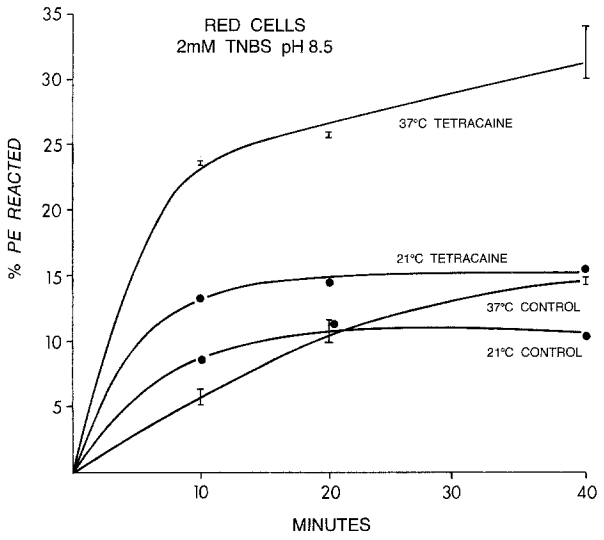

Fig. 1. Effect of tetracaine on the time course of reaction of red cell PE with TNBS. Red cells were reacted with 2 mM TNBS in NaCl-$NaHCO_3$ buffer, pH 8.5, after a preincubation in Hanks buffer, pH 7.4, in the presence or absence of 1 mM tetracaine for 30 min. Reactions were done at 37 or 21 °C for different time intervals as indicated. Analysis of phospholipids and their derivatives was carried out as described in the text. Tetracaine significantly enhanced the reaction with PE at both temperatures: $P < 0.01$, paired *t* test. Bars represent the SD of triplicate analyses

Results

In order to use TNBS as a probe for measuring perturbations of membrane PE and PS we used two different buffers at pH 7.4 and 8.5 and three different temperatures. In bicarbonate buffer at pH 8.5 the reaction of PE and PS proceeds to a larger extent due in part to the deprotonation of amino groups but also in part to perturbation of the membrane. The pH of 8.5 was chosen initially in order to increase the yield or trinitrophenyl product, and thereby making the analysis easier to carry out. At pH 8.5 about 25–30% of the total PE reacts with TNBS at 37 °C as compared to 6–8% at pH 7.4. However, pH 7.4 is the physiological pH and therefore most studies were done at this pH value. In order to minimize penetration of TNBS into the cell one would prefer to use a lower temperature of 21 or 0 °C. However, these are nonphysiological temperatures for the human red cell. Therefore, we decided to carry out our studies under various conditions but chose the physiological conditions of pH 7.4 and 37 °C for most experiments.

Table 1. Effect of washing red cells on the tetracaine enhanced labeling of PE with TNBS[a]

	% TNP-PE
Control	9.2±0.5
Tetracaine	14.0±0.9
Tetracaine+buffer wash (no BSA)	12.8±1.2
Tetracaine+buffer wash with BSA	9.6±0.8

[a] The experimental conditions are given in the text. Cells were treated with 1 mM tetracaine for 30 min at 37° in Hanks buffer, pH 7.4, and treated with 2 mM TNBS for 40 min at 37°. The values represent the mean±SD of triplicate determinations.

Treatment of cells with tetracaine at 21 or 37 °C allows more PE molecules to react with TNBS at pH 8.5 as compared to control cells (Fig. 1). The time course of reaction of PE with TNBS at pH 8.5 is biphasic, indicating that at least two populations of PE molecules are reacting with TNBS. The presence of the anesthetic agent in the membrane is required for the increased reactivity of PE towards TNBS. When tetracaine is added to erythrocytes at 0 °C, a temperature favoring the less penetrable gel phase of the membrane, there is no enhancement of the reaction of PE with TNBS. However, treatment of red cells with tetracaine at 37 °C will potentiate the reaction even when the cells are washed with albumin-free buffer and then reacted with TNBS at 0 °C (Table 1). When cells are treated with tetracaine and washed with buffer containing albumin, the tetracaine enhancement of TNBS labeling is abolished since albumin removes the tetracaine from the membrane. Moreover, treatment of red cells with tetracaine at 37 °C will enhance the TNBS reaction with PE even when the cells are cooled to 0 °C before the TNBS is added (Fig. 2). These results are consistent with NMR studies on the interaction of deuterated local anesthetics with model membranes by Boulanger, Schreier, Leitch and Smith (1980). They report a fast exchange of the anesthetic between solution and the membrane surface and a slow exchange in which the anesthetic is intercalated in the lipid bilayer.

