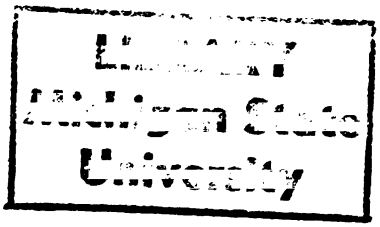
THESIS



This is to certify that the

thesis entitled

"An investigation on the interaction of heart drugs beta-adrenergic blockers and calcium blockers with membrane lipids using artificial model membranes"

presented by

Biao Shi

has been accepted towards fulfillment of the requirements for

Master of Science degree in Biophysics

Major professor

H. Ti Tien

Date May 13, 1985

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AN INVESTIGATION ON THE INERACTION OF HEART DRUGS BETA-ADRENERGIC BLOCKERS AND CALCIUM BLOCKERS WITH MEMBRANE LIPIDS USING ARTIFICIAL MODEL MEMBRANE

by

Biao Shi

A THESIS

Submitted to
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in partial fulfillment of the requirements
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Department of Biophysics

1985

ABSTRACT

AN INVESTIGATION ON THE INTERACTION OF HEART DRUGS BETA-ADRENERGIC BLOCKERS AND CALCIUM BLOCKERS WITH MEMBRANE LIPIDS USING ARTIFICIAL MODEL MEMBRANES

By

Biao Shi

The interaction of heart drugs beta-adrenergic blockers and calcium blockers, with the membrane lipids has been studied using the artificial model membranes to explore the mechanism of the action of these drugs. The experimental results show:

1. Many of the drugs induce the transmembrane potential on BLM. The magnitude of the induced potential is correlated with the lipid-solubility and the membrane effect of the drugs.
2. Many of the drugs increase the membrane fluidity of liposomes composed of various phospholipids. The fluidizing effect is also correlated with the membrane effect of the drugs.
3. These drugs inhibit the carrier-mediated cation transport. The inhibition is mainly due to a perturbation of the electrostatic properties of the membrane lipids elicited by the drugs.

These facts suggest that beta-blockers and Ca^{+2} blockers can induce several alterations of the physical-chemical properties of the membrane through their interaction with lipids. This lipid effect may contribute to some of the biological and pharmacological activities of the drugs directly or through the lipid-protein interaction.

Additionally, as the beginning of the study on the drug's action on membrane proteins, the potassium channels in sarcolemma vesicles from rat heart have been reconstituted in BLM.

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GENERAL INTRODUCTION

Cardiovascular diseases (heart diseases) are leading diseases, threatening people's health and life all over the world. Scientists have been making great efforts to develop new drugs to combat them. Calcium channel blockers (calcium blockers or Ca^{+2} blockers) and beta-adrenergic receptor blockers (beta-adrenergic blockers or beta-blockers) (Figures 1a and 1b) are two types of effective cardiovascular drugs (heart drugs) that have appeared in the last two decades. They are widely used today to treat a wide variety of heart diseases like angina, heart attack, arrhythmias and hypertension.

Calcium blockers are thought to combat heart diseases primarily by preventing Ca^{+2} entry through the excited myocardiac membranes (Flenkenstein-Grün, 1984). Because Ca^{+2} plays a critical role in muscle contraction, the decrease in the availability of Ca^{+2} slows the pumping rate and declines the contraction tension of the heart, and the blood demand and workload of the heart is reduced. The decrease in the intracellular Ca^{+2} level also leads the inhibition of the contraction of smooth muscles in the coronary artery walls, and thus allowing them to expand. This, in turn, increases the blood supply to the heart and protects arteries from suddenly clamping and closing off blood to the heart.

It is known that beta-adrenergic blockers act in a manifold manner (Shanks, 1984). Firstly, they compete with epinephrine and norepine-

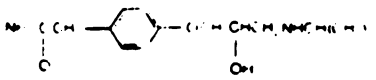
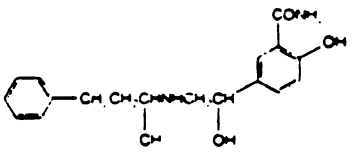
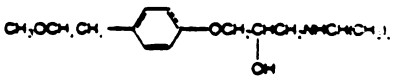
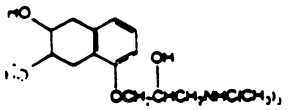
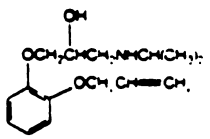
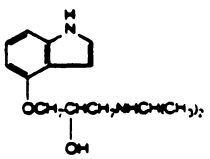
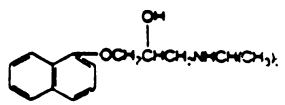
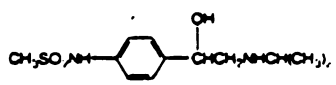
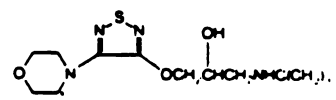
Atenolol	
Labetalol	
Metoprolol	
Nadolol	
Oxprenolol	
Pindolol	
Propranolol	
Sotalol	
Timolol	

Figure 1a. Chemical Structures of the beta-adrenergic receptor blockers.

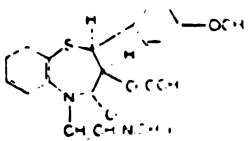
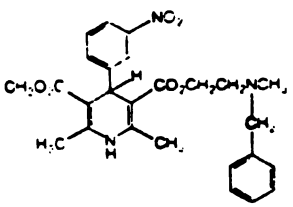
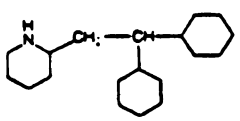
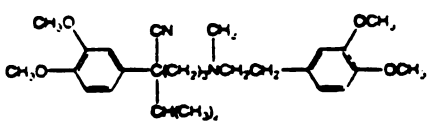
Diltiazem	
Nicardipine	
Perhexiline	
Verapamil	

Figure 1b. Chemical Structures of the calcium channel blockers.

phrine for beta-adrenergic receptors and prevent these neurotransmitters from stimulating the target cells (Lefkowitz, 1979). When deprived of such stimulation, the heart muscle pumps blood at a lower rate and requires less oxygen to function satisfactorily. This also helps to decrease the frequency and severity of heart diseases. In addition to the beta-receptor activity-related action, beta-blockers also function by blocking ion transport (Ca^{+2} and Na^{+}) through the interaction with the cell membranes (Messineo and Katz, 1979). In summary, both types of drugs are delivered to the periphery of target cells through the blood circulation, where they inhibit membrane channels or receptors directly or through other membrane components, exerting their pharmacological effects.

These drugs are receiving increasing pharmacological attention, but the precise mechanism of their action in molecular terms remains obscure. Because of the great signficance, they are being investigated extensively by pharmacologists, biochemists and physiologists in different approaches. Theoretically, these studies eventually will permit us to explore the molecular basis of the drug's action. Practically, we will be able to develop a novel approach for rapidly designing and screening more potent and safe heart drugs after our understanding the relationship between the drug's functions and their structures and properties.

As a fundamental type of biological macromolecules, lipids have been investigated much less than have proteins and nuclei acids. In the membrane biology, they have been assumed as inertly structural elements and as semipermeable barriers, and their implications for the membrane processes have been relatively ignored (Cullis et al., 1980). The

"mosaic fluid model" proposed by Singer and Nicolson (1972) endowed lipids with some influences on the membrane functions through lipid-protein interaction. For instance, the lipid salvation which results in the aggregation, capping or conformational change of proteins is required for activities of many membrane proteins (Sandermman, 1983). However Singer's concept is not complete, as to where lipids are restricted in providing a matrix in which protein are embedded. If, as argued by many researchers, lipids serve structurally as merely a supporting matrix, it would be most likely that a single species of lipid like phosphatidylcholine (PC), or a few species, could meet the requirement for all living membranes (deKruijff et al., 1981). However, the lipid compositions of biological membranes are extremely heterogeneous. Human red blood cell membrane alone contains at least 20 different molecular species of PC and a total of some 150-200 chemically different lipid molecules (Golde et al., 1967)! Little is known about the functional role of lipids in many membrane processes such as transmembrane transport, cell fusion and the interaction with proteins. The recent discoveries of lipid polymorphism (Verkleij et al., 1979) and the liposome helix (Lin et al., 1982) imply that lipids may be much more important in membrane functions than that we expected before. To appreciate the role of lipids, a modification of the fluid mosaic model—"metamorphic mosaic" model—was proposed (Cullis et al., 1980).

It is widely accepted that the plasma membrane, as being the place where a cell communicates with the external environment and other cells, is the major target site for a variety of drugs (Brasseur et al., 1984). For a large number of drugs, owing to the heterogeneity in chemical structures, the precise mode of interaction with the membrane components

(proteins or lipids) remains a matter of debate. It is known that membrane lipids, especially phospholipids can serve as the action sites for certain classes of drugs (Eibl, 1984). In this case, the chemical components and physicochemical properties of membrane lipids are the factors regulating and controlling the biological and pharmacological effects of drugs. One example is local anesthetics, a clear relationship between their therapeutic effect and their interaction with lipid components of the membrane has been established (Davio and Low, 1981), even though the detailed mechanism is still obscure.

There are different models proposed to explain the action of a drug on the plasma membrane:

1) Drugs act directly through specific protein components (enzymes, receptors and channels) in the membranes without the participation of lipid, and thus the drug's function is little mediated by a change in the property of the lipid matrix (Ohki, 1984). For specific interactions, the drugs usually have molecular structures complementary to the proteins electronically or stereochemically (Phadke et al., 1981). This is probably a common mechanism for many drugs. For instance, some hydrophilic drugs such as the antihistamine, cimetidine (Rooney et al., 1979), work according to this model. They bind to target membrane protein hydrophilically, being analogous to a typical substrate-enzyme interaction in aqueous media.

2) The final target of some drugs is the membrane proteins, but they affect the proteins only secondarily to their action upon the lipid matrix (Trudell, 1977). This lipid-protein cooperation model is thought to be quite common, and different ways are envisaged to link it to an effect of protein functions. Besides the lipid salvation mentioned

before, in which the activity of a protein is sensitive to the fluidity of either bulk lipids or annular lipids surrounding it, the binding of a charged drug may alter the charge state of the membrane. The electrical perturbation may change the gating states of channels, functional conformation of enzymes and affinity of receptors to ligands. An example is the histamine H_1 antagonist methydilazine (Lee, 1982) in which positively charged molecules show extensive lipid binding, and repel the cationic histamine molecules away from the membrane surface, thus reduce agonist availability in the vicinity of histamine receptor. Another alternative is the membrane pathway model. Herbette (1984) reported that propranolol, a beta-adrenergic blocker, has to partition into the lipid bilayer before searching the binding site on the membrane protein receptor by the lateral diffusion. The action site of a drug can also be at the lipid-protein interface. In the "lipid shape concept" (Hornby and Cullis, 1981), some membrane channels (e.g. Na^+ channel) are presumed to be protein-lipid complexes and the existance of lipids of a certain shape is necessary for the channel to acquire a conductive configuration. When the lipids are displaced by drug molecules of different shapes, the protein pore will be closed because of the loss of the "braces" supporting a proper architecture.

3) Membrane lipids play the predominant role in the drug's action. This not only refers to the lipid facilitation of the travel of many drugs acting intracellularly though the membrane. A classical example of the direct implication of lipids was proposed by Seeman (1972). In his membrane expansion theory for uncharged anesthetics, the drug molecules penetrate into the hydrophobic core of the lipid bilayer, expanding critical regions in the membrane and thus preventing the ionic

permeability. The antibiotic property of that polymyxin may also lie in the change in the membrane permeability due to the distortion of lipid bilayer (Hartmann et al., 1977). Electron microscopy demonstrated polymyxin induced separation of the bound lipids and free lipids and the appearance of domain structurals, leading to the formation of structural breaches on the separated lipid domains. The discovery of non-bilayer structures presents a new approach by which a drug may act through membrane lipids. These structures, like inverted micella and hexagonal (H_{II}) phase, have been proved to participate actively in some important membrane process such as the membrane fusion (Hui et al., 1981) and transmembrane transport of divalent ions (Verkleij et al., 1982). Some drugs, e.g. the antitumor agent, adriamycin (Goormaghtiph, 1982), are found to interfere with the transition between bilayer and non-bilayer lipid phases.

Of course, the variety of drug-membrane interactions can not be covered by such a few simple models. For instance, some drugs may be nondiscriminative by membrane components. They are directed to an interface between an aqueous solution and macromolecular structures on the membrane, regardless of proteins or lipids (Shibuta et al., 1984). Considering the complexity of biological membranes and the diversity of the structures and properties, we should say that all these models (and others) are possible. Different drugs, needless to say, function in different mechanisms, and a single species of drug may function in different ways, depending on the target membrane, the local physiological conditions and the drug's concentration and state.

The action of the heart drugs on the membranes is far from fully understood. For beta-adrenergic blockers there are two distinct

effects: specific and non-specific. In the specific effect, beta-blockers bind to beta-adrenergic receptors directly (Lefkowitz, 1979), leading to the attenuation of the response to the beta-agonist stimulation. In the non-specific effect, they cause a functional perturbation of the biological membranes. The target sites for the non-specific actions are believed to be lipid components (Hellenbrecht et al., 1973). The drug molecules partition into the bulk lipid matrix and decrease ion flux through interaction with membrane lipids, leading to the cardiac effects (Messineo and Katz, 1979). There has been a large body of evidence that the inhibition of Ca^{+2} uptake in cardiac cells by beta-blockers was independent of their beta-blocking activity, only representing the lipid effect (Noack et al., 1978). For example, d-isomers of these agents, which are devoid of beta-blocking activity, are equally effective against Ca^{+2} uptake (Evangelista et al., 1981). These membrane lipid effects were suggested to account for the pacemaker and negative inotropic properties of beta-blockers (Rauls and Baker, 1979), and contribute the antiarrhythmic and antiangina function of these agents (Shank, 1984). The lipid effect of beta-blockers also influences blood vessels. As we know, excessive reactivity of blood platelets may lead to atherosclerotic vascular diseases. Propranolol and some beta-blockers are found to be capable of normalizing hyper-active platelet aggregation in patients with coronary artery diseases (Frishman et al., 1974) and relieving angina pectoris (Weskler, 1977). This anti-aggregation effect correlates well with lipid solubility but not other properties of these agents (Iwamura et al., 1983), and Kerry et al. (1984) proposed that it is, most likely, due to the interaction of the drug with membrane phospholipids, which causes membrane disorgan-

ization and thus directly impairs the affinity of the receptor for agonists and/or the function of the Ca^{+2} ionophore.

The mechanism of the action of calcium blockers remains more obscure. Their potency, specificity and subtle structural-activity relations imply that the pharmacological function of Ca^{+2} blockers, at least some of them, is more relevant to the binding to recognition sites at or near Ca^{+2} channels. Such binding sites have been reported to be found on tissue extracts containing Ca^{2+} channel activities. For example, Norman et al. (1983) reported a binding site for dihydropyridin Ca^{+2} blockers located on a glycoprotein macromolecule of Mt. 200,000. On the other hand, some investigators believe that at least some Ca^{+2} blockers function by purely physico-chemical interaction with the cell membranes (Towart and Schramm, 1984). It is suggested that Ca^{+2} blockers may act at more than one site, rather than solely on the slow Ca^{+2} channels (Church and Zsoter, 1980). Some Ca^{+2} blockers, e.g. verapamil, have intracellular sites. They are facilitated to travel through the plasma membrane by lipid components (Pang et al., 1984), and then modulate Ca^{+2} level inside the cells through intracellular components such the channels on the sarcoplasmic reticulum (SR) (Galvin et al., 1982) or a Ca^{+2} binding protein like calmodulin (Epstein et al., 1982). Whether or not the interaction of Ca^{+2} blockers with membrane lipids is related to their Ca^{+2} blocking activities has not been studied and is uncertain. There have been some reports that the depression of Ca^{+2} uptake may also result from the general perturbation of the membrane lipids (Fairhurst et al., 1980). Galenhofen and Hermstein (1975) observed two mechanisms in the depression of Ca^{+2} transport by methylverapamil (D600): 1) the depression due to a specific protein at

low drug concentration, and 2) the depression due to the lipid effect at high drug's concentrations. Recently Erdreich and Rahamimoff (1984) found that verapamil affected Na^{+}-Ca^{+2} antiport system derived from heart sarcolemmal vesicles. The inhibition of Ca^{+2} uptake by verapamil could be reversed by adding an excess of PC, and so it is possible that the drug acts in the lipid phase and not just on the transporting molecules.

Calcium blockers so far examined are all appreciably membrane-active agents (Sasaki, 1984). For instance, they are able to inhibit the platelet aggregation through a modification of physicochemical properties of membrane phospholipids (Kiyomoto, 1980). Is the potent Ca^{+2} blockade by Ca^{+2} blockers a result of potentiation of the nonspecific membrane effect, and is the membrane-active property prerequisite to the potent Ca^{+2} blocking activity of the known Ca^{+2} blockers? There have been no answers. It is easy to understand the exertion of all beta-adrenergic blockers on beta-receptors in terms of their similar chemical structures. But the molecular architectures of the so-called "Ca^{+2} blockers", verapamil, diltiazem, dihydropyridines and others, have no outstanding common features (Henry, 1980) except that all possess a bulk hydrophobic mass with a secondary or tertiary nitrogen in the periphery. Usually, a wide spectrum of chemical structures means a lack of stereospecificity in the drug's action, and it may be indicative of the interaction of the drugs with membrane lipids rather than with specific proteins (Sasaki, 1980). This may be also explained by the existance of the different populations of Ca^{+2} channels or binding sites whose affinities are different for various Ca^{+2} blockers. This concept has been applied to interpret the

histological selectivity of Ca^{+2} blockers (Hof, 1984). As an example, Nachshen and Blaustein (1979) suggested that Ca^{+2} channels in neurons are less sensitive to Ca^{+2} blockers than their equivalents in myocardiac cells.

Since it appears that the interaction with membrane lipids represents an aspect of the action of the heart drugs, the detailed knowledge about it will be useful. Even if this interaction does not contribute much to their therapeutic functions but causes some side effects, the knowledge will help us to design new drugs devoid of the adverse effects which may restrict the clinical use of the drugs. As a possible application in this research, we can develop liposome carries, which are affinitive to both the drugs and the biological membranes, to deliver the drugs to the target tissues more efficiently (Finkelstein et al., 1978).

Complex structural and environmental factors make biological membranes in vivo not readily amenable to the investigation of a membrane process in physical-chemical terms. Model membranes such as planar bilayer lipid membrane (BLM) and lipid microvesicles (liposomes) provide good research tools for this goal. Besides the simplicity which make analysis easier, BLM simulates some of the important characteristics of biological membranes (Tien, 1985): it exists as "liquid-like" ultrathin structure associated with two coexisting liquid interfaces, and it is capable of separating dissimilar aqueous phases and functioning in a vectorial or directional manner. These advantages have shown the practical value of BLM in the area of drug research (Nelson et al., 1984).

The plan of the study of the heart drugs using artificial model membranes are in two steps:

1) Investigate the drug's action on the unmodified model membranes (membranes without the native proteins).

2) Isolate the protein channels and receptors from living tissues, incorporate them into the model membranes and investigate the drug's action on the reconstituted membranes.

In the first step, we have extensively and systematically studied the interaction of the two types of heart drugs (beta-adrenergic blockers and calcium blockers) with membrane lipids using unmodified BLMs and liposomes, as well as model membranes modified with artificial ionophore substances. The experimental results showed:

1) Many beta-blockers and Ca^{+2} blockers produced large transmembrane potentials. The magnitudes of the induced potentials are correlated with the lipid-solubility and the membrane effects of the drugs.

2) Many of the drugs increased the membrane fluidity regardless of the physical state of the lipid bilayer. The fluidizing effects are also correlated to the lipophilicities and the membrane effects of the drugs.

3) These drugs inhibitied the carrier-mediated cation transport but not the channel-mediated cation transport through BLM. This inhibitory effect is mainly due to an electric perturbation of the membrane elicited by the drugs.

These facts suggest that the heart drugs can induce several alterations of physicochemical properties of the membrane through their interaction with lipids. This lipid effect may be involved in or may

influence some of the biological and pharmacological functions of the drugs directly or through lipid-protein interactions.

It should be pointed out here that we only focus on the drug's action at the lipid level in this thesis. It does not mean, in any sense, that the role of membrane proteins should be ignored. On the contrary, proteins, as major performers of the membrane function, are responsible for the pharmacological effects, especially for more specific effects of many drugs. Lipids, not being informational macrobiological molecules, appear not to confer much specificity on a biological system according to today's concept. In the second step, we will investigate the interaction between the drugs and the related membrane proteins. So far we have reconstituted voltage-operated potassium channel and Ca^{+2}-dependent channel from rat cardiac sarcolemma into BLM. We feel that we can not delineate the mechanisms of the drug's action unless we make it clear in the different aspects and at the different levels.

