Effect of Local Anesthetics on Electrically Excitable Bilayer Lipid Membranes

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Abstract □ The effect of three local anesthetics on the action potential of electrically excitable bilayer lipid membranes was studied. The synthetic action potential of bilayers was abolished at drug concentrations roughly identical to those abolishing the action potential in nerves. The threshold depolarizing constant current pulse triggering the synthetic action potential was increased by slightly lower concentrations of these local anesthetics. It is suggested that the anionic channels, which are responsible for the rising phase of the action potential in bilayers, are affected preferentially by these drugs.

Keyphrases □ Anesthetics, local—effect on electrically excitable bilayer lipid membranes □ Lipid membranes, bilayer—electrically excitable, effect of local anesthetics

Action potentials generated in bilayer lipid membranes were like those observed with primitive cells (*Nitella* and ovae): they displayed anion–cation permeability transitions rather than sodium–potassium transitions as in nerves (1). It was hypothesized, however, that regardless of ion selectivity, the gating mechanism, *i.e.*, the molecular mechanism by which excitable membranes open and close for the passage of ions, might be identical in the bilayer and in the nerve (1–4). If this hypothesis is correct, the excitable bilayer should become a valuable asset in elucidating the molecular mode of action of some drugs that modify conduction along the nerve membrane.

While the mechanism of electrical excitability in nerves is still obscure, it is partially understood in bilayers (2–4), and the chemical components of electrical excitability in bilayers are known. Although much basic work is needed to establish the exact nature of the similarities existing between the nerve membrane and the excitable bilayer, it was interesting to compare systematically the effects of some drugs classes on these two systems. Local anesthetics were selected mainly because some available information suggests that these drugs might act (at least in part) on the gating mechanism of excitable membranes (5). This report presents the preliminary findings of this study.

EXPERIMENTAL

Material—The excitability-inducing material was a crude preparation obtained from *Aerobacter cloacae*, grown overnight in an egg white medium and stabilized by pan drying (1). Excitability-inducing material is not a well-defined substance. It is "predominantly a protein oligomer having a maximum specific activity at a molecular weight around 250,000 and a monomeric size presumably as low as 6000; it may also have some associated small molecular weight organic entity"[1]. The potency of this material may vary from batch to batch, and the concentrations necessary to render a bilayer electrically excitable vary accordingly. For a given batch of excitability-inducing material and the given concentration used initially, the concentration of protamine[2] necessary to "interact" with the excitability-inducing material in the bilayer varies little.

The membrane-forming solution contained 25 mg of sphingomyelin[3]/ml of chloroform–methanol–tocopherol[4] (3:2:5).

The 5 m*M* histidine buffer[5], pH 7.0, was prepared from a 0.1 *M* stock solution kept in the refrigerator. The inorganic chemicals used were from the best grade available, and the water was distilled twice.

The excitability-inducing material, protamine, lipids, and local anesthetic[6] solutions were always prepared fresh.

Method—The membranes were prepared by the hairbrush technique across a hole, about 1 mm in diameter, separating two compartments filled with the histidine buffer; one compartment, where all additives were introduced, was arbitrarily defined as the inside. Electrical stimulation and measurement of electrical properties were obtained with a pair of reference calomel electrodes, one on each side of the membrane. The instrumental arrangement, electrical circuits, and technical procedures were described previously (6).

The main steps to obtain an electrically excitable bilayer may be summarized as follows (1, 6, 7).

1. A bimolecular lipid membrane was formed from the membrane-forming solution ["the final compositions of the thinned membranes are unknown but they would be expected to contain both sphingomyelin and tocopherol" (7)], and the specific conductance of the membrane was checked (it had to be around $3–5 \times 10^{-9}$ mho/cm^2).
2. The excitability-inducing material solution was added to the inside compartment to a final concentration of about 0.5×10^{-4} g/ml, and small constant current pulses (with the inside electrode positive) were applied until a significant increase in conductance [to around $3–5 \times 10^{-5}$ mho/cm^2 (7)] was observed (meaning that the excitability-inducing material had adsorbed onto the membrane). Outgoing current pulses helped to drive the excitability-inducing material molecules to the membrane.
3. Potassium sulfate and potassium chloride were added to the inside compartment to final concentrations of 10 and 40 m*M*, respectively. On addition of the salts, a resting potential of more than 50 mv, negative inside, in good analogy with a cell, developed.
4. Constant depolarizing current pulses (inside electrode positive) were applied until the phenomenon of delayed rectification had fully developed (1–2 min) (1).
5. Protamine was carefully titrated into the inside compartment to a final concentration of about 1.5 μg/ml; after the protamine molecules had "interacted" with the excitability-inducing material molecules in the membrane (1–3 min), action potentials could be obtained. The threshold depolarizing constant current pulse necessary to trigger an action potential was then determined, and many action potentials were obtained subsequently to verify that the threshold of excitation did not vary.
6. A local anesthetic was added to the inside compartment to the desired final concentration. After about 1 min, depolarizing currents were applied, and the effects of the drug on the threshold of excitation and on the action potential were noted.