Since, at 0 °C, TNBS reacts primarily with the outer surface of erythrocytes (Gordesky, Marinetti & Love, 1975), it can be concluded from the data in Fig. 2 that tetracaine treatment exposes an additional 4% of the total PE in red cells and that this exposed PE is in the outer half of the bilayer. This conclusion is supported further by a kinetic analysis of the reaction with TNBS at pH 7.4 and 37 °C as shown in Fig. 3 and Table 2. A semi-log plot of the percent unreacted PE as a function of time can be resolved into fast-reacting and slow-reacting components, representing the reaction of PE in the outer and inner halves of the membrane bilayer, respectively. Tetracaine increases the amount of the fast reacting component from 4 to 8% of the total PE. The same increment of 4% is observed at 0 °C and pH 7.4 when TNBS reacts primarily with PE on the outer surface of the membrane. Experimental error is about ± 0.4%. The increase in the slow reacting component is about 2% at 120 min. The PE in the outer membrane surface exposed by tetracaine has reacted completely

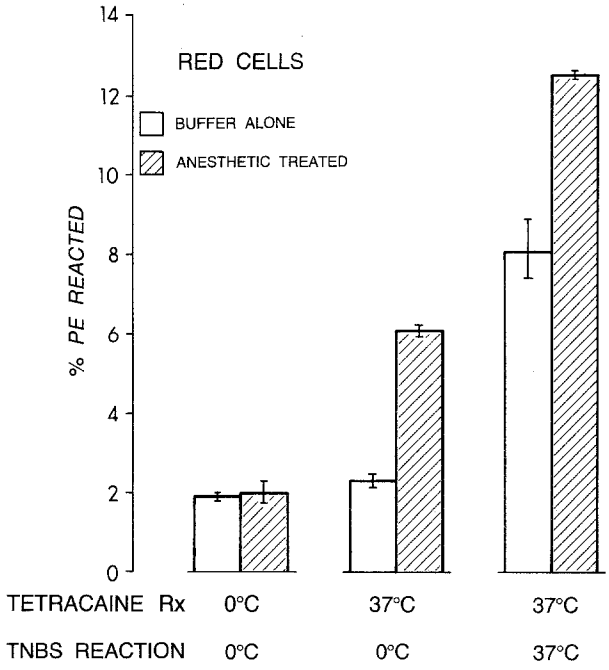

Fig. 2. Temperature dependence of the tetracaine effect on the TNBS labeling of PE in intact red cells. Red cells were incubated for 30 min with or without 1 mM tetracaine at 0 or 37 °C in Hank's buffer, pH 7.4. The cells were washed once with ice-cold buffer and then reacted with 2 mM TNBS in Hank's buffer, pH 7.4, for 30 min at either 0 or 37 °C. Reaction conditions are given in the text. Bars represent the SD of triplicate determinations

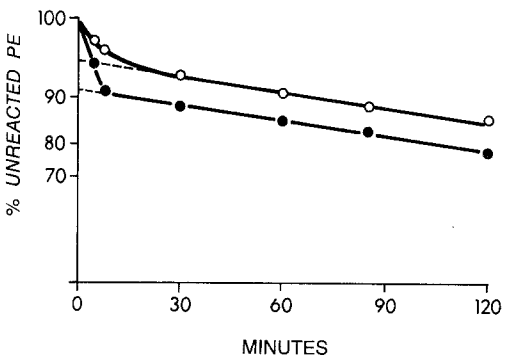

Fig. 3. Semi-log plot of time-course of reaction of PE with TNBS. Red cells were incubated at 37 °C for 30 min in Hank's buffer, pH 7.4, with (filled circles) and without (open circles) 1 mM tetracaine. The cells were washed once with ice-cold buffer and then resuspended in Hank's buffer, pH 7.4, containing 2 mM TNBS for the times indicated. Kinetic parameters were calculated from computer analysis of the data (Table 2)

Table 2. Effect of tetracaine on the rate and extent of reaction of erythrocyte PE with TNBS[a]

	Control	1 mM tetracaine
$t_{\frac{1}{2}}$ · fast component	4.0 min	3.7 min
% reaction	4.3	8.2
$t_{\frac{1}{2}}$ · slow component	599 min	536 min
% reaction	11.7	13.7

[a] The experimental conditions are given in the text.