PART I

THE DRUG-INDUCED TRANSMEMBRANE POTENTIAL ON BLM

The interaction of a drug with a lipid bilayer can be expected to produce various effects; and changes in the fluidity and electrical properties are the most common effects with important consequences for a wide variety of membrane processes (Lee, 1978). Electrical perturbation of BLMs is conveniently monitored by the measurement of electrical parameters, such as membrane potential, resistance, capacitance and dielectric breakdown using BLM (Tien, 1974). This strategy has been employed with various types of drugs. For instance, the influence of anesthetics on the membrane potential and resistance was studied on BLM by several groups (e.g., Tsofina et al., 1978). Schlieper and Medda (1980) used unplanar and unbilayer model membrane systems to investigate several beta-adrenergic blockers. There have been few experiments done with calcium blockers on the unmodified BLM. In the following experiment both beta-blockers and Ca^{+2} blockers were found to induce transmembrane potential but showed little influence on membrane resistance and capacitance.

MATERIALS AND METHODS

Experimental Arrangement

A schematic diagram showing the experimental arrangement for the formation and electrical measurement of BLM is drawn in Figure 2. It mainly consists of:

1) Outer container - made from a solid Lucite block with 2 adjacent drilled holes (one playing the role of outer chamber) and with 2 glass windows, one for illumination and another for observation.

2) Teflon septum - a 10 ml Teflon beaker with a small aperture punched through it. The diameter of the aperture was measured with a calibrated reticle and its area is 19.0 mm^2.

3) Stirrers - a pair of magnetic stirring bars, one in each chamber.

4) Calomal electrodes - a pair of KCl-saturated calomel electrodes are used to provide electrical contact with the membrane.

5) A pair of glass electrodes (Mankson), used for monitoring the solution pH and also for electrical measurement.

6) Digit pH meter (Larza).

7) High impedance electrometer (Keithly 610C).

8) Low impedance picoammeter (Keithley 41).

9) Low level capacitance meter (ICF/Electronic Model 1-6).

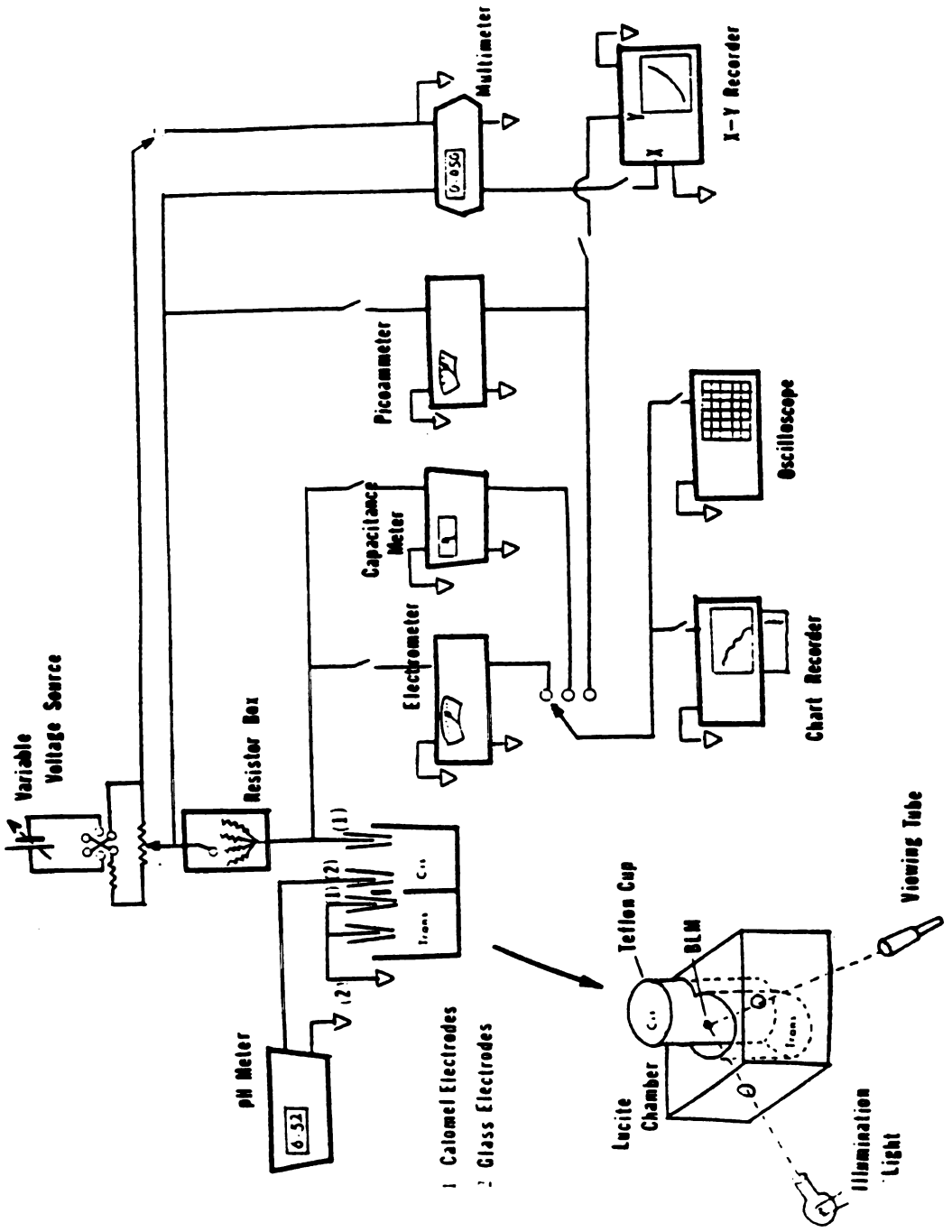
10) External variable voltage source.

11) Standard resistor box - it can be selected as 0 or 10^5 to 10^6 by a factor of 10.

12) Chart recorder (Omniscope).

13) X-Y recorder (MFE plotamatic 715M).

Figure 2. Schematic diagram of experimental arrangement used in BLM studies.


Variable
Voltage Source
Resistor Box
pH Meter
6.32
1 Calomel Electrodes
2 Glass Electrodes
Electrometer
Capacitance
Meter
Picoammeter
Multimeter
6.050
X-Y Recorder
Oscilloscope
Chart Recorder
Teflon Cup
BLM
Lucite
Chamber
Viewing Tube
Illumination
Light

BLM Forming Solution and Formation of BLM

The membrane forming solution (FS) consisted of 3.1% egg phosphatidylcholine (PC), 1.7% E.coli phosphatidylethanolamine (PE), 1.1% bovine phosphatidylserine (PS) and 1.0% oxidized cholesterol (OC) in W/V except for that described specially in some experiments. This formula gives a stable BLM with long lifetime (0.5 - 2 hrs) and is suitable for our measurements. Before application, all phospolipid-organic solvent solutions were evaporated, first in a stream of argon and then in vacuum. The procedure for oxidation of cholesterol followed Tien et al. (1966). The dried phospholipid and oxidized cholesterol were dissolved in n-octane or n-decane. The BLM was formed by usual technique (Tien, 1974) on the small hole of Teflon cup with the aid of a Hamilton repeating microsyringe. The formation of "black" membrane was monitored using a stereoscopic microscope.

Electrical Measurement

After the membrane had turned "black" a small volume of drug stock was injected into the inside of the Teflon cup with a micropipette and the same volume of the bulk solution was withdrawn for excluding any change due to hydrostatic pressure difference. The drug stock was prepared by dissolving a drug in the same buffer (0.01 M NaCl, 5 mm tris-glycine, pH 6.5 except for that described specially) used as the BLM bathing solution (BS), and the pH of the stock was readjusted so that the pH of bathing solution was kept constant after the addition of the drug, avoiding any interference from H^+ concentration gradient across the membrane. The introduction of the drug solution was followed by stirring with magnetic bars for a certain time to make the bulk solution homogeneous quickly.

The BLM separated two bathing solutions in which two electrodes were submerged. The side (Teflon beaker) to which drugs were added was designated as the inside (cis side), and electrode in the cis side was connected to the measurement instruments. The electrode in the outer chamber (outside or trans side) was connected to virtual ground, serving as a reference electrode. BLM resistance was measured by a voltage divider circuit (Tien, 1974). A known voltage (V_i) was applied across the BLM equivalent RC circuit, which is parallel to electrometer, and a standard resistor (R_i) in series, and the BLM resistance R_m was calculated from the electrometer reading of V_m (i.e., membrane potential) with the equation,

$$R_m = R_i \frac{V_m}{V_i - V_m} \qquad (1)$$

The membrane potential was measured by disconnecting the power source and R_i from the circuit. The time course was recorded with a chart recorder. In the pH effect experiment, pH in the bulk solution was monitored accurately by a pair of glass electrodes connected to a pH meter. The BLM formation system and electrodes were protected by a Faraday cage from the external noise. All the measurements were performed at room temperature ($22 \pm 1^{\circ}C$) and were repeated 2-3 times.

RESULTS

Many of the drugs tested (Figure 1), both beta-adrenergic blockers and calcium blockers, were able to induce large transmembrane potential while being added to the bulk solution on the cis side. This potential was due to the effect of drugs with BLM itself rather than the hydrostatic or mechanical perturbation which might arise at the moment of the

addition of chemicals and while stirring. This was demonstrated by a control in which the injection of an electrolyte solution, instead of drug stock, did not produce the effects. The potential has minus sign with respect to the cis side to which the drugs were added. And its magnitude is dependent upon the drug's concentration, the composition and concentration of the forming solution, and the pH, ionic species and strength of the bathing solution.

1. Dose-Response Relation

Three calcium blockers and seven beta-adrenergic blockers were examined by measuring the potential across BLM bathed in 0.01 M NaCl buffer (5 mM tris-glycin pH 6.5) (Figures 3 and 4). The drugs began to show their effect at about 10^{-6} M of the final concentration in the bathing solution and could induce quite high transmembrane potentials (-50 to -100 mV) at 10^{-3} M. The exceptions were sotalol and atenolol which produced small potentials (about -10 mV at 10^{-3} M). In the cases of all drugs, no significant change in current was measured while the potential was developing.

The dose-response experiment was performed by the successive addition of the drug solution without rupture of the membrane after the potential no longer changed. The curves in Figures 3 and 4 showed a quasi-linear relation between the induced potential and drug's concentration over the range from 10^{-6} to 10^{-3} M for the drugs. The capacities for inducing the potentials for various drugs are different. This can be estimated with the values of the induced potential at the same drug concentration and salt concentration (Table 1). It reflects the different abilities of drugs to interact with the membrane lipids.

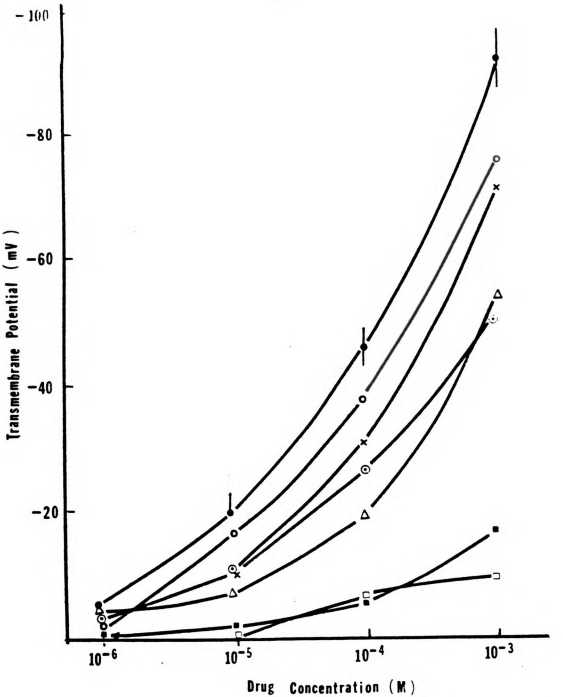


Figure 3. Variation of the induced potential with the concentration of beta-blockers: (●) propranolol, (○) labetalol, (×) oxprenolol, (Δ) metoprolol, (⊙) pindolol, (▪) atenolol and (□) sotalol.

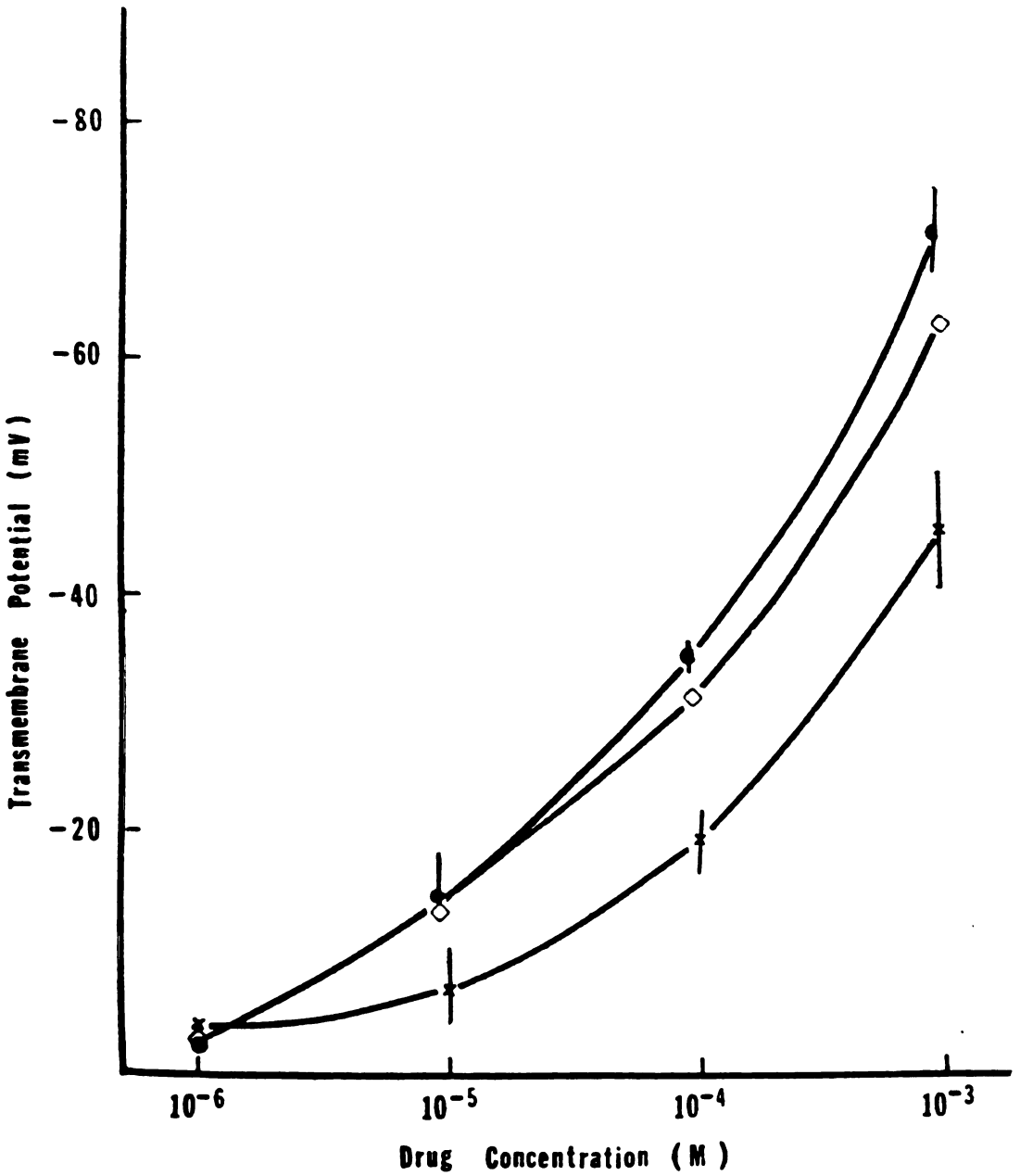


Figure 4. Variation of the induced potentials with the concentrations of calcium blockers: (●) verapamil, (◇) nicardipin and (×) diltiazem.

Table 1: The potentials induced by various drugs at 1.0mM in 1.0mM NaCl in BLM of various composition.

Drug	(A) potentials (mv) on PC+ PE+OC+PS BLM	(B) potentials (mv) on PC+ PE+OC BLM (mv)	Ratio B/A
verapamil	-75.0 ± 3.0	-45.5 ± 2.5	0.61
nicardipin	-61.5		
diltiazem	-49.0 ± 5.0	-37.0 ± 1.0	0.76
propranolol	-96.5 ± 4.5	-53.0 ± 2.0	0.55
labetalol	-73.5 ± 3.0	-50.0 ± 2.0	0.68
oxprenolol	-70.0 ± 1.5	-48.0 ± 4.0	0.64
metoprolol	-64.0	-38.0	0.59
pindolol	-55.0		
atenolol	- 9.5 ± 2.5		
solalol	- 7.0	- 5.0	0.71

Some values are the average of the potentials from two repeats + standard deviation of the mean.

While developing the transmembrane potential, the drugs showed little influence on BLM resistance (Figure 5). The slight apparent decreases in the membrane resistance in Figure 5 were due to not only the introduction of the drugs but also to a spontaneous change during the lifetime of BLM which at time is difficult to avoid completely. The results for the heart drugs can be compared with the behavior of 2-2-dinitrophynel (DNP), a well known phosphorylation uncoupler. DNP also generated a transmembrane potential (Lea and Croghan, 1969). We measured about +45 mV potential across BLM in presence of 10^{-3} M DNP in cis side, but the development was accompanied by a tremendous decrease (3 orders) in BLM resistance.

2. Effects of Ionic Strength on Drug-Induced Potential

Verapamil, a representative of calcium blockers, which is widely used in dilating blood vessels, was chosen for further study in the series of experiments described below.

The ionic strength in bulk solution affected the induced transmembrane potential in a unique way. In the experiment shown in Figure 6, verapamil concentration was fixed at 10^{-3} M. Up to 0.1 M of electrolyte KCl, the potential had practically no change with the variation of the salt concentration. Over 0.1 M, it was depressed dramatically with the increase in KCl concentration until it was almost totally eliminated by highly concentrated electrolyte (about 1 M). This phenomenon was also observed with solutions of other monovalent metal cations (Na^+ and Li^+). And it was true for other drugs tested. Some results are shown in Figure 7. Exceptions were sotalol and atenolol, where the effect of ionic strength was not significant, because their

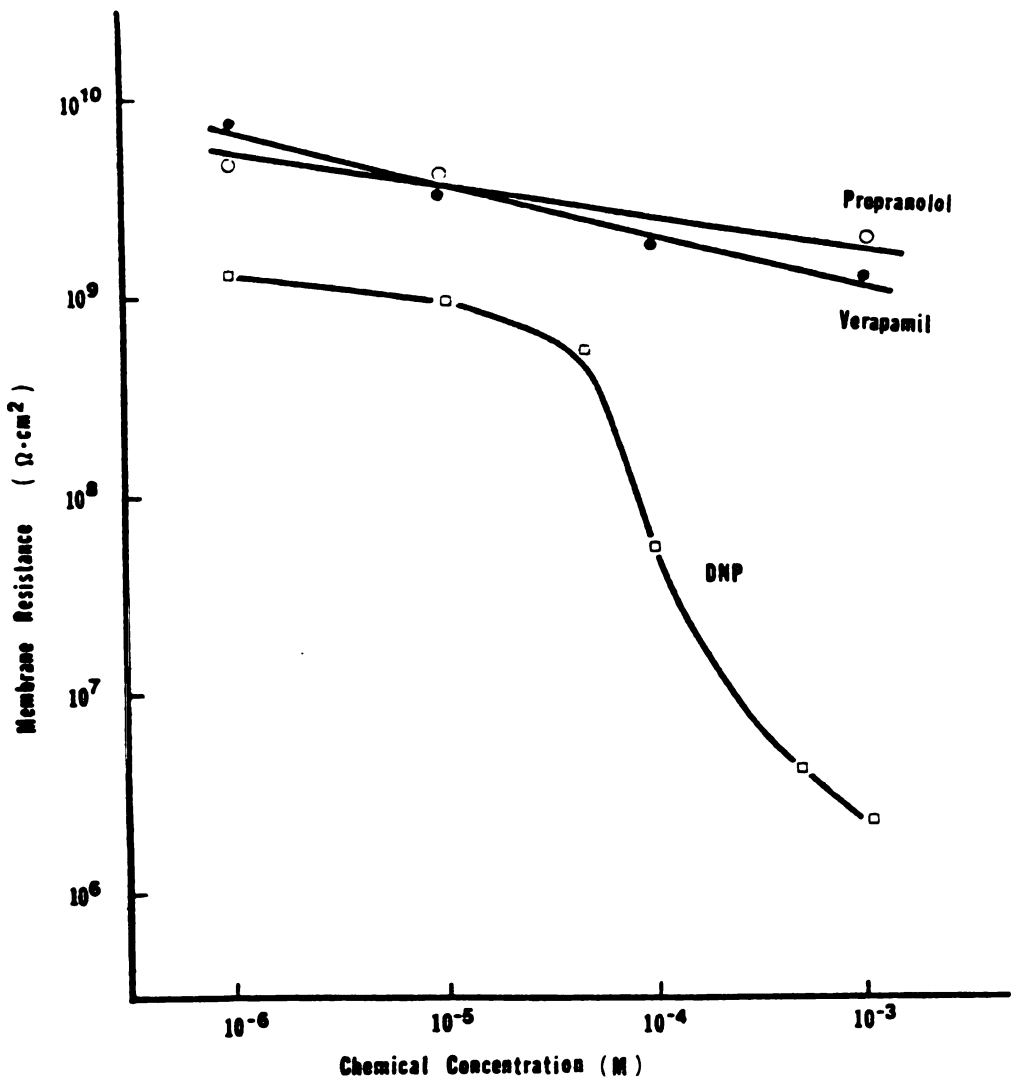


Figure 5. Variation of BLM resistance with the concentrations of the drugs and DNP. (●) verapimil, (○) propranolol, (□) DNP. Membrane solution in DNP experiment was the same as that in the drug experiment. The bathing solution in DNP experiment: 0.1MNaCl.