Many experiments were performed with the three local anesthetics. Indeed, "like their counterparts in living systems, [electrically excitable bilayers] are extremely sensitive to small changes in their environment so that experiments must be repeated several times before the main response pattern can be distinguished among the minor fluctuations" (7).

THEORY

Since both the delayed rectification and the action potential occurring in bimolecular lipid membranes must be considered, a brief description

[1] J. Bukovski, Eastern Pennsylvania Psychiatric Institute, Philadelphia, PA 19129, personal communication.

[2] As the sulfate, Schwarz/Mann.

[3] Sigma.

[4] Chloroform, reagent, Merck; methanol, certified, ACS, Fisher; DL-α-tocopherol, Sigma.

[5] L-Histidine, Nutritional Biochemicals.

[6] Procaine hydrochloride USP, New York Quinine and Chemical Works; tetracaine and lidocaine, Pfaltz and Bauer.

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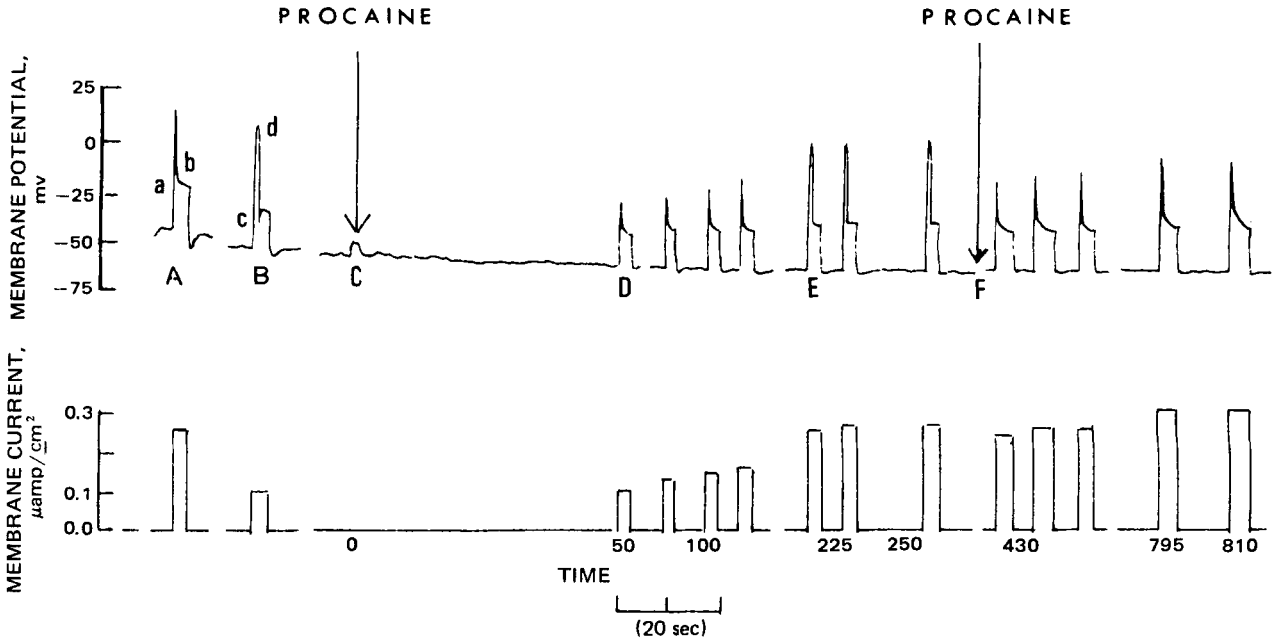