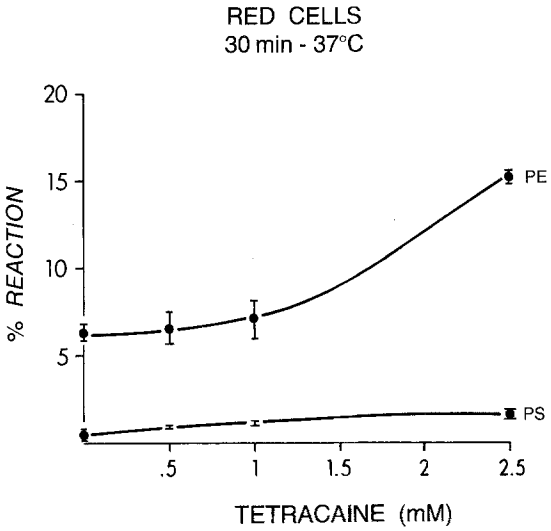

Fig. 4. Effect of different concentrations of tetracaine on the reaction of red cell PE with TNBS. Red cells were incubated at 37 °C for 30 min in Hanks buffer, pH 7.4, containing different concentrations of tetracaine. The cells were washed in cold buffer and then reacted with 2 mM TNBS at 37 °C for 30 min in Hanks buffer, pH 7.4. Bars represent the SD of triplicate determinations

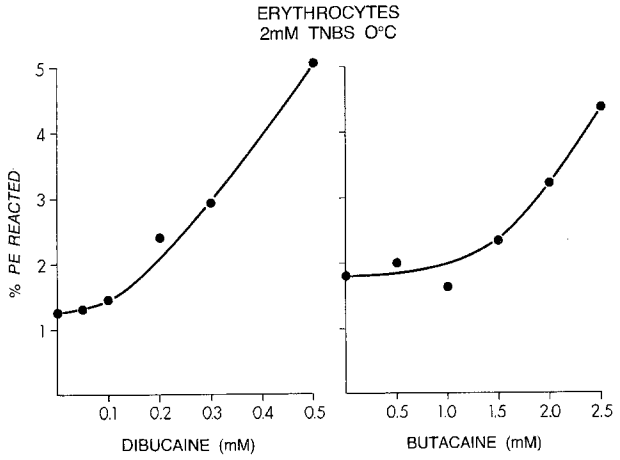

Fig. 5. Effects of dibucaine and butacaine on the labeling of red cell PE with TNBS. Red cells were incubated in Hanks buffer, pH 7.4, at 37 °C for 30 min with either dibucaine or butacaine at the indicated concentrations. The cells were washed with cold buffer and reacted with 2 mM TNBS at 0 °C for 30 min in Hanks buffer, pH 7.4, for dibucaine-treated cells and 45 min for the butacaine-treated cells. Each point represents the average of duplicate determinations

with TNBS by 30 min, whereas the PE on the inner membrane surface reacts only to a small extent by 120 min. PE in the intact red cell membrane is asymmetrically distributed with 5–10% being on the outer surface, 75–80% being on the inner surface, and about 10–15% being tightly bound to membrane protein (Marinetti & Crain, 1978).

Concentration profiles for tetracaine, dibucaine, and butacaine are shown in Figs. 4 and 5. Under the conditions of the incubation, cell lysis was less than 0.5%, although at higher concentrations of anesthetic agent, and especially at 37 °C for the TNBS reaction, extensive hemolysis can occur. The sharp increase in labeling of PE at higher concentrations of anesthetic agent may be due in part to cell lysis.

To show that the very small amount of cell lysis does not correlate with the anesthetic effect on the TNBS labeling, the % PE reaction is shown with the degree of hemolysis as measured by the moles of hemoglobin released in the supernatant (Fig. 6). In all cases the amount of cell lysis was very small, being less than 1%. Procaine had no effect on the TNBS reaction but caused more cell lysis than did tetracaine, butacaine, and lidocaine.