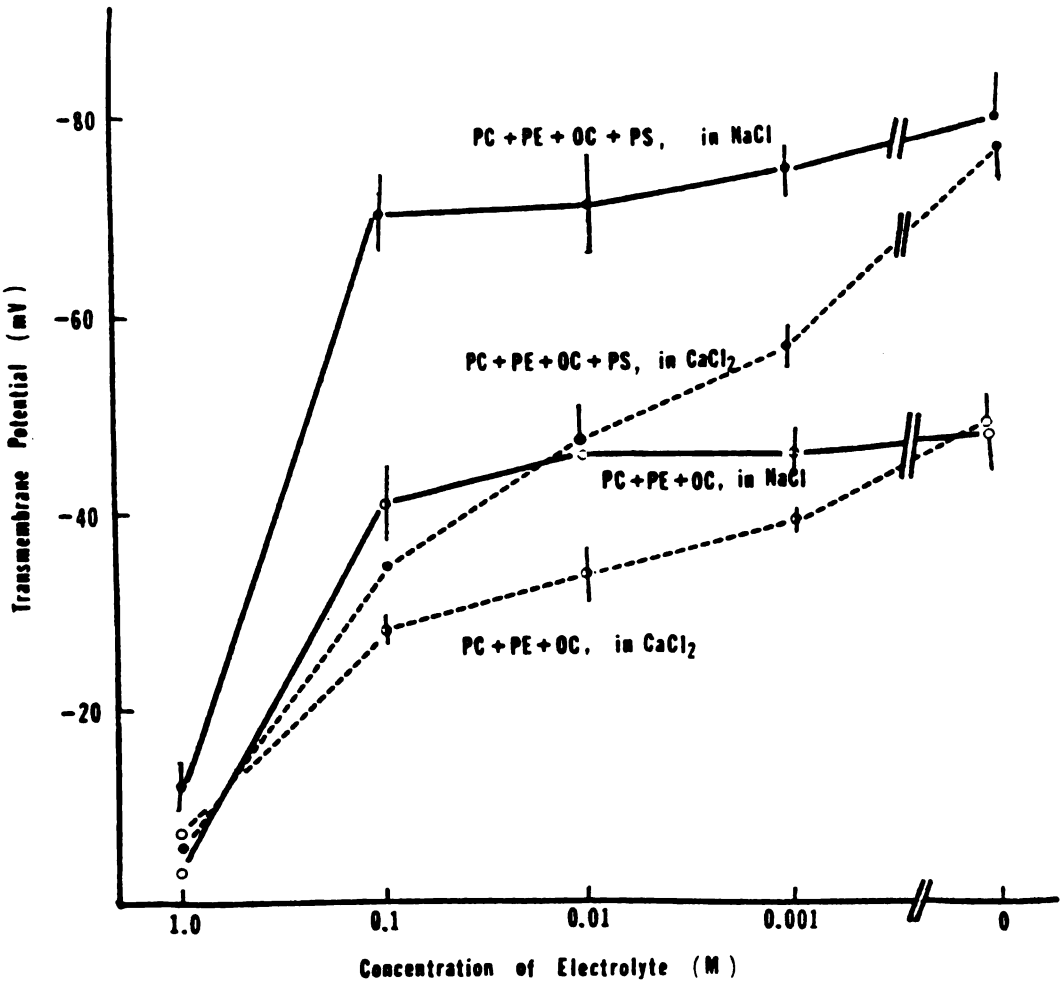


Figure 6. The transmembrane potentials induced by veraparmil 0.001M on neutral BLM and PS-containing BLM under various ionic strength and species. PC + PE + OC + PS BLM was formed in NaCl (●—●) and $CaCl_2$ (●--●) bathing solution. PC + PE + OC BLM was formed in NaCl (○—○) and $CaCl_2$ (○--○) solution.

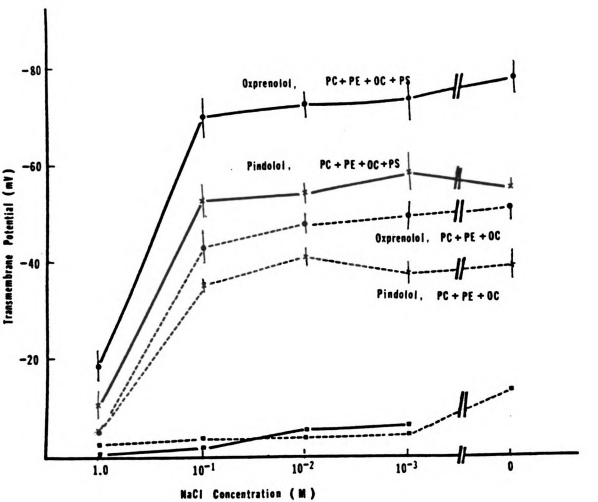


Figure 7. The transmembrane potentials induced by oxprenolol (●), pindolol (×), and sotalol (■) on neutral BLM and PS-containing BLM at various ionic strength: PC + PE + OC + PS BLM (——) and PC + PE + OC BLM (----). Drug concentration was 0.001M.

induced potentials are too low to be much affected, and such small changes are difficult to measure accurately.

3. Effects of Ionic Species on Drug-Induced Potential

The ionic species is another important factor modulating the drug's effect. Divalent metal cations, especially Ca^{+2}, are distinct from monovalent cations in influencing the drug-induced potential (Figure 6). When electrolytes were administered at the same level, verapamil produced the much smaller potentials across the BLM bathed in $CaCl_2$ than those bathed in NaCl or KCl solution. Furthermore, one can see the dissimilarity in the curves of potential vs. salt concentraton in Ca^{+2} and in Na^{+} bulk solution. The induced potential decreased significantly even though not as greatly in the presence of Ca^{+2} but experimentally showed little change in the presence of Na^{+}. These facts imply an antagonism of Ca^{+2} against the drug molecules. This antagonizing effect was found with BLMs of different formula, but it seems more prominent on PS-containing BLM. Other divalent cations like Mg^{+2} also diminished the drug-induced potential, but not a potently as Ca^{+2}.

4. Effects of Lipid Composition Upon Drug-Induced Potential

These heart drugs generally induced much higher potential on the BLM containing phosphatidylserine (PS) than on the BLM without PS (Figures 6 and 7). To evaluate the influence of PS, the induced potential was measured on two types of BLM namely 1) PC + PE + OC + PS, and 2) PC PE + OC at 10^{-3} M verapamil and 10^{-3} M NaCl. The two potentials and their ratio are listed in Table 1 which shows that the potentials on BLM without PS are only 50-75% of BLM containing PS.

PS is a negatively charged phospholipid owing to -COOH group of serine residue (pK = 2.2) and thus it tends to increase the charge

density on the polar region of the membrane. To confirm the correlation of the charge with the induced potential, the acidic lipids cardiolipin and ganglioside were used to substitute PS in the forming solution. As expected, these 2 acidic lipids also enhanced the verapamil's effect, although not effectively as PS (Figure 8). The correlation was supported strongly by the following quantitative experiment in which we prepared a series of forming solution in such a way that PC and OC were fixed at the usual level (3.1% and 1.0%), but PS concentration was varied from 0.01-1.0%. The measurement revealed that the potential induced by verapamil increased as a linear function of PS content in the forming solutions (Figure 9).

Contrary to this, neutral phospholipid PC of the high concentraton diminished the drug-elicited potential as shown in a similar experiment (Figure 10) where OC was 0.1% and PC varied from 0.1 to 10% in the forming solutions.

5. Effects of pH on Drug-Induced Potential

Figure 11 describes diagrammatically the induced-potential as a function of the pH of the bathing solution. A distinguishing feature in the pH effect curve is that the maximum potential appeared at about pH 8 on PS-containing BLM. On the neutral BLM, the peak value shifted to the right (higher pH) slightly. The pH values of both are close to pK of verapamil (8.45) (Lüllmann and Wehling, 1979). The potentials decreased when pH is changed to a more alkaline or a more acidic milieu. Other heart drugs gave the similar potential vs. pH curves. For instance, metoprolol has pK of 9.68 (Newton and Kenza, 1978) and its peak potential was obtained at around pH 9.0 (Figure 11). These facts

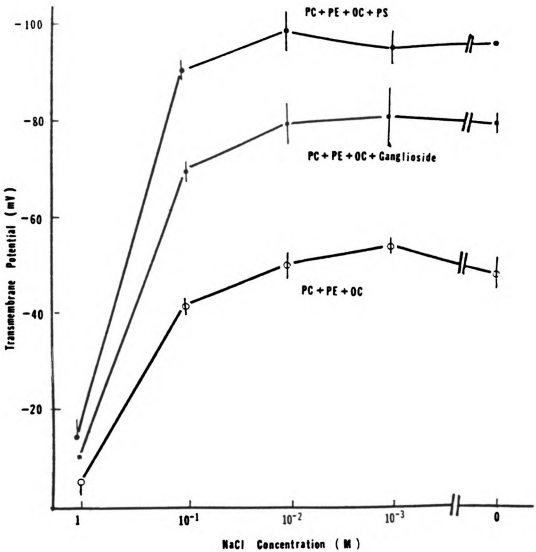


Figure 8. The transmembrane potential induced by propranolol 0.001M on BLM of various composition: PC + PE + OC + PS BLM (●), PC + PE + OC + ganglioside BLM (■), PC + PE + OC BLM (○). The concentrations of PC, PE, and OC were as usual. PS or ganglioside concentration was 1.1%.

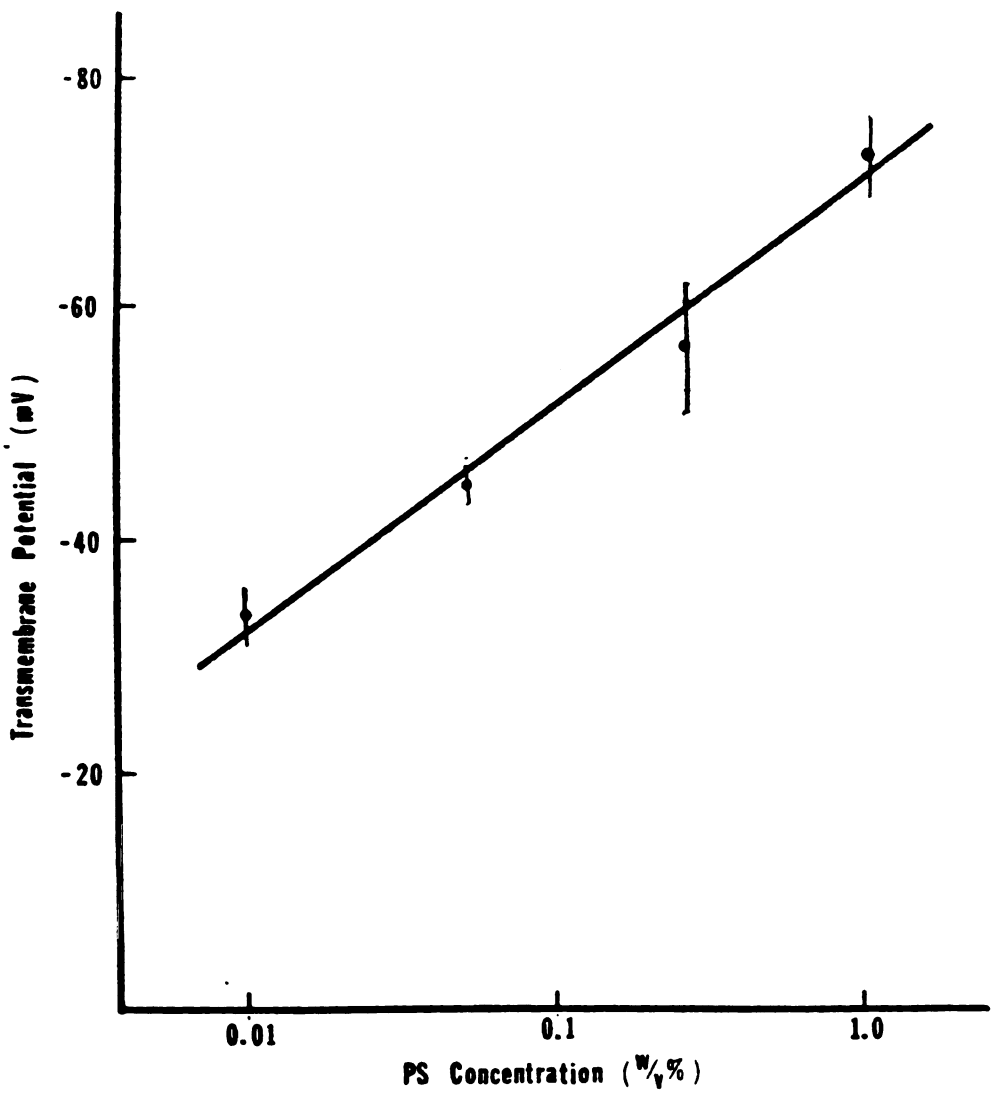


Figure 9. Variation of the induced potential with PS content in BLM forming solution. The forming solution was composed of 3.5% PC, 0.5% OC plus PS. The drug used was verapamil (0.001M).

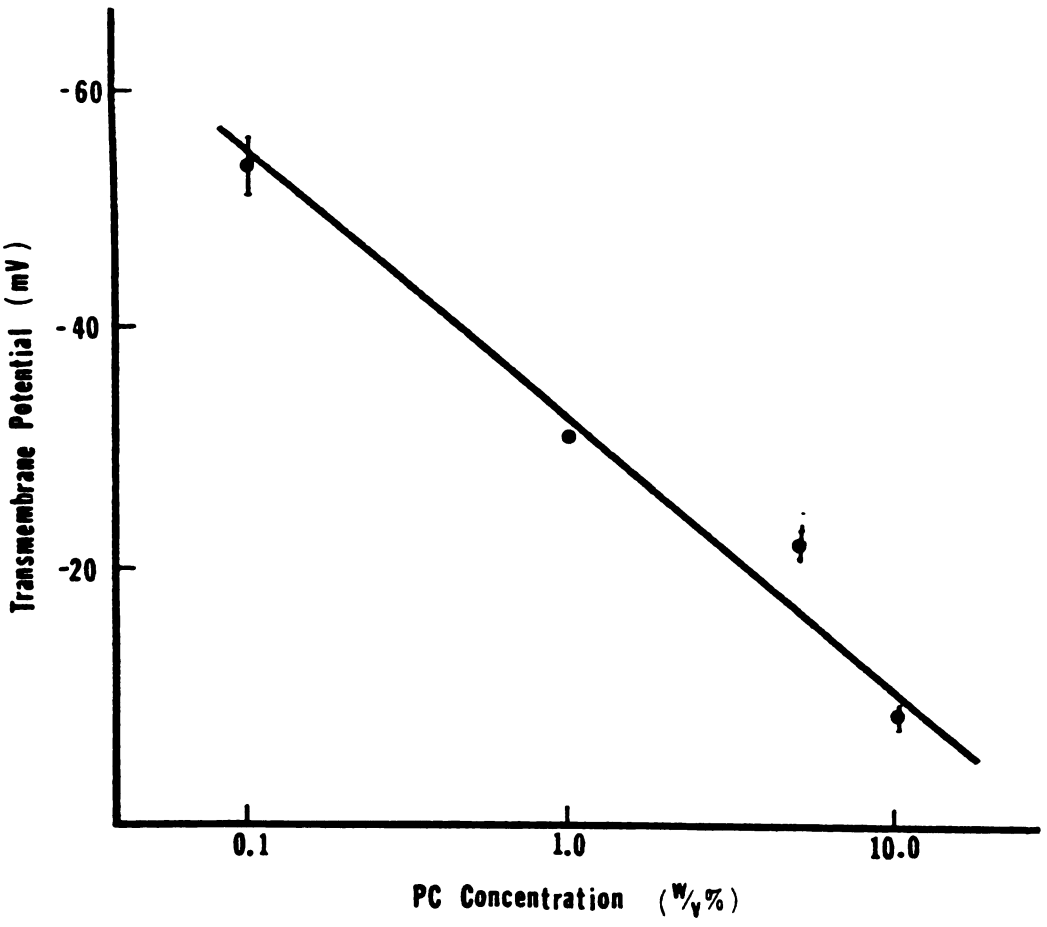


Figure 10. Variation of the induced potential with PC concentration in BLM forming solution. The forming solution was composed of 0.1% OC plus PC. The drug used was verapamil (0.001M).

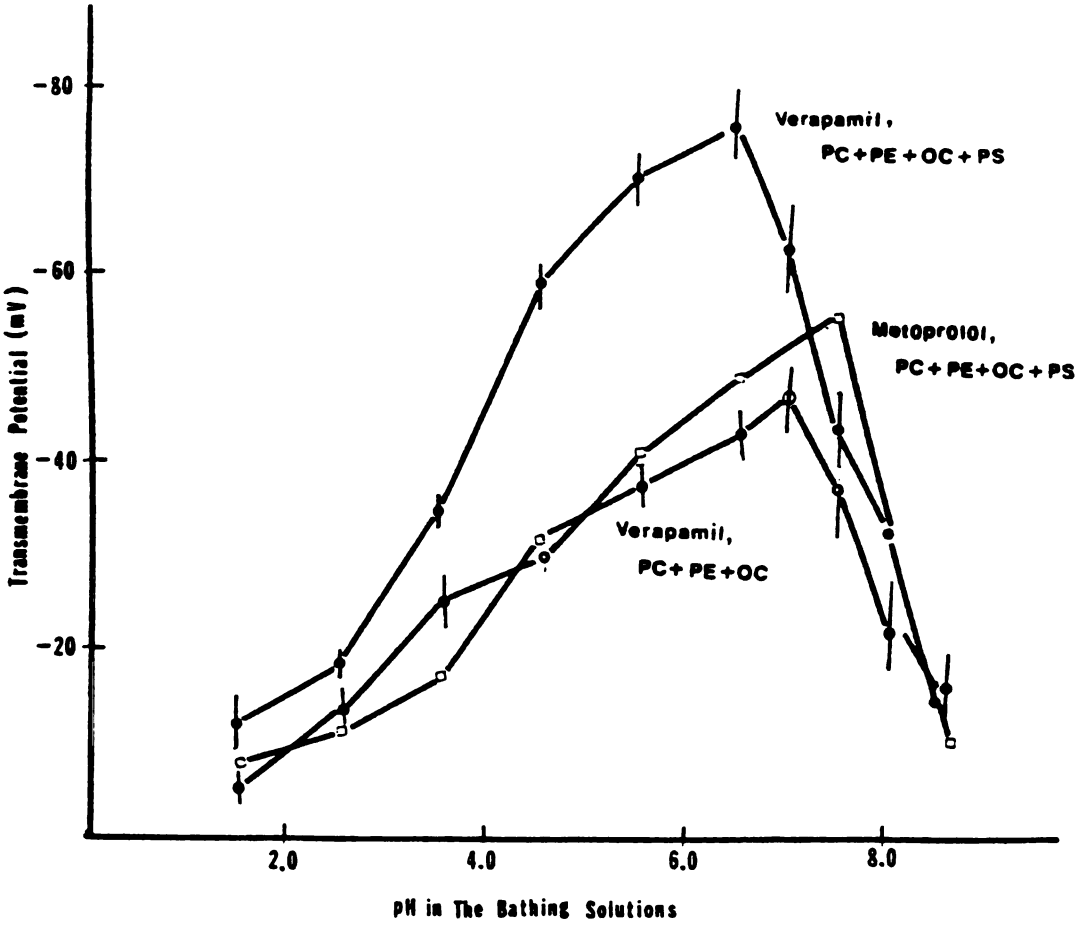


Figure 11. Variation of the induced potential as a function of pH of the bathing solution. Potential induced by 0.001M verapamil on PC + PE + OC + PS BLM (●) and on PC + PE + OC BLM (○) and potential induced by 0.001M metaprolol on PC + PE + OC + PS BLM (□).

indicate that the uncharged forms of these drugs seem to be the functional species responsible for the transmembrane potential.