Figure 1—*Effect of procaine on electrically excitable bilayer lipid membranes. At A, only the excitability-inducing material was present in the bilayer, and the phenomenon of delayed rectification was obtained in response to a depolarizing constant current pulse. At B, both excitability-inducing material and protamine were present in the right proportions in the bilayer, and a depolarizing constant current pulse of threshold intensity triggered an action potential. At C, procaine was added to the inside compartment to a final concentration of about 3 mM (arbitrarily chosen as time zero and shown on the time scale). After about 1 min (at D), four successive depolarizing current pulses, of gradually increased size, were applied; the delayed rectification phenomenon was obtained in response to these current pulses. At E, depolarizing pulses of much increased size were applied to the membrane, and action potentials could then be restored. At F, the concentration of procaine was brought to 5 mM, and increasing depolarizing current pulses were applied again; only the delayed rectification phenomenon was obtained in response to these pulses.*

of the main electrical and molecular events thought to underlie these phenomena is presented to help in understanding the effects of the local anesthetics.

Delayed Rectification—After its addition to the inside compartment, the excitability-inducing material goes to the membrane where it adsorbs and, under the proper experimental conditions, forms channels. Its gating characteristics are (1, 7, 8): (*a*) in the absence of an electric field, its channels remain open; but in the presence of a field, they close; (*b*) these channels permit the passage of positive current from the excitability-inducing material-rich side to the other side; and (*c*) the gate selectivity of this particular channel-forming system (both excitability-inducing material and lipids) is cationic and is without discrimination between ions of this class.

Before the addition of the salts, the voltage gradient across the membrane is zero, and the "excitability-inducing material channels" are open and permit an important increase in the specific conductance of the membrane. After the addition of the salts to the inside compartment, some cations (but not anions) escape through the opened channels to reach the outside compartment; as a consequence, a voltage gradient is developed. This voltage gradient, in turn, causes the gates to close.

A depolarizing current, which brings the voltage near zero, causes the gates to reopen; cations then flow from the inside to the outside, and the voltage gradient tends to be restored. This voltage-restoring effect occurs after a small time delay necessary to depolarize the membrane and to open the gates. Experimentally, it is important to note that there is no inflection in the voltage–time curve of the first phase of the phenomenon (a in Fig. 1) and that the rectification process occurs, in a continuous way, all over b (Fig. 1) as the potential moves toward a new equilibrium value.

Action Potential—Protamine "interacts" with *a portion* of the excitability-inducing material channels in the membrane ["it adsorbs in or near the channel" (9)] and changes the selectivity of that portion of the initial excitability-inducing material–lipid system from cationic to anionic (here, also, the selectivity applies to anions as a class). Now, the bilayer has two different kinds of channels, anion selective and cation selective, which behave (kinetically, at least) similarly to the sodium and potassium channels of nerves (1–4, 9).

A depolarizing current of threshold intensity activates the rapid forming anionic gates, allowing the passage of anions and the depolarization of the membrane. The cationic gates (like the potassium gates of nerves) are activated more slowly and manifest their effect after the peak of the action potential has been attained. The fall in potential they initiate acts as a stimulus for the closing of the anionic channels (starting at d in Fig. 1), and the membrane potential is thus restored rapidly. (For simplification, the inactivation of the channels is not considered here.)

In bilayers, anions and then cations flow from the same side of the membrane during the action potential; in nerves, sodium and then potassium flow from opposite sides of the membrane. Experimentally, two inflection points are essential features of the phenomenon: during the rising phase of the action potential (c in Fig. 1) and during the falling phase of the action potential (d in Fig. 1)[7].

RESULTS AND DISCUSSION

The effects of procaine (added to the inside compartment) on the synthetic action potential of bilayers are shown in Fig. 1. At low drug concentrations (0.2–1.0 m*M*), no significant effect could be detected. As the concentration was increased to about 3 m*M*, depolarizing currents of the intensity previously needed for excitation failed to trigger the action potential. Much higher depolarizing currents were then necessary. Upon further increase in the concentration of the local anesthetic (to about 5 m*M*), the synthetic action potential was definitely abolished, regardless of the intensity of the depolarizing currents used.