To avoid the effects of biological variability among different samples and ages of blood, the local anesthetics were assayed on the same batch of erythrocytes. The concentration of anesthetic varied and represents a compromise between its exerting an effect on the membrane and its effect on cell lysis. Using the enhancement of TNBS reaction as an assay and considering the concentration of anesthetic, the following order of potency was obtained: dibucaine > tetracaine > butacaine $\gg$ lidocaine $\geqq$ procaine (Fig. 6). The same order is observed for anesthetic action in blocking nerve conduction (Blaustein & Goldman, 1966). It is noteworthy that the anesthetics exert their effect on making the lipids more reactive at concentrations comparable to those reported to produce anesthesia (Luduena & Hoppe, 1956). Labeling of PS was also enhanced by local anesthetics. 1 mM tetracaine increased significantly the amount of PS which is reactive to TNBS from 1.2 ± 0.4 (SD) % to 2.0 ± 0.3% ($P < 0.01$).

In order to see whether PE and PS molecules on the inner membrane surface or more deeply buried in the hydrophobic domain of the membrane were perturbed by anesthetics, cells were exposed to tetracaine and then reacted with the penetrating hydrophobic probe FDNB. As seen in Fig. 7, tetracaine treatment led to an enhanced reaction of both PE and PS with FDNB. These effects are of greater magnitude than that obtained with TNBS since many more molecules of PE and PS are localized on the inner surface of the membranes and are available to react with FDNB. Since FDNB penetrates the cell

membrane it may alter its structure more than TNBS. The effect of tetracaine on the enhanced labeling of PS is particularly interesting since a major part of this phospholipid appears to be tightly bound to membrane protein (Marinetti & Crain, 1978). Tetracaine may disrupt PS-protein bonds. It is noteworthy that PS is believed to be required for the ATPase of the Na^+ pump (Wheeler & Whattam, 1970) and is believed to be involved in nerve excitation (Cook, Low & Ishijima, 1972).

Discussion

Local anesthetics inhibit sodium conductance in nerves both by frequency-dependent block (Courtney, 1980) and by closed channel block. The latter is dependent upon the lipid solubility of the anesthetic and is the mode of action discussed here. The membrane expansion theory of Seeman (1974) and the lateral phase separation theory of Trudell (1977) provide physical modes for anesthetic action and are consistent with a lipid site of action. The nature of the changes in the lipid state which are responsible for anesthesia is not completely understood. Our present experiments were aimed to elucidate the role of membrane phospholipids in the action of anesthetics. Evidence has accumulated that some membrane proteins, particularly those catalyzing transport, require a closely associated lipid annulus for their function (Brasitus, Schachter & Mamouneas, 1979). It is likely that many intrinsic membrane proteins require a lipid complement to maintain certain conformations necessary for normal function. Local anesthetics may disrupt lipid-protein interaction directly or indirectly, converting a critical population of phospholipids from the gel to liquid crystalline state. Lee (1977*a*) reports that for charged lipids, variations in hydrogen ion and metal ion concentrations can cause changes between gel and liquid crystalline states at constant temperature. Divalent cations raise transition temperatures of charged lipids and hence would be expected to stabilize the gel phase. The interaction of local anesthetics with phospholipids of erythrocyte membranes seems to be antagonized by calcium ions (Low et al., 1979). Insertion of anesthetics into the membrane can displace bound calcium, some of which may be associated with phospholipids. Since PS is anionic it has the capacity to bind calcium.

Phospholipids around some membrane proteins are more disordered and less fluid than the bulk lipids (Lee, 1977*b*). These lipids may be good candidates for the site of anesthetic action. Although the sodium transport channel in nerves is the most publicized target of local anesthetics, other membrane proteins may be affected and undergo changes in their interaction with phospholipids and indirectly modify the sodium channels. Our data suggest that these potential protein and lipid changes are detectable by altered susceptibility to labeling by TNBS and FDNB.