Generally, the pH giving peak potentials in our experiments does not quite agree with the pK of drugs in aqueous solution reported in literatures, often being 0.5-1.0 unit lower than the latter. This difference can be explained as follows: firstly, pH at the interface between the membrane and soluton is not in accord with that in the bulk solution. According to the Boltzmann distribution of a charged particle in a varying field, H^+ concentrations at the membrane surface $[H^+]_s$) and in bulk solution ($[H^+]_b$) are related by the equation (Lee 1977),

$$[H^+]_s = [H^+]_b \, e^{-F\psi_o/RT} \qquad (2)$$

Correspondingly,

$$[pH]_s = [pH]_b + 0.434 \frac{F\psi_o}{RT} \qquad (3)$$

where ψ_o is surface charge potential on the membrane. It can be readily seen that for negatively charged BLM ($\psi_o < 0$), the pH at the membrane surface is always lower than that in bulk solution. In our case, assuming $\psi_o = -0.020$ V (calculated from $\psi_o = 51.4 \, \mathrm{Sinh}^{-1} \, 137 / c$), pH at the membrane surface is 0.35 pH unit lower). Secondly, as pointed out by Schreirer et al. (1984), the pK value for a ligand in the membrane phase may differ from that in aqueous phase if the ligand or/and the membrane possess ionizable groups.

DISCUSSION

1. Origin of the Transmembrane Potential

The origin of a transmembrane potential on BLM was discussed in detail by Tien (1974). An observed transmembrane potential in a BLM

system may be due to one or a combination of the followings:

1) Diffusion potential which is associated with the movement of charged particles through BLM under a concentration gradient.

2) Surface charge potential which arises as a result of the absorption and redistribution of charge on the membrane surface.

3) Dipole potential which originates from a change in dipoles of membrane lipids or absorbed ligands.

In our experiment, if the transmembrane potential is due to the diffusion of charged particles—protonated drug molecules or protons in this case, it would be accompanied by a large decrease in the membrane resistance (Foster and McLaughlin, 1974), but such change was not observed. More convincingly, a diffusion potential is expected to be depressed around pK of a drug where its molecules exist predominately as a neutral form. This is in direct contradiction with the actual observation in pH effect where the potential had its maximum around the pK (Figure 11). It is true that some drugs are found to travel through BLM, but their diffusion species are the neutral molecules. This is different from DNP which was shown to travel through BLM in a negatively charged form HA_2^- (Finkelstein, 1970), and its induced potential is mainly a diffusion component which is followed by a tremendous decline in BLM resistance (Figure 5). The potential induced by DNP can be predicted by the Nernst equation, which is used to describe the diffusion potential when a concentration gradient of DNP is across BLM,

$$\Delta\Phi = \frac{RT}{ZF} \ln \frac{[C]_{out}}{[C]_{in}} \qquad (4)$$

But in the presence of the concentration gradient of the drugs, the potential developed did not follow equation (4). For example, for a

10-fold difference in verapamil concentration on two sides, only a potential of -15 mV, instead of the theoretical value of the diffusion potential -58 mV, was measured. All the facts seem to rule out the diffusion component as a major part of the drug-induced potential.

In the case where there is no charge diffusion, a transmembrane potential must be related to a difference between two potentials at bifaces. The surface charge potential is usually suggested as a preferential candidate for the potential induced by amphiphalic drugs (Singer, 1977). However this component, which is based on the Gouy-Chapman double electrical layer model (Ohki, 1976), can not totally account for our experimental data. When Gouy's theory is applied to a BLM system, we have (Haydon and Myers, 1973),

$$\sigma = \frac{No}{F}\left(\frac{2eRTC}{}\right)^{1/2} \quad \mathrm{Sinh}^{-1} \quad \frac{ZF\Psi_G}{2RT} \qquad (5)$$

For unit-unit electrolyte simply,

$$\Psi_G = \frac{2KT}{e} \quad \mathrm{Sinh}^{-1} \quad \left(\frac{136.6\sigma}{c}\right) \qquad (6)$$

where ψ_G: Gouy's surface potential (mv)

K: Baltzman constant

T: Temperature (^oK)

e: Electronic charge

σ: Surface charge density (Å^{-1})

c: Electrolyte concentration in bulk solution (M)

Taking 60 Å^2 as average area for lipid molecule (Fettiplace et al., 1971), the surface charge density on BLM was estimated as 1/450 Å^2 from PS percentage in total lipids (14%). By using equation (6), we calculated the Gouy potentials at the different concentrations of

unit-unit electrolytes and plotted a theoretical curve (Figure 12). It is clear that the calculated surface charge potential does not correspond to the experimental data. At low salt concentration, the measured value is much smaller than expected by the theory. More outstandingly, in contradiction with what was predicted by Gouy's model, the drug-induced potential remained constant practically, rather decreased while salt concentration ranged from 0-0.1 M. Therefore some factors other than the Gouy component should be taken into consideration.

A jump in membrane potential can also arise from an alteration of the oriented dipole in the polar group region of BLM (Hladky and Haydon, 1973). Generally an overall surface potential (Galvani potential) is the sum of Gouy's potential ψ_G and dipole potential (Tien, 1985),

$$\Phi = \psi_G + 4n\mu_d \qquad (7)$$

where n is the number of dipole per cm^2, Vd the overall dipole moment of the constituent molecules in BLM. The dipole potential can be changed by means of the insertion of either the charged (Haydon and Myers, 1973) or neutral molecules (Anderson et al., 1976) into the membrane. The interaction of neutral molecules with zwitterionic lipids may alter the dipole potential by changing either the single dipole moment or the orientation of the dipoles. The following facts support the hypothesis that the drug elicits the transmembrane potential through affecting the dipole potential:

1) Unlike Gouy potential, the dipole potential is independent of electrolyte concentration (Hladky and Haydon, 1973) and this is seen in our experiment (Figures 6 and 7).

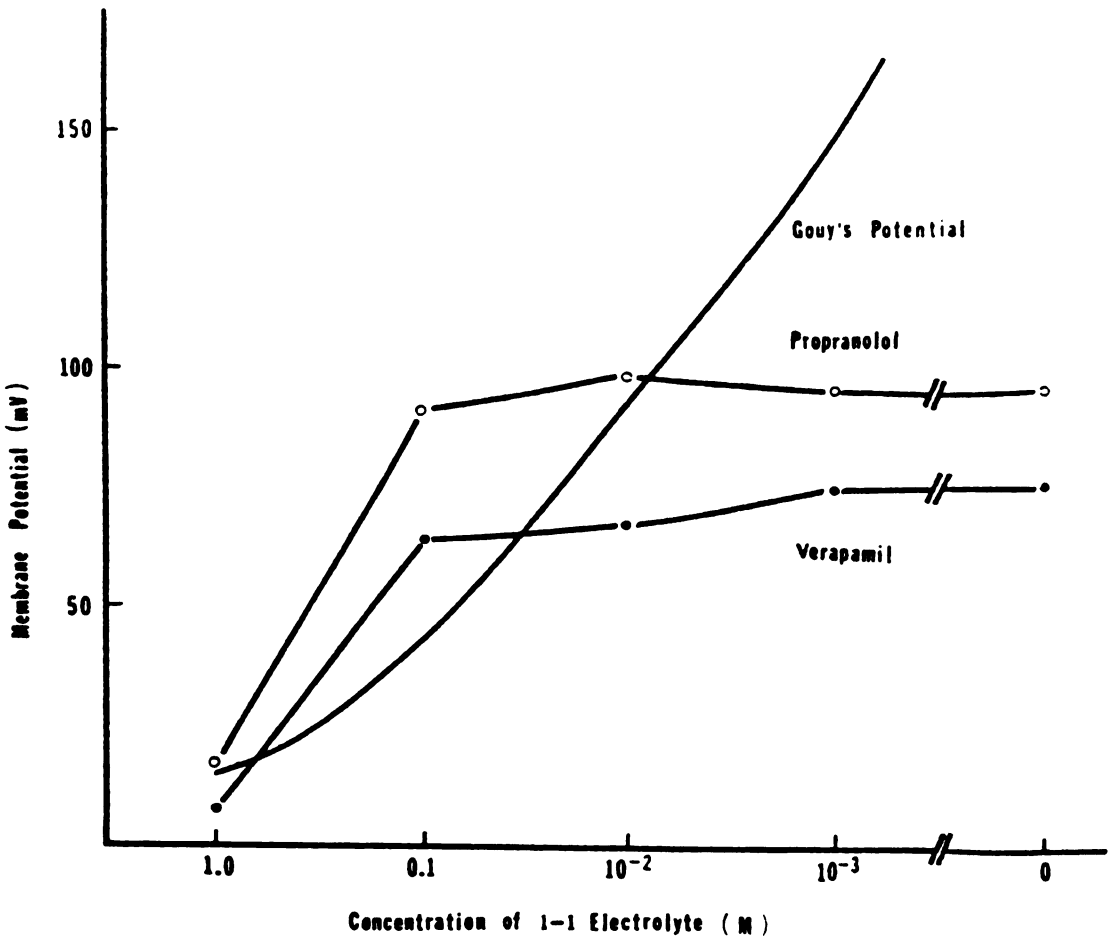


Figure 12. Comparison of Gouy's surface charge potential and the drug-induced transmembrane potential: the potential induced by 0.001M verapamil (●) and 0.001M propanolol (○) on PC + PE + OC + PS BLM. Gouy's potential was calculated using equation (5), assuming the surface charge density of $1/450\text{Å}^2$.

2) Unlike Gouy's potential, the dipole potential is located within polar-layer region, extending very little, if at all, into the aqueous phase adjacent to the membrane (McLaughlin, 1977), so that a ligand needs to penetrate into the bilayer to alter the dipole. Thus, the higher dipole potential depends on the availability of more lipid-soluble ligands. This is coincident with the observation in the experiment on pH effect.

From the arguments above, the origin of the transmembrane potential can be partially explained as follows: the drug molecules alter the orientated dipole of polar phospholipids on the side on which they are present, and so make the dipoles on the two sides asymmetrical, and the elimination of the symmetry of dipoles results in the observed potential across BLM.

2. Relation Between the Induced Potential and the Drug's Membrane Effect

As summarized in Table 2, the order of the magnitudes for drug-induced potentials is parallel to the order of the drug's lipophilicities. P_{corr} in the table stands for the correlated partition coefficient of a drug in octanol-H_2O system, which usually served as a measure of the hydrophobility of a chemical. P_{corr} is calculated from the apparent partition coefficient (P_{app}) with equation (Wang and Tien, 1980),

$$P_{corr} = P_{app}/(1-\alpha) \qquad (8)$$

Here is the ionization degree of a drug, and

$$\alpha = 1/1+ \text{antilog } (pK-pH) \qquad (9)$$

The further analysis was done by plotting log P against the potential (Figure 13). Linear regression of the data by least square fit showed a

Table 2: Comparison of the drug-induced potentials and lipophilicities.

Drug	PKa	log P_{app}	log P_{corr}	Induced Potential on PC+PE+OC+PS BLM
propranolol[a]	9.45	1.24	3.29	-96.5
labetalol[a]	9.45	1.13	3.18	-73.5
oxprenolol[b]		0.118	2.17	-70.0
metoprolol[a]	9.68	-0.25	2.04	-64.0
pindolol[b]		-0.328	1.73	-55.0
sotalol[d]		-1.45	0.60	-7.0
atenolol[a]	9.45	-1.62	0.43	-9.5
verapamil[c]	8.45	1.70	2.74	-75.0
nicardipin				-61.5
diltiazem				-49.0

P_{app} is the apparent partition coefficient and P_{corr} the corrected partition coefficient of a drug in octanol/water system (see text). The values of PKa, log P_{app} are taken from (a) Wang and Lien (1980), (b) Harada et al. (1981), (c) Lullmann and Wehling (1979), (d) Hellenbrecht et al. (1972).

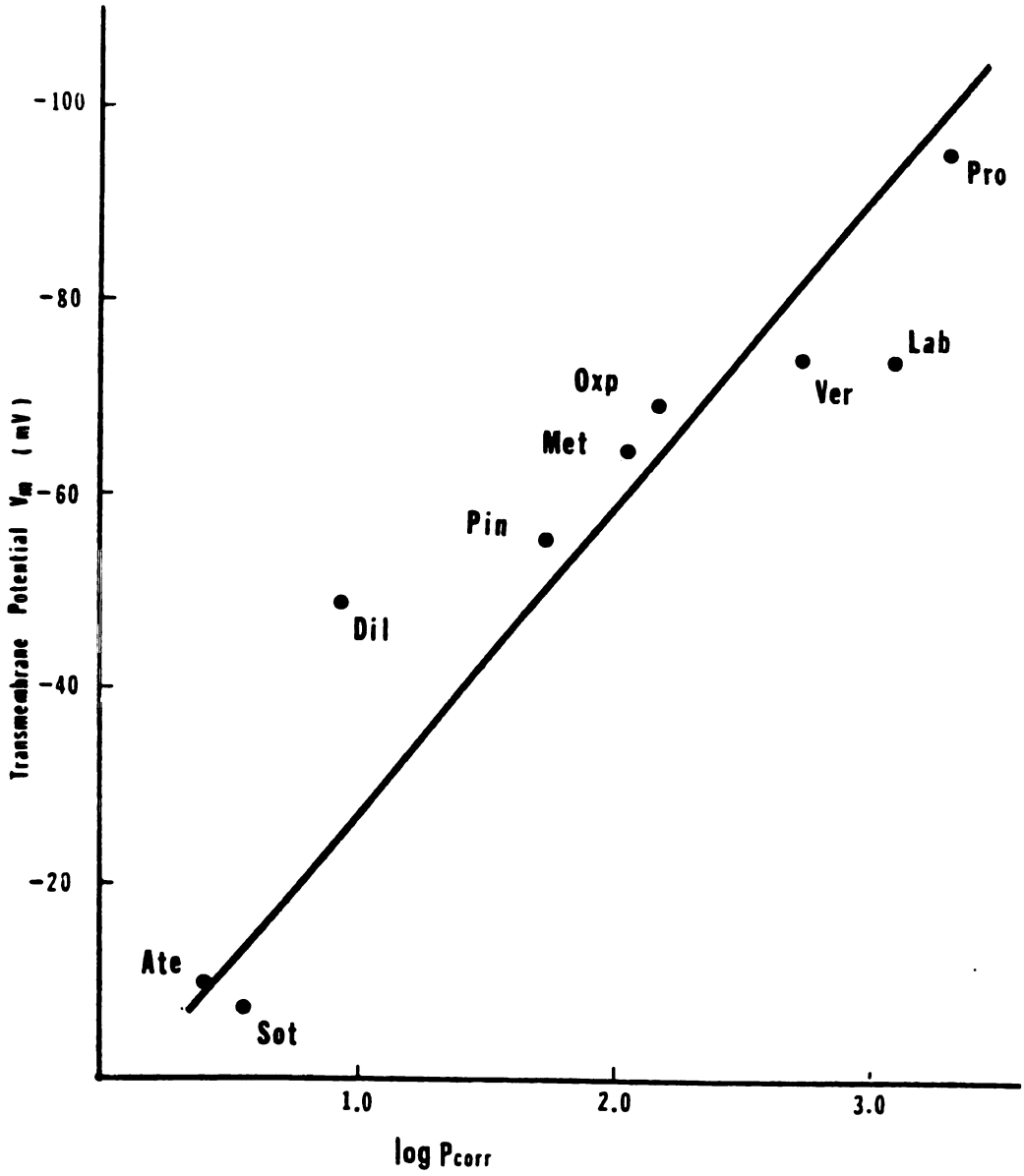


Figure 13. Repression curve--correlation between the induced potentials and partition coefficient of the drugs. P_{corr} is the corrected partition of a drug in octanel/water system. The data is treated by least squares fit and the regression curve is plotted. The curve has regression coefficient of -0.961 ($p<0.05$).

good correlation between the two parameters ($r = -0.961$, $p < 0.05$). This correlation indicates that the drug's action occurs on the membrane rather than the aqueous "unstirred layer" adjacent to the membrane surface. In other words, the observed potential results from a direct interaction between drug molecules and membrane lipids. This correlation also suggests that this interaction is mainly governed by hydrophobic effect, even though a contribution from electrostatic force can not be totally excluded.

In the present experiment, many of beta-adrenergic blockers but not all were able to produce rather high transmembrane potential. It is not difficult to link the relative potential-inducing capacities of these compounds to their chemical structures. Most of the beta-blockers tested are the analogs of propranolol, being amphiphilic molecules containing a hydrophobic ring system and a hydrophilic side chain (Figure 1a). Investigation using NMR (Kulkarni et al., 1979), ESR (Phadke et al., 1981) and neutron diffraction (Herbette, 1983) have revealed that propranolol has an extended configuration while inserting into a lipid bilayer. Its naphatalene moiety partitions into the hydrocarbon core, and the amine side chain is positioned in the head group region. This intercalation allows it to disturb the oriented dipoles of phospholipids and reduce the surface charge, resulting in a potential difference across BLM. This interpretation is valid for lobetalol, oxprenolol and metoprolol. On the other hand, sotalol and atenolol are also "propranolol-like" drugs but they bear additional hydrophilic groups attached to their aromatic rings. It seems plausible to ascribe the poor potential-inducing ability of these two agents to this merely structural dissimilarity: the hydrophilic group prevents

the drug molecules from penetrating into lipid phase. This speculation is strongly reinforced by the application of timolol. It resembles the hydrophilic property of sotalol and atenolol by containing polar ring moieties and so failed to induce a large transmembrane potential.

Beta-adrenergic blockers can be subgrouped into two classes according to their action sites. Some of them (e.g., propranolol) possess both beta-receptor blocking activity and general membrane effects, and some (e.g., timolol) show strong beta-receptor blocking activity but minimal general membrane effect. Usually the latter relieve the symptoms of heart diseases more specifically but less potently at the same dose. It has been proved that the order of the inhibitory effects of beta-blockers on Ca^{+2} uptake (Temple et al., 1974) and the negative inotropic effects of these agents (Harada et al., 1981) followed the order of their abilities to perturb the membrane lipids. For instance, in Messineo and Katz's (1979) experiment the velocity of Ca^{+2} influx to RS vesicles was eliminated 50% by 0.65 mM propranolol but required 11 mM timolol for the same effect. Herbette et al. (1981) reported that on SR membrane the magnitude of the inhibition of Ca^{+2} uptake by beta-blockers depended on both lipid solubility of the drugs and the physical state of fatty acid chains of membrane lipids. On the basis of neutron diffraction analysis, they suggested that the drugs interfere with the function of Ca^{+2} channel protein indirectly via the bulk lipid matrix, and other sites of drug interaction, namely with the lipid annulus and directly with channel protein, do not occur.

The induced potential in our experiment is clearly correlated with the ability of the drugs to block Ca^{+2} uptake and other membrane effects. Propranolol and exprenolol, as the most powerful inhibitor of

Ca^{+2} uptake (Temple et al., 1974) produced the higher potentials. The poor inhibitors sotalol atenolol and timolol induced the small change in the transmembrane potentials. The middle size potentials correspond the status of metoprolol and pindolol as the moderate inhibitors. Thus the measurement of the induced transmembrane potential on BLM provides a method of estimating the potency and selectivity of beta-blockers.

There is little known about the effect of calcium blockers on electrical properties of BLM. In this experiment, the members of this group that were used all tended to generate a potential across BLM. Some of Ca^{+2} blockers like verapamil may interact wih membrane lipids in a mechanism similar with that of beta-blockers, but it is dangerous to extrapolate this to all compounds of Ca^{+2} blockers because of their diverse chemical structures.