Careful examination of Fig. 1 reveals, however, that the delayed rectification was not significantly modified, suggesting that the local anesthetic exerted its effects preferentially on the anion-selective channels of the excitable bilayer. (A 50-fold increase in the protamine concentration did not restore the action potential, thus minimizing the possibility that the only effect of the local anesthetic was the competitive displacement of this basic protein from its coupling with the excitability-inducing material and, consequently, the destruction of the anionic gates.) The concentrations of procaine, tetracaine, and lidocaine necessary to abolish the synthetic action potential were 2–5, 0.1–0.25, and 1–5 m*M*, respectively; in nerves, the concentrations of these drugs abolishing the action potential are 3.7, 0.1, and 3.8 m*M* (10).

From these observations, a few analogies between the effects of local anesthetics on excitable bilayers and nerves become apparent. The

[7] The recording paper can be unrolled at a faster rate (by a factor of 5 or 10) during an experiment, which enlarges the time scale and helps in the identification of the two inflection points.

concentrations of these drugs abolishing the synthetic and the nerve action potential are roughly identical. In both cases, the excitation threshold is raised (see Ref. 11 for this effect in nerves). With nerves, it is well established that the sodium channels, responsible for the rising phase of the action potential, are generally more sensitive than the potassium channels to the presence of local anesthetics (10, 12, 13); with excitable bilayers, the observations presented in Fig. 1 suggest that the analogous channels, the anionic channels, responsible for the rising phase of the action potential also are affected preferentially by the presence of the drugs.

At this stage of the investigation, no attempt was made to define the kind of molecular effect local anesthetics might exert on the anionic channels of bilayers [*i.e.*, these drugs, for example, may act directly on these channels or indirectly through an effect on the lipid matrix (14)]. Rather, the hypothesis that local anesthetics may specifically affect the gating mechanism of excitable membranes in general is emphasized. Procaine does exert some effect on the sodium gating currents of nerves (5), thus affecting their molecular mechanism of gate formation. Another observation consistent with this idea is that tetrodotoxin, which exerts its effects on the "component" of the channels responsible for sodium selectivity of nerves [*i.e.*, this poison selectively blocks the sodium conductance (15) without affecting the sodium gating currents (16)] had no effect on the synthetic action potential of bilayers[8] (where different ion selectivities are involved).

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Binding of Butylated Hydroxyanisole to Human Albumin Using a Novel Dynamic Method

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Received March 7, 1977, from the *Department of Pharmacy, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612.* Accepted for publication November 2, 1977.

Abstract □ To study the interaction of butylated hydroxyanisole with various body tissues, a fully automated dynamic method was developed for the determination of plasma protein binding constants at 37°, using membrane filtration equipment designed for dissolution rate studies. Appropriate equations were derived for the calculation of the free drug concentration from comparative diffusion rates across sealed dialysis sacs. A monoexponential equation described the diffusion in the absence of proteins, and a biexponential equation was fitted to diffusion from the drug–protein complex. The Scatchard and double-reciprocal plots were developed for butylated hydroxyanisole. A high degree of reproducibility was obtained for the calculation of protein binding constants ($K = 2.4 - 2.9 \times 10^4$ and $n = 1.4 - 1.32$). The magnitude of these binding-constants suggests that any change in protein binding can have a significant effect on the distribution of butylated hydroxyanisole throughout the body, such as may be brought about by the common variations in the amount ingested.

Keyphrases □ Butylated hydroxyanisole—binding to human albumin studied using membrane filtration equipment □ Binding—butylated hydroxyanisole to human albumin, studied using membrane filtration equipment □ Antioxidants—butylated hydroxyanisole, binding to human albumin studied using membrane filtration equipment

Phenolic antioxidants, butylated hydroxyanisole and butylated hydroxytoluene, are widely used in various products (1) to which the American public is exposed frequently. Although these compounds are generally regarded as safe (GRAS list), they cause enzyme induction (2–9), accumulate in the body tissues (9–13), exhibit dose-dependent elimination (10, 14, 15), affect cellular growth and organization (16–26), and induce various other effects (27). However, no tissue interaction studies have been reported that characterize the toxicity of these compounds.

This paper reports plasma protein binding characteristics of butylated hydroxyanisole using membrane filtration equipment designed for dissolution rate studies. This novel method allows quick estimation of binding parameters at physiological temperature and is totally automated. Because of its advantages over other methods

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