The effect which we observe on PS is particularly noteworthy since this acidic phospholipid is asymmetrically localized in several cell membranes, is tightly associated with membrane proteins, and has a

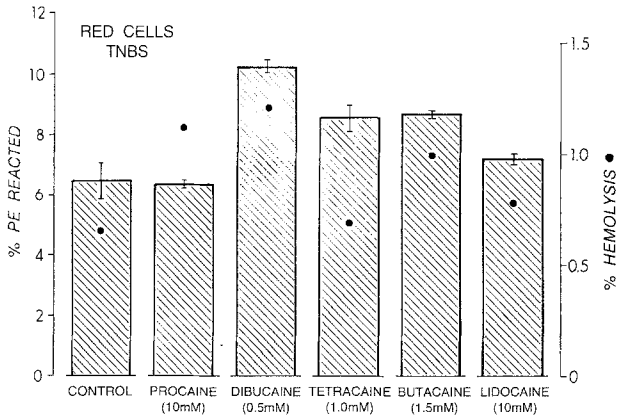

Fig. 6. Effects of local anesthetics on the reaction of red cell PE with TNBS. Reactions were done as described in the text. Red cells were incubated in Hanks buffer, pH 7.4, at 37 °C for 30 min without or with the different anesthetics at the concentrations indicated. The cells were washed with cold buffer and then reacted with 2 mM TNBS at 37 °C for 30 min in Hanks buffer, pH 7.4. After reaction, hemoglobin in the supernatant was analyzed. Bars represent the SD of triplicate determinations

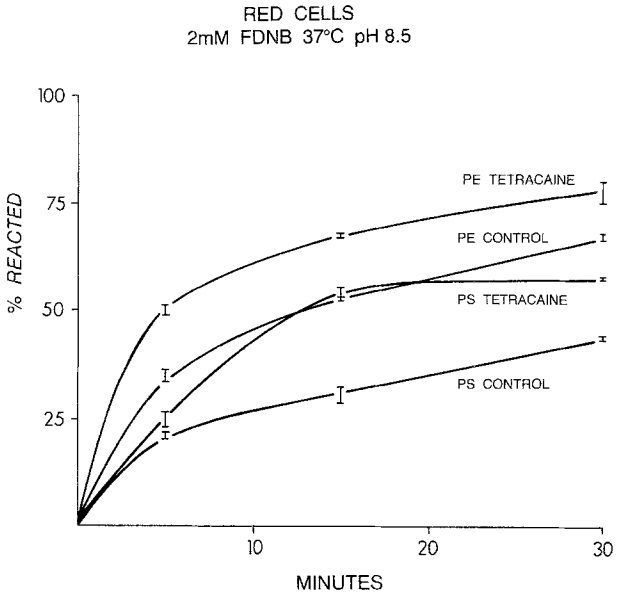

Fig. 7. Effect of tetracaine on the reaction of red cell PE and PS with FDNB. Red cells were incubated at 37 °C for 30 min in NaCl-$NaHCO_3$ buffer, pH 8.5, in the presence or absence of 1 mM tetracaine. The cells were washed and reacted with 2 mM FDNB at pH 8.5 for different time intervals as described. Tetracaine significantly enhanced reaction of both PS and PE: $P < 0.01$, paired t-test. Bars represent the SD of triplicate determinations

strong affinity for calcium ions. This latter property of PS we believe to be important and relevant to nerve conduction since our data on the effect of anesthetics on PS coincide with the effects of anesthetics on calcium release from membranes observed by Low et al. (1979). The order of potency of anesthetics and the concentrations used are the same that Low et al. observe and are the same as the action of anesthetics in blocking nerve conduction. These results lead us to postulate a chemical molecular mechanism of action of local anesthetics in which these agents intercalate in the membrane and disrupt lipid-lipid and/or lipid-protein interactions such that Ca^{++}-PS bonds are broken and Ca^{++} is released. The breaking of Ca^{++}-PS bonds leads to an electrical uncoupling of Na^{+} channels which are normally connected by these Ca^{++}-PS arrays, and this leads to a block in nerve conduction.

Our hypothesis accounts for the role of Ca^{++} in nerve conduction and the restoration of nerve conduction in anesthetic treated nerves by added Ca^{++} or by pressure. It accounts for why relatively large amounts of anesthetic agents are required to block nerve conduction. The potency of the anesthetic agent is related to its partitioning in the membrane bilayer and also to its ability to disrupt Ca^{++}-PS bonds and/or to disrupt PS-protein interactions. Since the density of Na^{+} channels in membranes is low (about 100/μm^2) and since the concentration of PS is about 10^7–$10^8/\mu m^2$ this explains why anesthetics are not effective in the pM or nM range as would be expected if these bound directly to the Na^{+} channel. PS decarboxylase inhibits nerve conduction (Cook et al., 1972). The loss of carboxyl groups of PS would eliminate some Ca^{++} binding to the membrane, uncouple Na^{+} channels and block nerve conduction.

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