Since many heart drugs show capacity of developing transmembrane potentials, a question arises logically as to whether or not this electrostatic effect is implicated in the drug's function. Such a mechanism has been employed in the blockade of ionic channels by beta-adrenergic blockers (Schlieper and Medda, 1980). It is generally agreed that calcium blockers inhibit Ca^{+2} influx through acting on voltage-operated channels (VOC) (Lanvin et al., 1983), but relatively little is known about these channels in terms of molecular insight. They are supposed to be protein pores with negatively charged groups which are particularly intriguing for divalent cations and serve as selectors to distinguish between different cations. They are also endowed with "voltage sensors" which confer voltage dependence on channel opening and closing (Triggle, 1982). The precise mechanism for which heart drugs regulate the gating of VOC is not clear. It is known

that Ca^{+2} blockers do not act on VOC by directly plugging the channel pore in a manner analogous to tetrodotoxin (TTX) blockade of Na^{+} channels (Janis et al., 1984). Therefore, they may bind allosterically to a channel and trigger a conformational change, making the channel insensitive to voltage. As another possibility, they may create an electrostatic field in the environment of VOC to influence the gating of the channel. Biological membranes are ultrathin structures with less than 100 Å thickness (Robertson, 1981), so that a drug can build up an extremely high electrical field (10^5 V/cm) by means of developing a membrane potential, which is enough for changing the gating of VOC through moving "sensors" in the direction of the field. The calculation made by Lundström (1977) indicated that the electrical potential due to the surface charge and dipoles is of the same order of the actual voltage difference across the excitable membrane. Recently Erdreich and Rahamimoff (1984) reported that part of the inhibitory effect of verapamil on Ca^{+2} uptake by cardiac sarcolemma is due to its action on the membrane potential, even though it is not known how this effect is exerted.

The speculation will be challenged by an argument about the specificity of the drug's action. There are several groups of membrane active drugs which can induce electrical change on the membranes. Why do Ca^{+2} blockers inhibit Ca^{+2} uptake potently but others do not? We do not know whether the selectivity of drugs is only determined by their action on the specific proteins or if they can be also discriminated by lipid components in an unknown manner, even the latter seems not very meaningful in the today's concept. As far as the induced potential is concerned, there is no fundamental difference between beta-blockers and

calcium blockers (Figures 3 and 4, and Table 1) because the BLMs used are pure lipid bilayers, lacking of specific protein channels and receptors for the two groups of drugs. The results from such model membranes are not sufficient to explore the action of the drugs on the related proteins directly or via lipid-protein interaction.

3. Facilitation of PS in Drug's Action

In our experiment, all the heart drugs induced 0.5 - 1 fold higher potential on PS-containing BLM than on neutral BLM (Table 1). One can simply ascribe this enhancement to the presence of the net charge on the former. pK values of the drugs tested was between pH 8 and pH 10, and thus a considerable proportion of the drug molecules in the experimental pH (6.5 -7.0) are protonated. These protonated drug molecules neutralize the negative charge on cis side of the PS-containing BLM, thus creating a greater potential difference than those on neutral BLM. The net negative charge on PS-containing BLM also facilitates the positively charged drug in penetrating into the bilayer to alter the dipole potential. However this interpretation is only partially true. As discussed before, the interaction between the drugs and membrane lipids is mainly hydrophobic and the drug species for developing potential is mainly in the neutral form. In Figure 11, we can see that the ratio of the potentials on PS-containing and neutral BLM appeared to be approximately the same at high pH and low pH, indicating that the enhancement of potential due to PS has little dependence on the charge state of the drug's molecules. In other words, the electrostatic interaction bewtween the drugs and lipids is not the major factor regulating the potential.

Lüllmann and Wehling (1979) measured the binding of varous amphiphathic drugs (including verapamil, propranolol, metoprolol and atenolol used in this experiment) to liposomes composed of different polar lipids and they found an affinity order of PC < PE < PS. They suggested that the preferential binding of drugs to PC-containing membrane is mainly due to the formation of a non-tight packing membrane structure. Owing to a large hydration radius of serine residue, PS bears a larger polar head group compared with PC. Thus lipid molecules are kept distant from each other by the steric hindrance and electrostatic repulsion between adjacent serine molecules (Rojus and Tobius, 1965), and there is a large area not occupied by lipids but available for the intercalation of drug molecules. Surewicz and Leyko (1981) reported that the partition coefficient of propranolol in PS bilayer is more than 20 times higher than in PC bilayer. The steric effect due to the intrinsic lipid-lipid interacton can explain why not only charged but also uncharged drug species are easier to insert into the PS-containing BLM. This steric effect model is also suppoted by the experiment in Figure 9 where the drug-induced potential decreased at high PC-concentration which is favorable to the formation of a tight-packing structure (Schlieper and Medda, 1980).

4. Antagonism of Ca^{+2} Against Drug's Action

The antagonism of divalent metal cations, especially Ca^{+2}, against beta-blockers (Schlieper and Steiner, 1982) and Ca^{+2} blockers (Fairhurst et al., 1980) has been known for some time. The drug-induced inhibition of Ca^{+2}-dependent responses in cardiac and smooth muscles can be overcome by the elevation of Ca^{+2} itself. Since Ca^{+2} does not block the binding of the drugs to Ca^{+2} channels (Krafte et al., 1985), the

antagonism may be related to other membrane components. Langer (1980) reported that the force of heart contraction depends on the physical state of glycolipids and glycoprotein attached to lipid bilayer, which act as binding sites for Ca^{+2} with their sialic acid residues. Negatively charged lipids like PS, PI and cardiolipin are known to be also binding sites for divalent cations (Barrett, 1981). There is a suggestion that Ca^{+2} must first bind to these sites before it crosses the membrane via channels. Indeed Ca^{+2} is found to compete for the same sites on phospholipids with cationic drug molecules (Browning and Akutsu, 1982).

The role of membrane lipids in the interaction between Ca^{+2} and heart drugs is confirmed by our experiment in terms of the depression of the induced potential in the presence of Ca^{+2}. In the neutral BLM, the antagonism is the result of electrical screening, and in PS-containing membrane, a relatively prominent effect may be caused by a steric hindrance rather than the reduction of negative surface charge, as pointed out by Schleiper and Steiner (1983). Ca^{+2} is known to be able to bridge between a pair of PS molecules (Gregory and Ginsherg, 1984), and in this way it fills the intermolecular space between PS and blocks the entrance to the interior of the lipid bilayer. X-ray diffraction showed that the spacing of PS-Na^{+} complex is 78Å but the spacing of PS-Ca^{+2} complex is only 53 Å in PS-membrane. In order to test the steric effect, we designed the experiment shown in Figure 14. The same quantity of drug was added into Ca^{+2} and K^{+} solution of the same normal concentration separated by BLM, and in this case the apparent potential should be intermediate between the potentials developed at two halves of bilayers. The induced transmembrane potential was found to be larger on

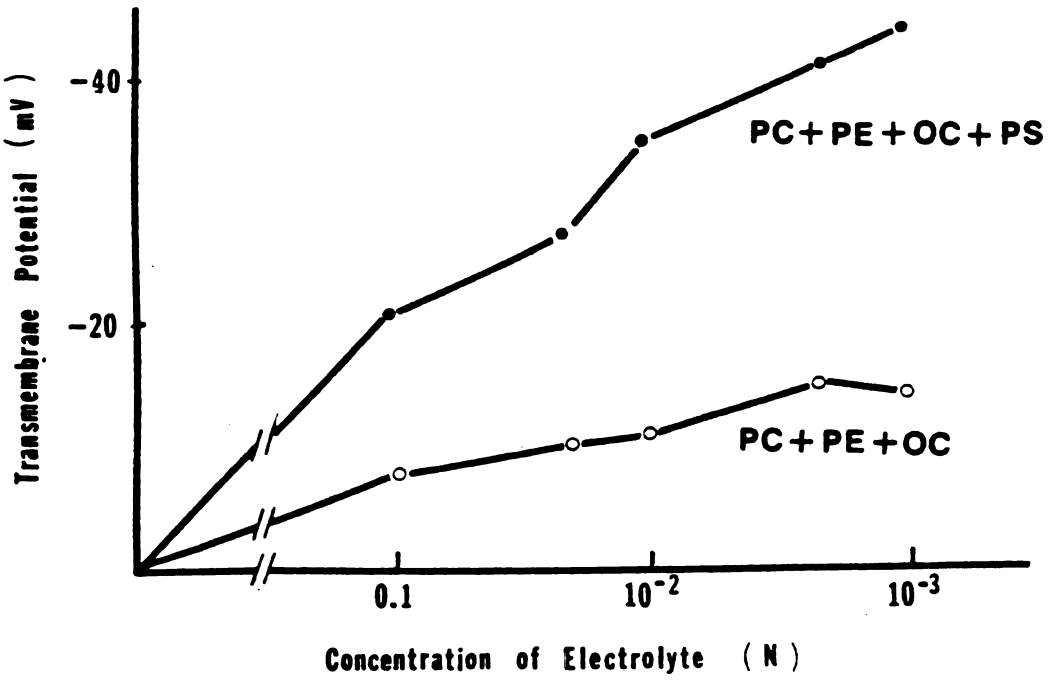


Figure 14. Verapamil-induced potentials across BLM separating different electrolyte solutions: KCl solution in <u>cis</u> side and $CaCl_2$ in <u>trans</u> side. The electrolyte solutions were added into the distilled water bulk solution after the formation of BLM and their final concentrations were equal in normality. The potential was induced by 0.001M verapmil on BLM composed of PC + PE + OC + PS (●) or only PC + PE + OC (○).

PS-PC-OC BLM than on the PC-OC BLM, implying the dissimilarity of antagonizing mechanism of Ca^{+2} and K^{+} on the former and the similarity on the latter. This is difficult to be interpreted solely by screening by which Ca^{+2} and Na^{+} should have approximately the same effect because there is no significant difference in charge density on the membrane surface in both cases.

PART II

EFFECTS OF DRUGS ON IONOPHORE-MEDIATED TRANSPORT

As mentioned before, the reconstitution experiment is the best way to explore the mechanism of the membrane channels using BLM. Ca^{+2} channels have been incorporated into BLM recently in several laboratories (e.g., Ehrlich et al., 1984; Coronado and Affolter, 1985). Prior to the reconstitution experiment, we used "artificial" ionophore substances to probe the effect of heart drugs on membrane transport. These substances, most of which are antibiotical products of microorganisms, have been widely employed to study membrane transport processes (Tien, 1985) because their mechanism models have been well established. Of course, caution should be observed while extrapolating the results of such experiments to the events on the biological membranes. However if we consider the drug's action through membrane lipids, there might be some resemblances between the drug's effect upon the artificial ionophores and native transport proteins, so that the ionophore-probing investigation may offer some clues for understanding the real events that happen in vivo. As examples of such applications, nonactin, a monovalent metal cation carrier, was used to study local anesthetics (McLaughlin, 1975), and the Ca^{+2} carrier, A23187, was used

to study calcium blockers (Malaisse, 1979) and beta-adrenergic blockers (Weksler et al., 1977).

Valinomycin and gramicidin A are well-known antibiotic ionophores. They facilitate the translocation of monovalent cations across the membrane in different mechanisms and they were chosen for this experiment. As a mobile carrier, valinomycin forms a complex with K^+ and diffuses back and forth through the lipid bilayers. Gramicidin A, an outstanding representative of channel formers, increases the membrane permeability to ions by forming a structural pore spanning lipid bilayer.

MATERIALS AND METHODS

The measuring apparatus is basically the same as that described in Part I. The steady-state current (I_m) through the BLM was measured using an electrometer or low impedance picoameter while a voltage (V_m) was applied. The membrane conductance (G_m) was calculated from the current-voltage (I-V) curve recorded by an x-y recorder:

$$G_m = \frac{I_m}{V_m} \tag{10}$$

To ensure that G_m had reached a stable value, all I-V curves were recorded at 10 minutes after the addition of chemical.

Valinomycin and gramicidin were dissolved in 99% ethanol. The volume of the antibiotics stock added was less than 0.2% of the total volume of the bulk solution to avoid the perturbation of BLM due to ETOH, which was proved with the appropriate.

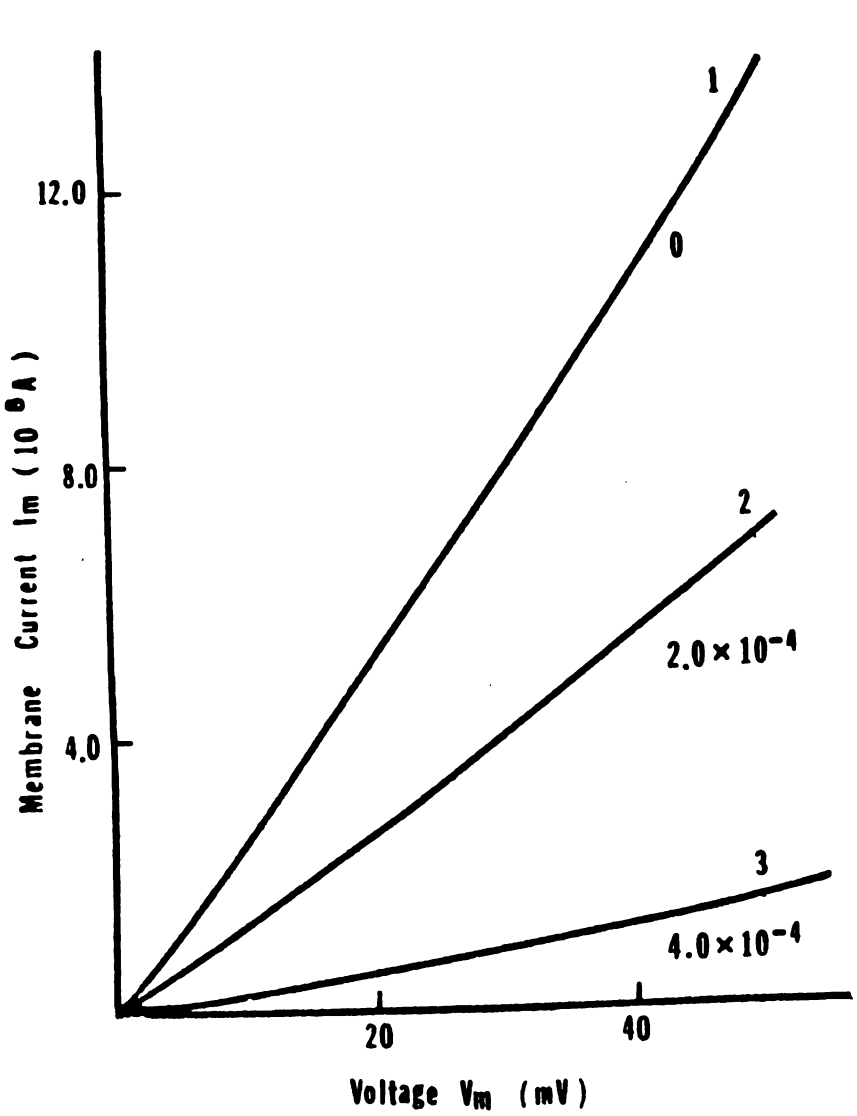
RESULTS

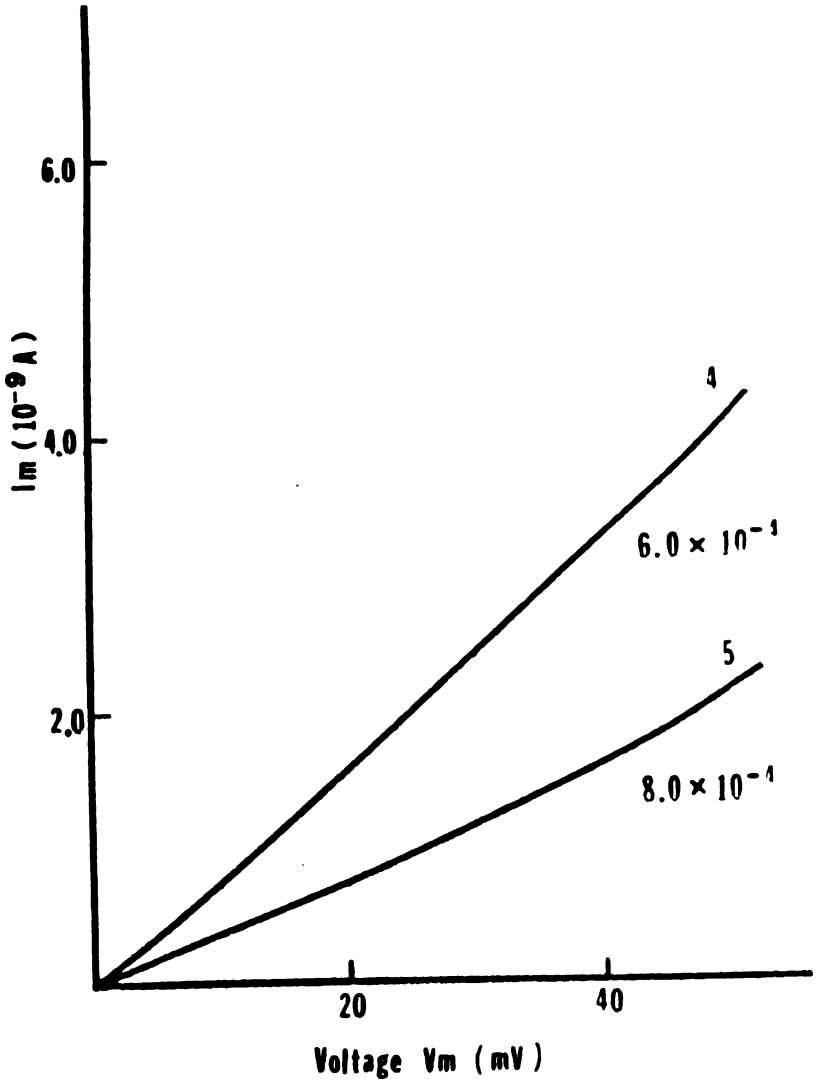
1. The Effect of Propranolol on Valinomycin-Mediated K^+ Transport

The addition of 10^{-8} M valinomycin into 0.1 M KCl buffer solution (5 mM HEPES pH 6.5) on the cis side increased the conductance of BLM (5% natural lecithin plus 1% OC in n-decane) by 2-3 orders (from 10^{-9} $\Omega^{-1}cm^{-1}$ to 10^{-7} to $10^{-6}\Omega^{-1}cm^{-1}$). After K^+ conductance (G_{K^+}) no longer changed, a certain amount of propranolol was introduced and the membrane current was decreased. I-V curves at different drug concentrations are plotted in Figure 15, whose slopes give the membrane conductance G_{K^+}. In the controls performed with propranolol of the same concentration but without valinomycin, G_{K^+} did not change or increased slightly, confirming that the decrease in G_{K^+} resulted from the inhibition of valinomycin-mediated translocation.

Over the range of 10^{-4} to 10^{-2} M, G_{K^+} in log scale appeared as a linear function of propranolol concentration. At the higher concentrations, the saturation of the drug's effect was observed. Two things should be noticed here: 1) The maximum reduction of G_{K^+} caused by propranolol was generally about 10-fold. The membrane conductance could not be restored to its original level before the addition of valinomycin. Because propranolol was in a great excess of valinomycin with molar ratio of 10^4 - 10^6:1, it is not likely that propranolol inhibits K^+ transport by interacting with valinomycin directly, which is expected to abolish K^+ current totally. 2) The absolute concentration of propranolol for blocking K^+ transport is rather high (about 10^{-4} M), 2-3 orders higher than those for affecting the specific proteins but at the same order as those for perturbing membrane lipids. These facts suggest

Figure 15. Current-voltage curves (I-V curves) or valinomycin-mediated K transport at various propranolol concentrations. Membrane solution: 5.0% natural lecithin (plant) plus 1.0% OC. Bathing solution: 0.01M KCl, 5mM HEPES (pH 6.5). Valinomycin concentration: 1.0×10^{-8}M. Propranolol concentrations are shown in the figure.


12.0
8.0
4.0
Membrane Current Im (10 ⁻⁹ A)
1
0
2
2.0 × 10⁻⁴
3
4.0 × 10⁻⁴
20
40
Voltage Vm (mV)


6.0
4.0
2.0
Im (10⁻⁹ A)
4
6.0 × 10⁻⁴
5
8.0 × 10⁻⁴
20
40
Voltage Vm (mV)

that propranolol, most likely, interferes with the carrier's function through effects on the lipid bilayer.

The experiment was repeated with 2,4-dinitrophynol (DNP) to examine the effect of propranolol on a negatively charged permeant. At a concentration of 10^{-5} M DNP reduced the membrane conductance by about 1 order. Unlike the valinomycin-K^+ complexes, the diffusion of DNP through the BLM was accelerated by propranolol (Figure 16). The drug influences the transport of two species in an opposite and symmetrical manner.

2. Effects of Other Drugs on Valinomycin-Mediated K^+ Transport

Several Ca^{+2} blockers and beta-blockers were tested using valinomycin as probe (Figure 16). Verapamil, diltiazem, exprenolol and metoprolol were found to be capable of depressing K^+ conductance, in more or less degree, whereas sotalol, atenolol and timolol lacked this ability. Again, here we see a parallel relation between the depression of G_{K^+} and the drug's lipid effect. To clarify this, we measured valinomycin-dependent G_{K^+} in the absence and presence of the drugs and design them as Go and Gl respectively. The ratio Go/Gl serves as a parameter to evaluate the ability of a drug to inhibit G_{K^+}. The comparison (Table 3) showed that there is the correlation between the ratio Go/Gl and lipophilicities and induced transmembrane potentials of the drugs.

We repeated the experiment using Ca^{+2} carrier A23187 as a probe, [The method followed Case et al. (1974). Forming solution: PE, bathing solution: 10 mM $CaCl_2$, 5 mM Ca^{+2} citrate pH 5.0] and did not find any significant inhibitory effect of the drugs. This is probably because: 1) The ionophore efficiency of A23187 is much lower than valinomycin

Figure 16. Effect of heart drugs on the diffusions of valinomycin-K^+ (Curve 1-4), $Ca(A23187)_2$ (Curve 5) and DNP (Curve 6) through BLM. The forming solution and bathing solution in valinomycin and DNP experiments were the same as those in Figure 15. In A23187 experiment, the forming solution: 2.0% PE, (W/V in n-octane), bathing solution: 10mM $CaCl_2$ and 5mM Calcium citrate. pH 5.0, the drugs tested were propranolol (●), verapamil (●), metoprolol (□) and sotalol (◆). The concentration of drugs was 0.001M.

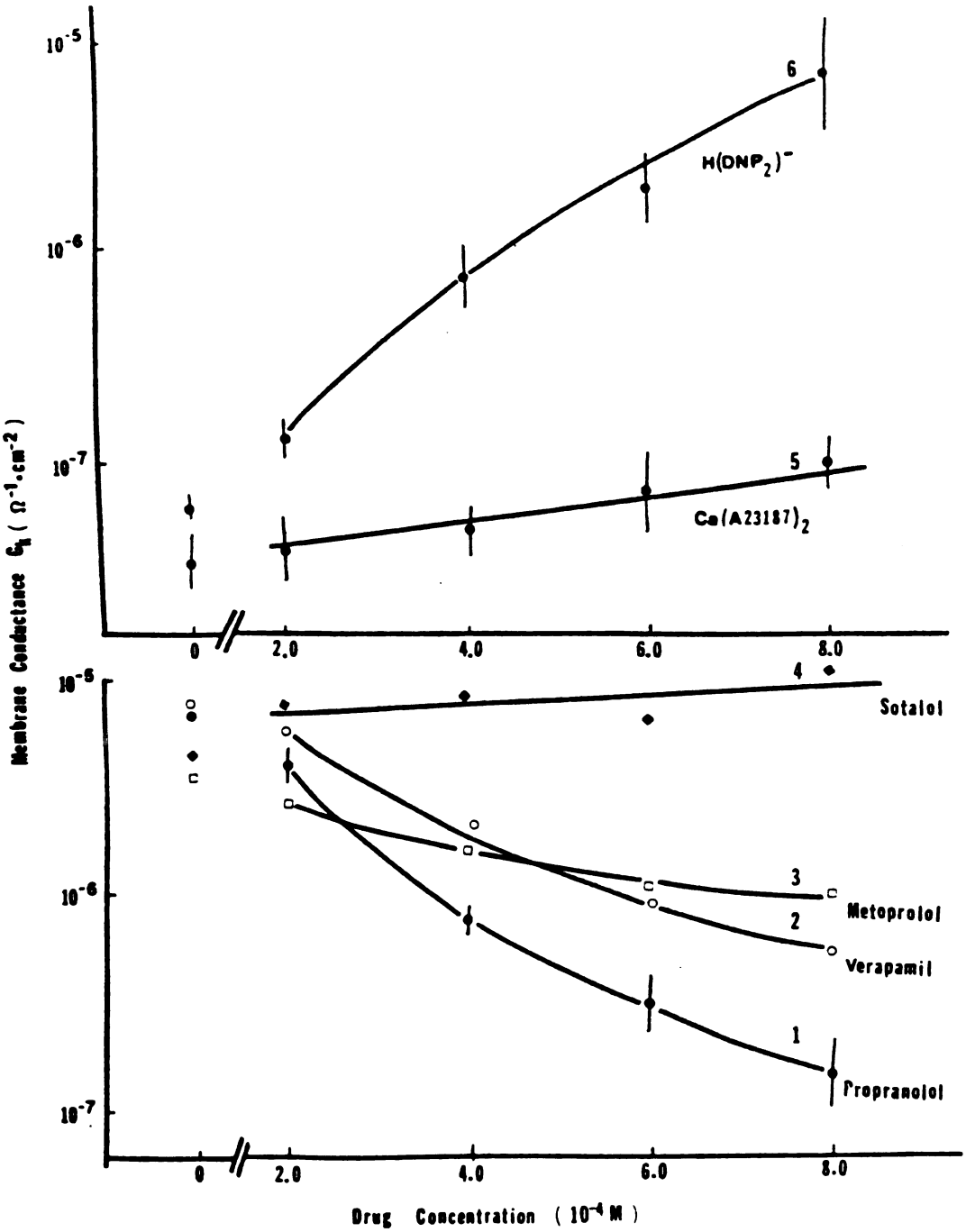

Membrane Conductance G_M ($\Omega^{-1} \cdot cm^{-2}$)
10^{-5}
10^{-6}
10^{-7}
6
$H(DNP_2)^-$
5
$Ca(A23187)_2$
0
2.0
4.0
6.0
8.0
4
Sotalol
3
Metoprolol
2
Verapamil
1
Propranolol
Drug Concentration (10^{-4} M)

Table 3: The ability of various drugs to inhibit valinomycin-mediated K^+ conductance and the comparison of the ability with drug's lipoplilicity and induced potentials.

Drug	G_o/G_1	log P_{corr}	Induced Potential (MV) on PC+PE+OC BLM
propranolol	9.20	3.29	-53
verapamil	5.60	2.74	-45
oxprenolol	4.30	2.17	-48
metoprolol	2.50	2.04	-38
sotalol	0.88	0.60	- 5
atenolol	0.82	0.43	- 9.5 (in PC+OC+PE+PS BLM)

G_1 is BLM K^+ conductance at 0.001M drugs and G_o is K^+ conductance in the absence of the drugs (see text).

(Szabo et al., 1969). In our case, maximum increase in Ca^{+2} conductance was less than 10-fold at 10^{-5} M A23187, and the small change in calcium conductance induced by the drugs was difficult to detect quantitatively. 2) More importantly, A23187 carries Ca^{+2} in an electrically neutral form.

3. Effects of Drugs on Gramicidin A-Mediated K^+ and Na^+ Transport

The behavior of beta-blockers and Ca^{+2} blockers is quite different on gramicidin A-modified BLM. Gramicidin A is more potent than valinomycin in facilitating K^+ transport. To obtain the comparable results, it was necessary to change the membrane conductance by the same order for both antibiotics. 10^{-10} M gramicidin is equivalent to 10^{-8} M valinomycin in 2-3 order enhancement of G_{K^+}. Of all the drugs tested, only propranolol was able to inhibit gramicidin-facilitated K^+ and Na^+ transport, but its effect was much smaller than that in the valinomycin experiment. None of the rest of the drugs showed any effect upon the gramicidin-dependent G_{K^+} and G_{Na^+}, irrespective of their effects on valinomycin's function. It is not surprising to have such different results if we presume that the inhibition of the drugs on the ionophores is through the membrane lipids. Apparently, the efficiency of the mobile carrier valinomycin greatly depends on the physicochemical state of lipid, but the channel former gramicidin is relatively insensitive to a change in the lipid bilayer (Krasne et al., 1971).

4. Ion Transport at Various Ionophore Concentration

Membrane conductance was measured at varying gramicidin concentration in the absence and presence of propranolol. The results are shown in Figure 17 where one can note two features in terms of the comparison between the 2 curves:

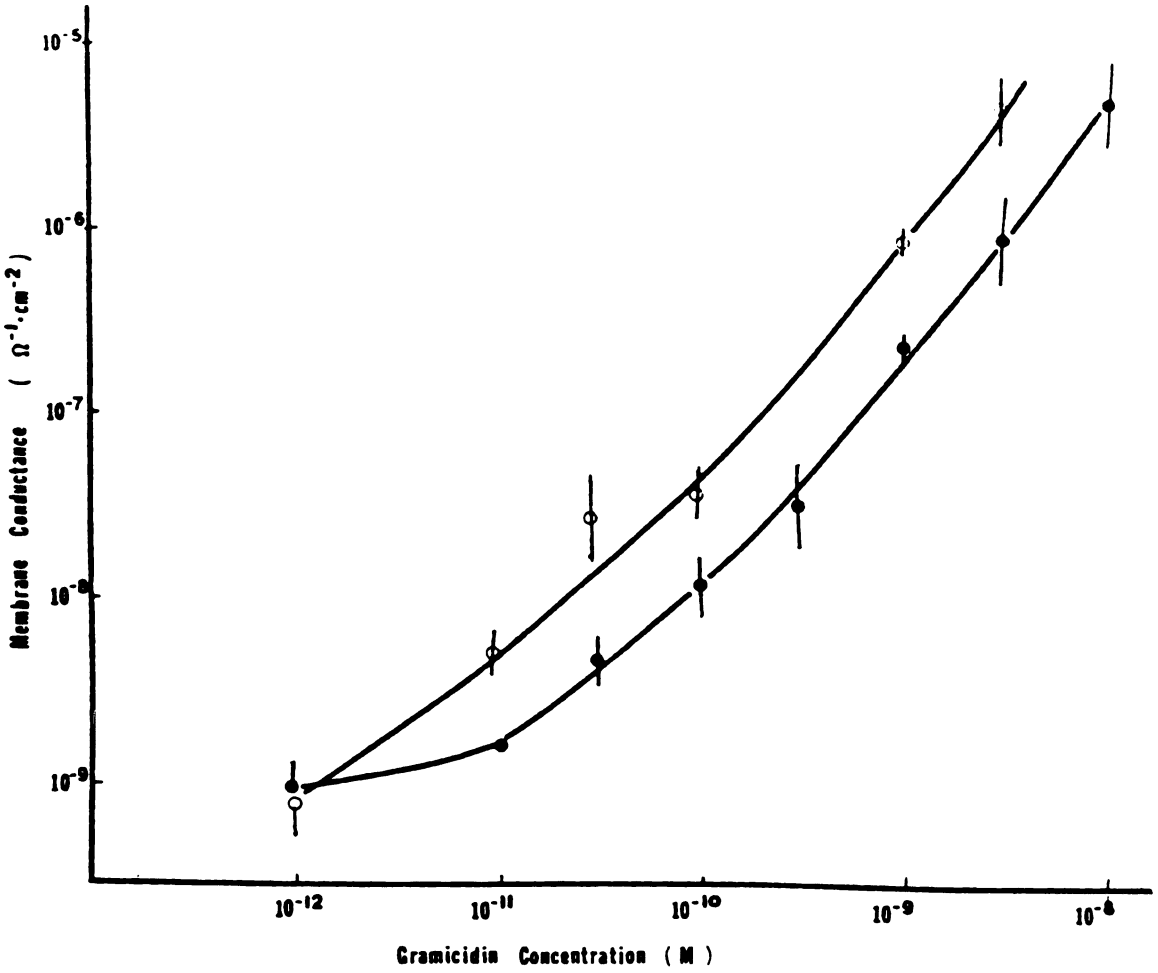


Figure 17. Variation of BLM K^+ conductance with gramicidin A concentration in the presence of 1.0 x 0.001M propranolol (●) and the absence of propronolol (○).

1) G_{K^+} changed in the same pattern with the variation of gramicidin concentration from 10^{-11} to 10^{-8} M, regardless of the presence of propranolol. In both cases G_{K^+} tended to increased with the same increment corresponding a given quantity of the ionophore. This is clearly seen from the two parallel curves, implying that the transport function of gramicidin is independent of the drug.

2) At a given concentration of gramicidin, G_{K^+} is lower in the presence of propranolol, but the difference is not very large. This difference may reflect the interferance of the drug on the translocation of a small portion of ions through the the lipid phase or the "marginal effect": a slight deformation of the pore structure by the disturbance of the lipid matrix (Gomperts, 1976).

As expected, the two curves looked different when valinomycin was used (Figure 18). The two curves rise with different slopes. The result showed that G_{K^+} changed much more slowly in the presence of propranolol, the difference in G_{K^+} for the cases became larger with the increase in valinomycin level.

5. Influence of Ionic Strength on the Drug's Action

The inhibition of the drugs on valinomycin-facilitated K^+ transport is dependent upon ionic strength, and it is depressed greatly at high salt concentration. When valinomycin and propranolol were administered at a fixed level, the ability of the drugs (e.g., propranolol and verapamil) to block the K^+ transport was reversely proportional to K^+ concentration. In order to understand the mechanism, we kept K^+ concentration constant (10^{-3} M) and changed the ionic strength of the bulk solution by varying the Na^+ concentration. The same antagonizing phenomenon was observed (Figure 19). Because valinomycin has very low

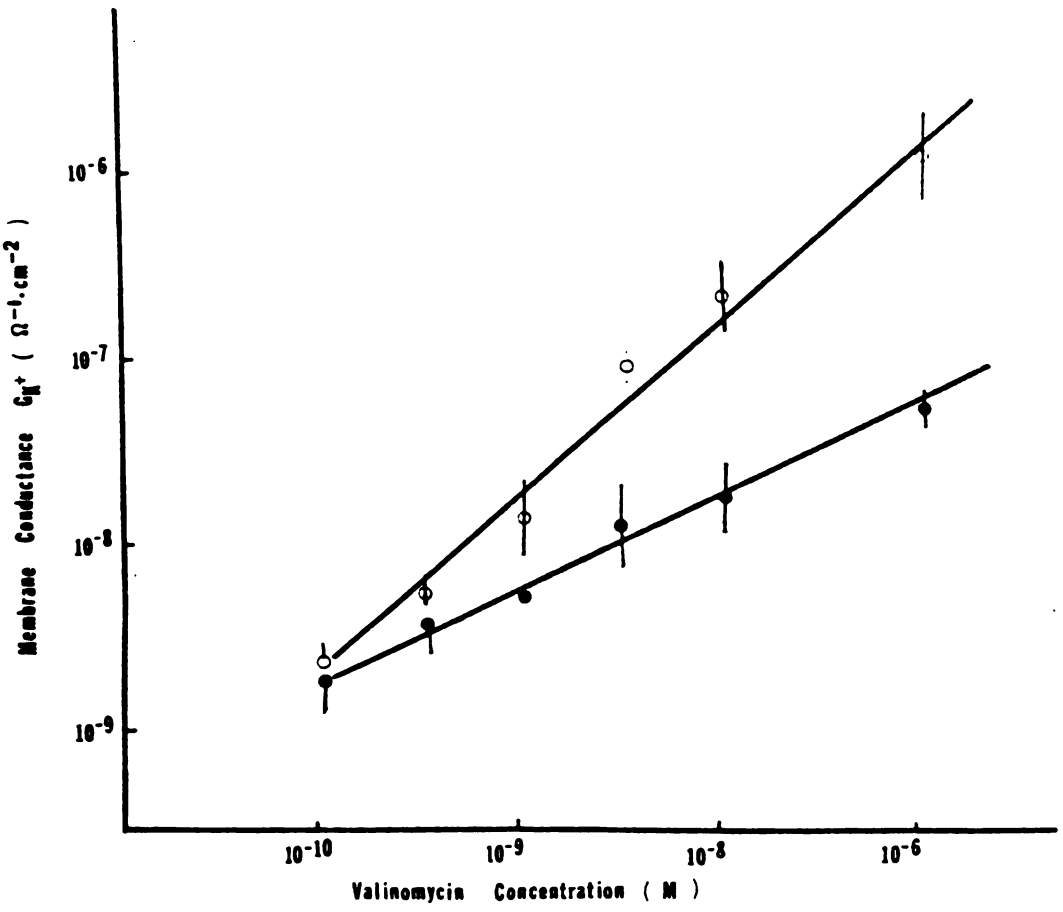


Figure 18. Variation of BLM K^+ conductance with valinomycin concentration in the presence of 1.0 x 0.001M propranolol (●) and the absence of propranolol (○).

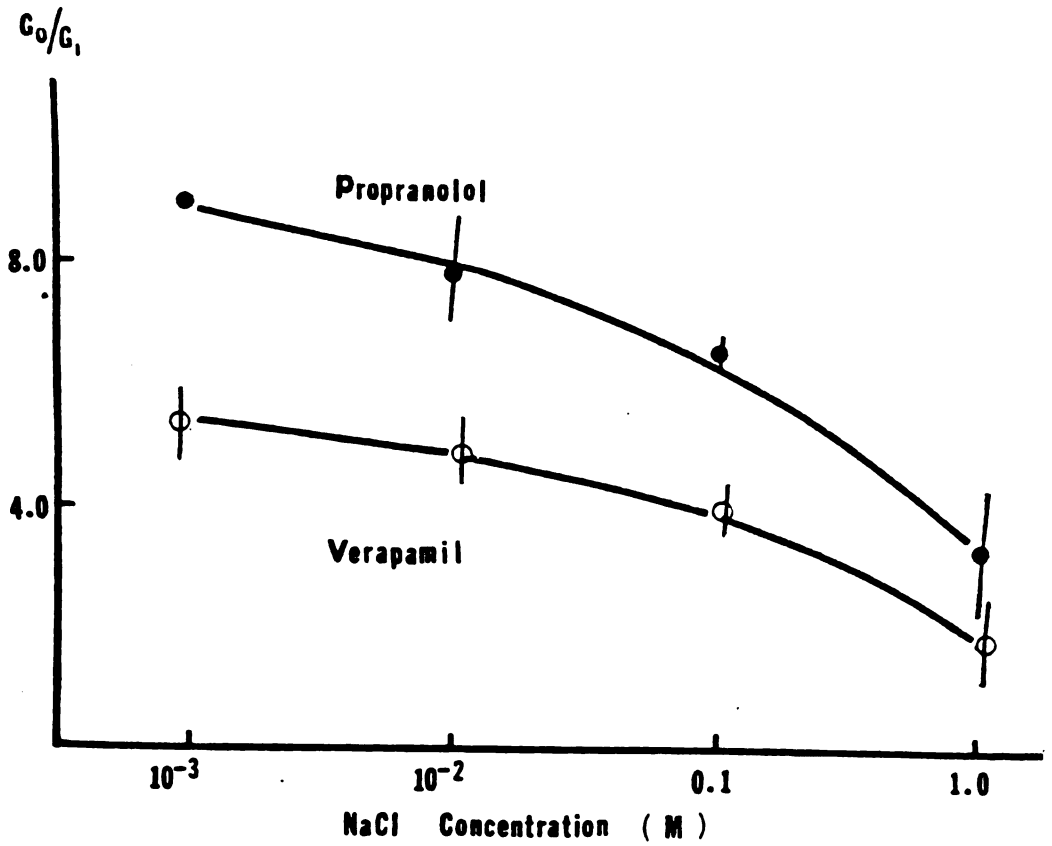


Figure 19. The effect of electrolyte concentration on the ability of drugs to block valinomycin (1.0×10^{-8}M) - mediated K^+ transport. Go: the membrane K^+ conductance in the absence of propranolol. G_1: membrane conductance at 1.0 x 0.001M propranolol (●) or verapamil (○). K^+ concentration in the bulk solution was fixed at 0.001M.

affinity for Na^+ compared with K^+ (1:300) (Mueller and Rudin, 1967), Na^+ had little chance to bind to the carrier and to interfere with the formation of the K^+-valinomycin complex. Furthermore, the partial substitution of Na^+ to K^+ (at the very high Na^+ concentration) in the complex could not influence the total current through BLM.

DISCUSSION

In a brief summary, the cation transport through the BLM is inhibited by beta-adrenergic blockers and calcium blockers. The primary event of the inhibition occurs at the lipid level owing to the following experimental facts:

1) The drugs only act on the mobile carrier-mediated cation transport, and having little effect on the counterpart facilitation by channel forming mediator.

2) There is a correlation between the ability of the drugs to decrease the carrier-dependent membrane conductance and their interaction with membrane lipids.

3) The concentration of the drugs for blocking the ion translocation is of the same order as that for perturbing lipid bilayers, but 2-3 orders higher than that for affecting proteins specifically.

Relating these facts to the results in Part I, we can directly ascribe the inhibition of carrier transport to the drug-induced change in electrostatic properties of the membrane: the elimination of negative charge and development of the transmembrane potential make it unlikely for the positively-charged valinomycin-K^+ complex to enter into the lipid bilayer and travel through it. However, caution should be exercised here because the change in the membrane conductance may be due

to some other factors, such as dielectric and fluidity of the membrane (McLaughlin, 1975).

An unmodified BLM presents a very high energy barrier for the transport of an ion from aqueous solution to the bilayer phase (Tien, 1985). This energy barrier can be estimated with the Born equation (Finkelstein and Cass, 1968),

$$E = -\frac{Z^2 q^2}{8 E_o A}\left(\frac{1}{E_m} - \frac{1}{E_w}\right) \qquad (11)$$

Here A is the ion radius, Z the ion valence, q the charge, and E_o the dielectric constant in vacuum. E_m and E_w are the dielectric constants of the membrane and aqueous phase respectively, having usual values of 2 and 80. According to equation (11), an increase in the membrane dielectric constant (E_m) leads to a reduction of the energy threshold and makes the ion diffusion easier. The involvement of the membrane dielectric in the alteration of the membrane permeability seems to be ruled out by our capacitance measurement in which the capacitance was only slightly increased (generally 10% to 20%) by the addition of drugs irrespective of their capacities of blocking K^+ conductance (Table 4). The total measured capacitance (C_t) in a BLM system can be expressed in terms of the membrane capacitance (C_m) and the capacitance of the two double electrical layers (C_d) (Lauger et al., 1967),

$$\frac{1}{C_t} = \frac{1}{C_m} + \frac{1}{C_d} = \frac{1}{E_m/4\pi t_m} + \frac{2}{E_d K/4\pi} \qquad (12)$$

where E_d is the dielectric constant of the double layer, t_m the membrane thickness and 1/k Debye-Hückel length. For 0.1 M 1-1 electrolyte, 1/k is about 10 Å, so that the second term in equation (12) can be neglected in the practical purpose (Tien and Diana, 1967) and we have,

Table 4: BLM capacitance C measured at 0.001M various drugs.

Drug	C_0 (Capacitance in control, 10^{-9}F)	C_1 (Capacitance at 10^{-3}M drug, 10^{-9}F)
verapamil	3.8	4.0
diltiazem	4.2	5.6
nicardipin	3.5	4.0
perhexiline	5.2	5.2
propranolol	3.5	4.2
labetalol	3.4	3.8
oxprenolol	5.5	5.7
metoprolol	5.0	5.5
pindelol	4.0	5.2
nadolol	4.8	5.6
dimolol	5.0	4.0
sotalol	4.0	4.2
atenolol	4.8	5.2

The capacitance was measured using low level capacitance meter. The membrane solution used was the usual one: PC + PE + OC + PS and bathing solution was 5mM KCl, pH 7.0.

$$C_t = C_m = \frac{E_m}{4\pi t_m} \qquad (13)$$

From equation (13), we can see that the constantance of the apparent capacitance (C_t) simply means no change in the membrane dielectric constant E_m assuming that the membrane thickness keeps constant.

The membrane premeability is also linked with the membrane fluidity. A permeant moves more with difficulty in a frozen lipid area. It is easy to deduce that the fluidicity-associated membrane conductance is independent of the charge state of a carrier (McLaughlin, 1975). In other words, the positive permeant (G^+), negative permeant (G^-) and neutral permeant (G) are not discriminated by a change in fluidity, so that the membrane conductance for G^+, G^- and G always change in the same direction with an alteration of membrane fluidity. This differs from the potential-associated membrane conductance for which G^+ and G^- conductance change in the opposite direction, and a decrease in G^+ conductance is always accompanied by an increase in G^- conductance. Another difference between the two types of permeability change is that the fluidity-associated one is not sensitive to ionic strength of aqueous solution.

In our experiment, the diffusion of the negative species $HDNP_2^-$ was accelerated while valinomycin-K^+ transport was slowed down in the presence of the heart drugs (Figure 16). The third diffusion species, A23187, is known to form an electrically neutral complex $Ca(A23187)_2$ to carry Ca^{+2} across the BLM (Vuilleumier et al., 1977), and its transport rate basically was not influenced by the drugs. It was also found that the inhibitory effect of the drugs on the carrier's transport function was diminished at the high salt concentration (Figure 19). According to

the two criteria, it is likely that the electrostatic perturbation rather than the fluidity change of the membrane is the major factor responsible for the carrier-mediated transport. However, on the basis of BLM electrical measurement alone, it is still not certain whether the heart drugs affect the membrane fluidity.

One question left is whether or not the depression of carrier-facilitated ion transport encountered here really mimics the mechanism of the heart drugs in vivo. This does not seem to be the case. Even the existence of the mobile carrier transport system in the biological membrane is a matter of debate; in all cases for which the detailed structure-function information is available, the evidence favors of a channel-type mechanism for the biological transport process (Zubay, 1983). Weskler et al. (1977) reported that propranolol inhibited the enhancement of A23187-induced Ca^{+2} uptake by platelets, but the natural calcium transport proteins known so far are channel formers.

PART III

EFFECT OF DRUGS ON MEMBRANE FLUIDITY

For the last decade, the effect of drugs on membrane fluidity has been studied extensively using model membranes. Therapeutically different groups of drugs, including anesthetics (Papahadjopoulos et al., 1975), antitumor agents (Goldman et al., 1978), aspirin (Ohki et al., 1980), cholinergic antagonists (Wang et al., 1983), and antibiotics (Murphree et al., 1981) were found to be capable of inducing changes in the membrane fluidity and phase transition temperature (T_t) in a greater or lesser degree. The effect was thought to play critical roles in the biological functions of certain types of drugs. Such investigation has not been extended much to beta-adrenergic blockers and calcium blockers except individual examples such as propranolol. Thus it is necessary to test them by measuring their effect on membrane fluidity. Electron spin resonance (ESR), as a standard technique in this field, was chosen for this purpose.

MATERIALS AND METHODS

Extraction of Lipid From Rat Heart

The procedure for extracting lipid from rat heart followed a modification of Folch's method (Folch et al., 1957), briefly:

1) Blend fresh rat hearts using Sorvall omni-mixer and then homogenize them in 2:1 chloroform-methanol mixture (v/v) to a final dilution 20-fold the volume of the tissue sample.

2) Clarify the tissue extracts by filtering.

3) Mix the filtrate thoroughly with 0.2 of its volume of phosphate buffer (10 mM, pH 6.5) by stirring violently for 10 minutes, and then leave the solution overnight.

4) Carefully remove the upper solution (H_2O plus MeOH) and interfacial fluff without disturbing the bottom solution. The lipid extracts in CH_3Cl at the bottom phase are ready for the next procedure after being dried.

Preparation of Liposome

The lipid dispersions used in ESR measurement were prepared by mechanically shaking. Synthetic or natural lipids were dissolved in double-distilled and molecular sieve (M-514, Type 4A) treated chloroform. The resulting solution was dried under a stream of argon for 4 hours. Residual organic solvent was removed from the sample by evaporating it in vacuum for another 4 hours. The dried lipids were added into phosphate buffer containing 0.2 mg/ml spin label compound TEMPO. The final lipid concentration was 8%, so that the ratio of lipids to TEMPO was maintained at 400:1. Such a low TEMPO level avoided the broadening of spectral lines due to spin-spin interaction (Azzi and Montecucco, 1977). A high ratio of lipid to drug (2:1 mole ratio) in the dispersion avoided the probable cluster formation around the drug molecules and the inherent loss of the information regarding interaction at the molecular level (Phadke et al., 1981). The mixture was dispersed

by shaking for 5 minutes using a vortex rotamixer. Drugs were added into the resulting milky dispersion (lipid vesicle solution containing a spin label) and the sample was left overnight at the ambient temperature for the equilibrium.

ESR Spectra Measurement

Spin label measurements were carried out on a Varian E112 X-band spectrometer with a quartz flat cuvette as the sample tube. A 100 KHz field modulation and the detection unit was used for detection purposes. The temperature was controlled by a nitrogen stream flowing through a Varian variable controller and was monitored with a calibrated thermocouple attached to an Omega digital meter with an accuracy of $\pm$ 0.2°C. A typical setting for a spectrum was: modulation amplitude = 2.5 G, magnetic field strength = 3220 Gauss, microwave power = 11 mw (below saturation) and frequency = 9.15 GHz. The sample was allowed to equilibrate in the machine for 5-7 minutes before each measurement.

RESULTS

TEMPO (2.2.6.6-tetramethyl-piperdine-1-oxyl) is known as a good reporter about the physical state of membrane lipids. The partition of TEMPO in the membrane phase and aqueous phase is directly dependent of the degree of fluidity of the hydrocarbon area in lipid bilayers (Shimshick and McConnell, 1973). This partition results in a split of the high field peak into two components: hydrophobic component (H) and hydrophilic or polar component (P) (Figure 20) because of the different hyperfine splittings (A) and g factors of TEMPO in two media due to the

different polarities (Azzi and Montecco, 1977). The ratio of height of these two peaks is referred as TEMPO spectral parameter f,

$$f = \frac{H}{H + P} \tag{14}$$

and it provides a good measure of the fraction of the spin label dissolved in lipid bilayer. The larger values of f correspond to the higher fluidity of the membrane lipids.

1. Effects of Drugs on TEMPO ESR Spectra

Several beta-adrenergic blockers and calcium blockers were tested using TEMPO as the probe. Some x-band ESR spectra are given in Figure 20. One can note that on the room temperature, the lipid phase peak H of natural lecithin (plant) become more pronounced in the presence of some drugs, indicating increases in the membrane fluidity. The f parameter was calculated from equation (14) and its changes were related to the ability of drugs to interact with membrane lipids (Table 5). Propranolol and verapamil enhanced f values about 25-30%. The increase induced by metoprolol was smaller (13%), and sotolol and timolol showed little effect on the lipid fluidity. Ca^{+2} blockers (verapamil and diltiazem) appeared to have stronger effect on membrane fluidity than what is expected from their partition coefficients compared to those of beta-blockers. This may be due to their lower pK and chemical structures different from those of beta-blockers.

2. Temperature Scanning Measurement

The temperature scanning spectra of spin label can provide more general information about the physical state of the membrane. Different lipids, namely, synthetic dimyristoyl PC (DMPC), natural lecithin (plant) and heart lipid extracts were used in this experiment. DMPC

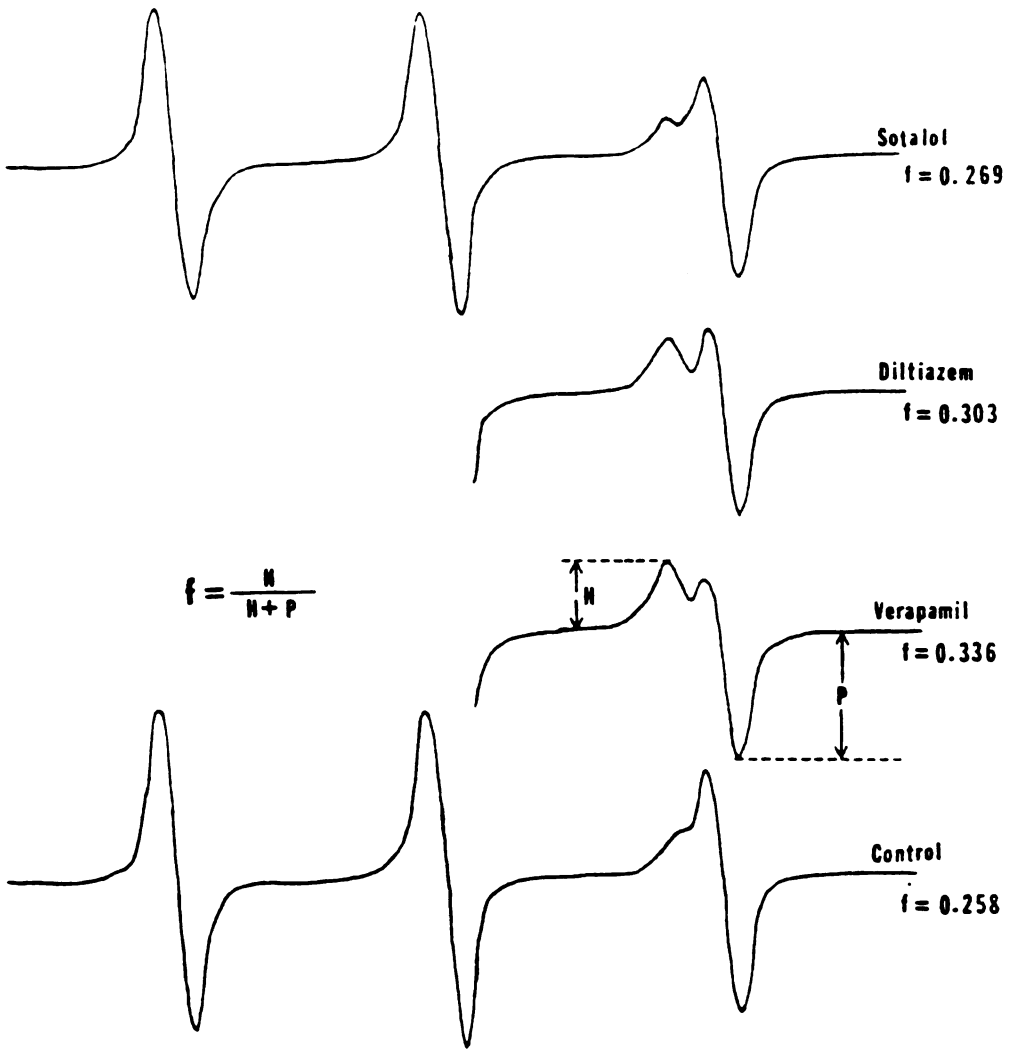


Figure 20. TEMPO ESR Spectral—effect of various drugs on partitioning of TEMPO in natural lecithin liposomes. TEMPO ESR spectra were measured at 22.0 C (± 0.2 C). The concentration of drugs was 0.10M.

Table 5: Changes in TEMPO spectral parameter f induced by various drugs on natural lecithin liposomes.

Drug	f	Δf	log P_{app}	Induced Potential (mv) on PC + PE + OC BLM
control	0.263 ± 0.005			
verapamil	0.336	28%	2.74	-45
propranolol	0.332 ± 0.002	26%	3.29	-53
diltiazem	0.303 ± 0.010	15%		-37
metoprolol	0.297 ± 0.004	13%	2.04	-38
sotalol	0.279 ± 0.010	6%	0.60	- 5
timolol	0.254 ± 0.017	-3.5%		

Drug concentration was 0.10M. f was measured at 22.0°C (± 0.2°C) $f = (f_{drug} - f_{cont})/f_{cont} \times 100\%$. f values in the table were the average from two repeats + standard deviation of the mean.

liposomes showed an abrupt phase transition over a narrow temperature range (< 5°C) (Figure 21). In the control, the midpoint of the translation was about 22.5°C. The presence of verapamil did not broaden the transition, but it decreased the transition temperature (T_t) greatly, and 0.1 M verapamil shifted T_t to 12.5°C from 22.5°C. Another prominent effect was the disappearance of the pretransition region of DMPC. The transition of PC from a gel to a liquid phase takes place in two steps. The pretransition region, corresponding to the premelting peak on DSC spectra, represents the tilt state of the head group of PC according to Chapman's interpretation (Chapman and Urbina, 1974). Thus the loss of the pretransition region may be explained by the interaction between the lipid polar head and the cationic part of verapamil.

When the scanning measurement was repeated with natural lecithin liposomes, there was no sharp change in the f parameter from 0 to 60°C irrespective of the presence of verapamil, suggesting that no distinct phase transition occurred (Figures 22 and 23). It is unlikely that the transition happened below zero or above 60°C because f was already rather small or large in these cases. The lack of sharp transition is not surprising if we consider the fact that natural lecithin consists of chemically various species with different T_t due to the heterogeneity in the degree of saturation and the length of hydrocarbon chains. This heterogeneity confers the membrane a broad transition in which the solid phase and liquid crystalline phase coexist. In this experiment, H peak appeared at a rather low temperature, indicating that a portion of the hydrocarbon chains was already melted. The measurement was not practicable below -5°C due to freezing of the sample solution. The transition seemed to be finished at about 40°C beyond which f did not change

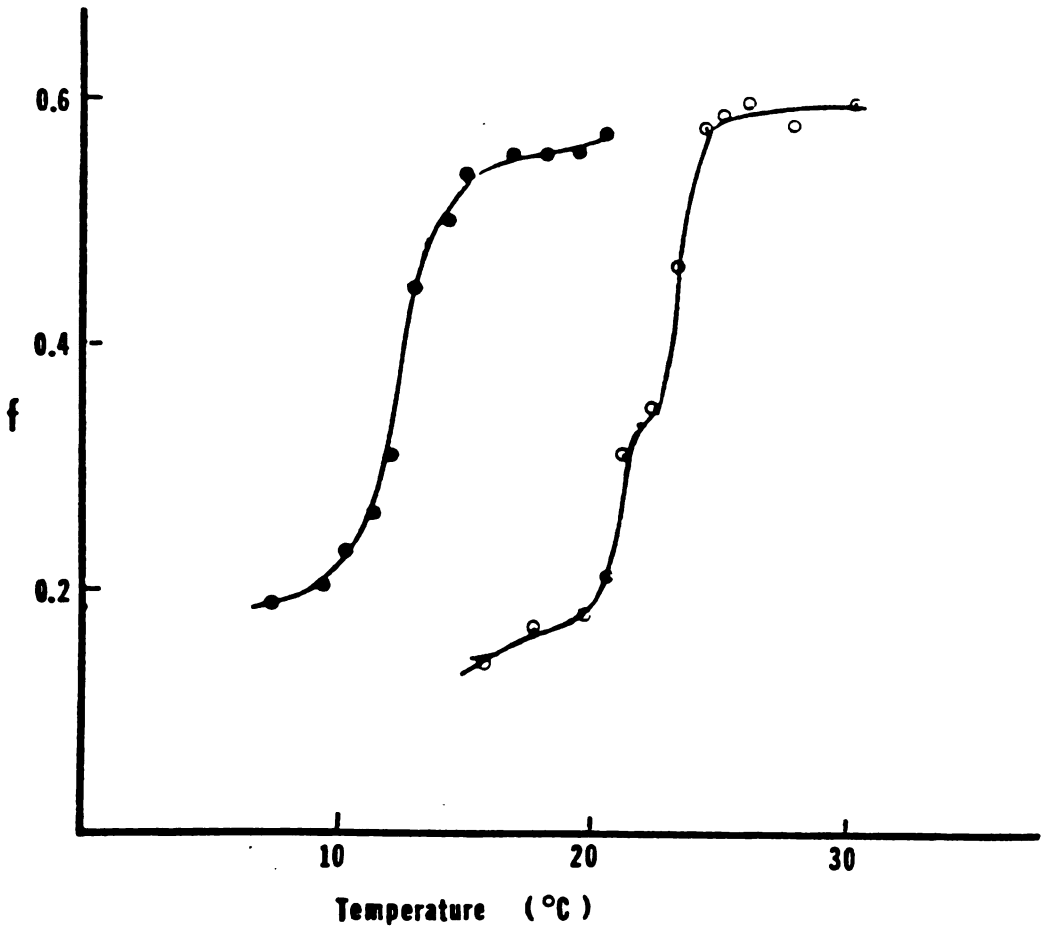
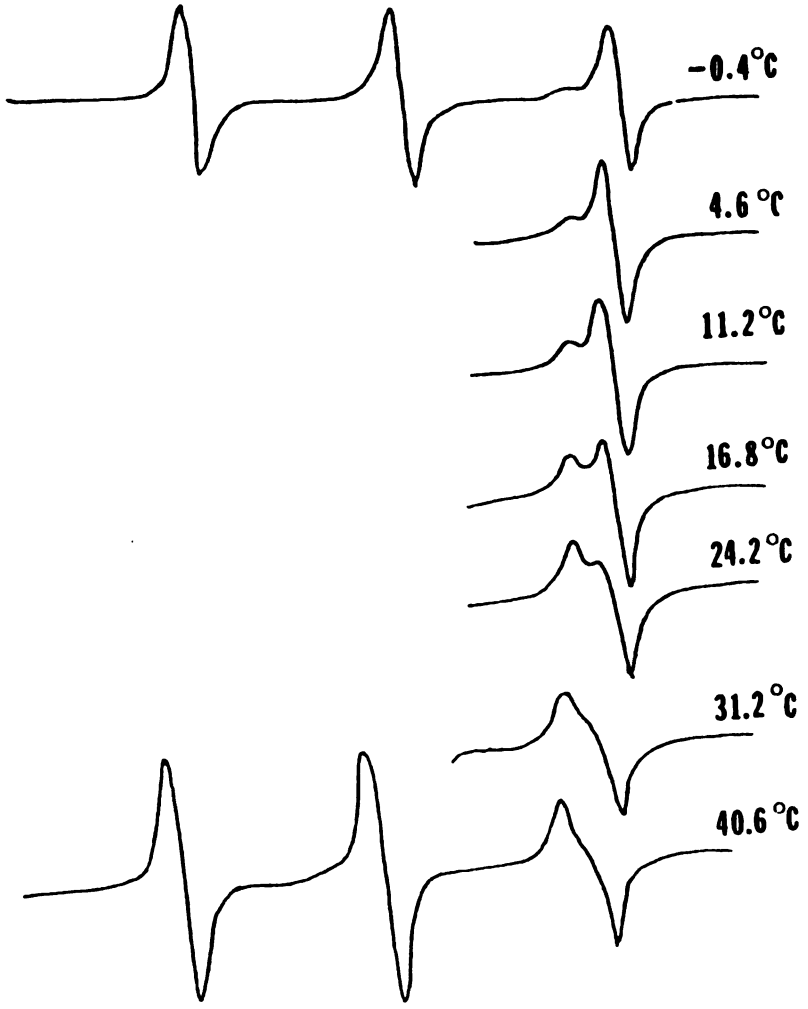
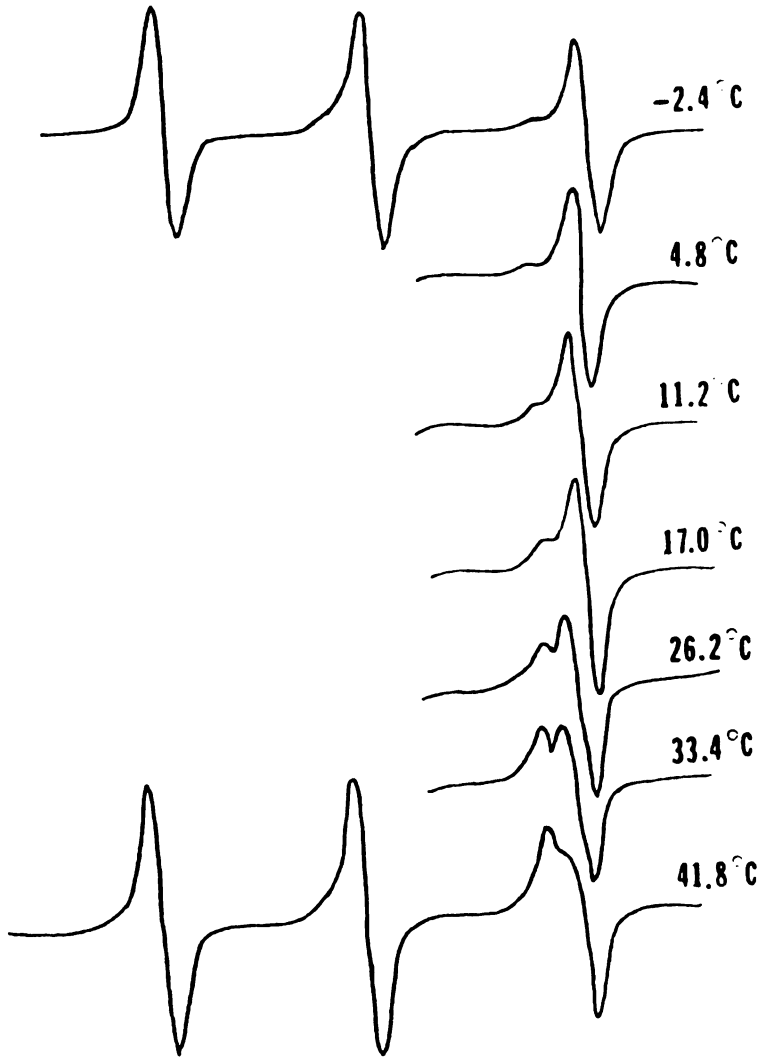


Figure 21. Variation of TEMPO spectral parameter f in temperature scanning—effect of verapamil on the fluidity of DMPC liposomes. f was measured at 0.10M verapamil (●) and n the control (○) (see text).

Figure 22. TEMPO ESR spectra in temperature scanning--Effect of verapamil on partitioning of TEMPO in natural lecithin liposomes: (a) the spectra at 0.1 M verapamil and (b) the spectra in the control.



a. At 0.10M Verapamil



b. Control

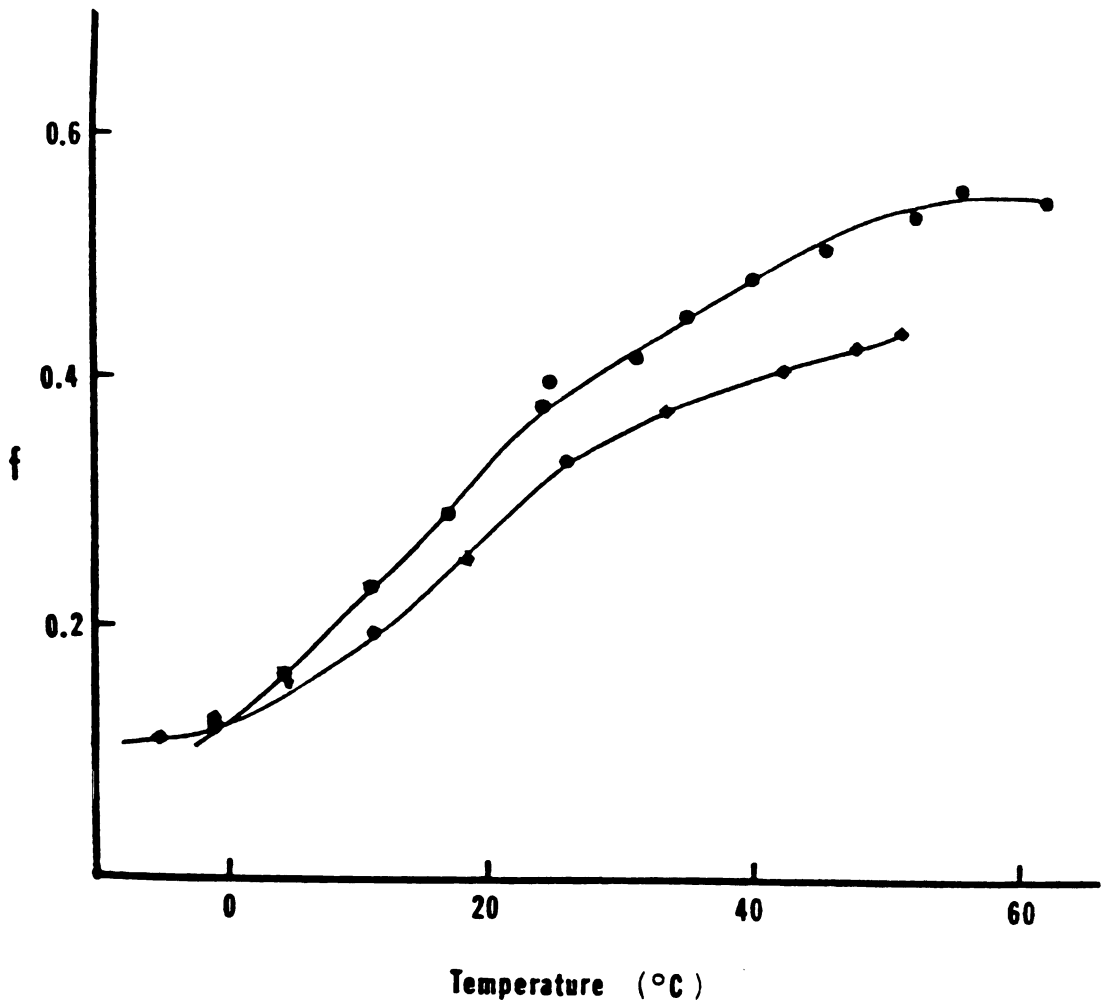


Figure 23. Variation of TEMPO spectral parameter f in temperature scanning—effect on verapamil on the fluidity of natural lecithin liposomes. f was measured at 0.10M verapamil (●) and in the control (■).

significantly. Due to the lack of a discriminable transition, we could not detect the decrease in T_t caused by verapamil on natural lecithin liposomes. However, the scanning experiment demonstrated clearly that verapamil tends to increase the membrane fluidity over a wide temperature range. Verapamil also fluidized the liposome prepared from rat heart lipid extracts, and in this case the phase transition became even broader (Figure 24).

3. Effects of Drug Concentration and pH

In the dose-response experiment (Figure 25), the concentration of verapamil required to produce a detectible change in f was very high. Below 0.01 M, no change in spin label spectra was detected. From 0.01 M to 0.1 M, the fluidity increased rapidly in a non-linear manner with the drug's concentration. The measurement was not extended to concentrations higher than 0.1 M because of the limitated solubility of the drug in aqueous solution.

On the other hand, the effect of verapamil on the membrane fluidity was not influenced by varying pH from 6.5 to 3.0 (Figure 26). The f began to rise at pH 3.0, but this was a pure pH effect independent of the drug as demonstrated by the control. This is probably due to the ionization of the phosphate group of PC in the acidic media (Trauble and Eibl, 1974). The more significant pH range is around the pK of the drugs (8.45 for verapamil) where one would expect to observe some interesting things. But it is very difficult to do the spin label measurement at such high pH since verapamil solubility in water decreases dramatically above pH 6.5.

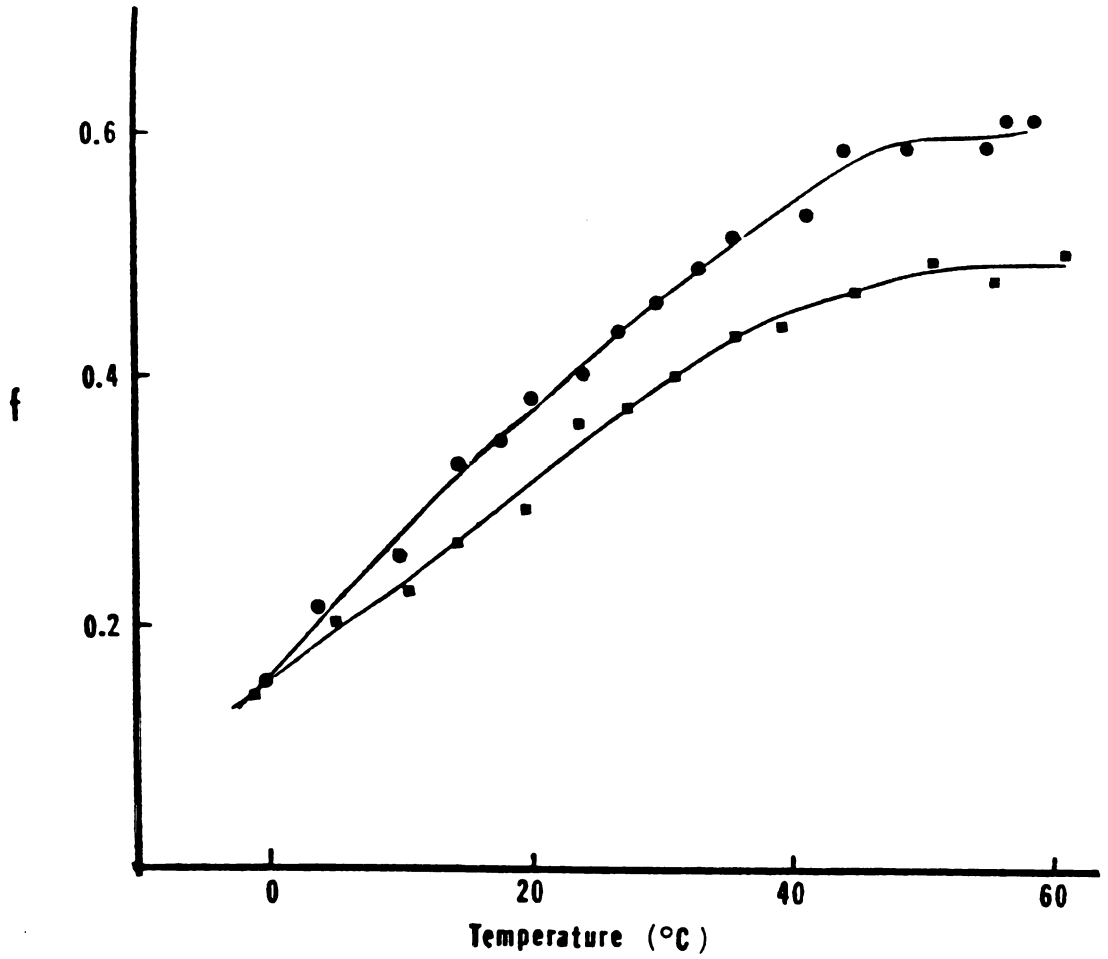


Figure 24. Variation of TEMPO spectral parameter f in temperature scanning—effect on verapamil on the fluidity of the liposomes prepared from rat heart lipid extracts. f was measured at 0.10M verapamil (●) and in the control (▪).

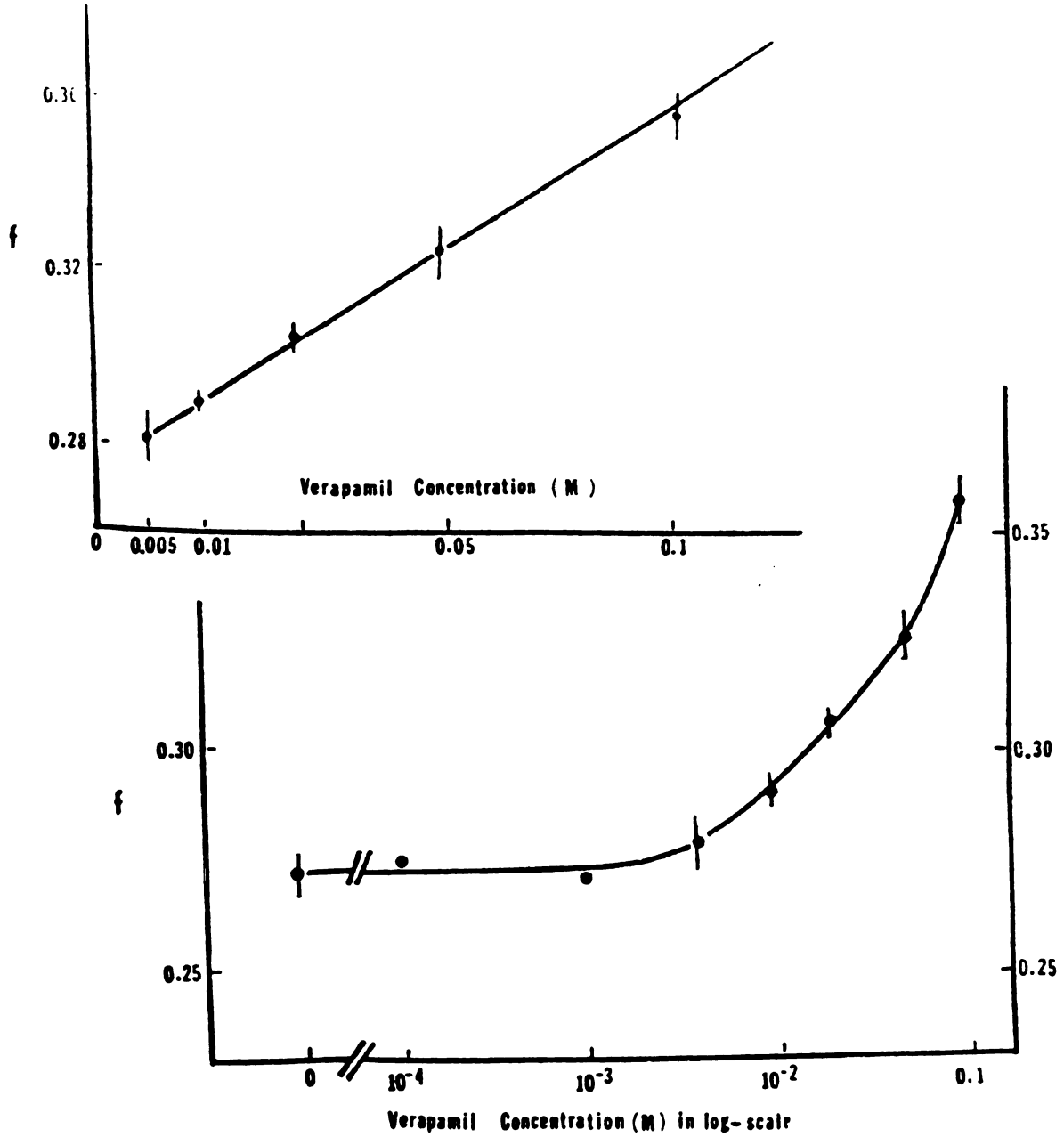


Figure 25. Variation of TEMPO spectral parameter f as function of verapamil concentration. TEMPO spectra were measured at 22 C (± 0.2 C). The concentration of verapamil in the bottom panel is varied from 10^{-4}M to 0.1M and is in log-scale, and the concentration in the upper panel is varied from 0.005M to 0.1M.

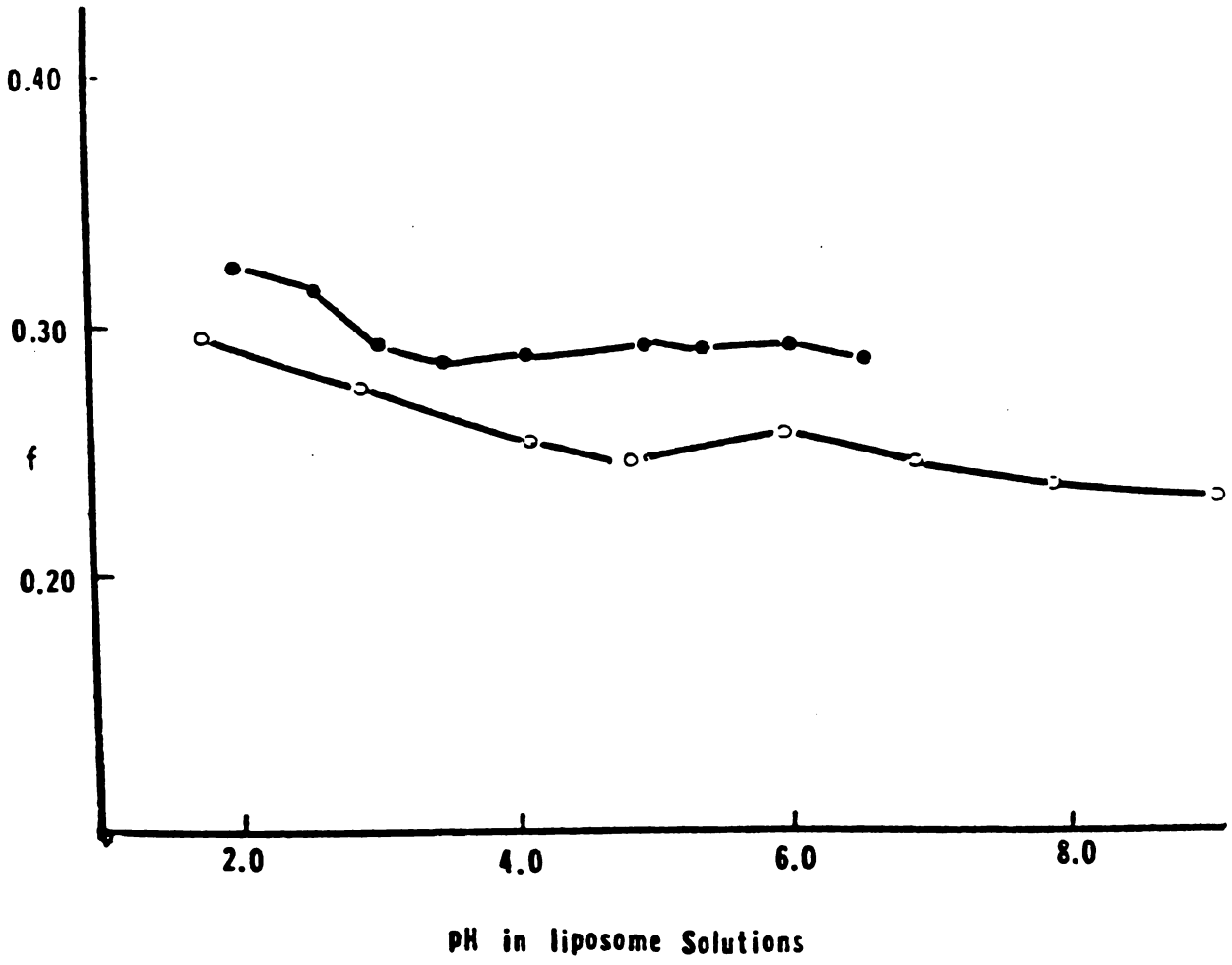


Figure 26. TEMPO spectral parameter f at various pH of natural lecithin liposome solutions. f was measured at 0.10 M varapamil (●) and in the control (○). The experimental temperature was 22 C (± 0.2 C).

DISCUSSION

The fluidizing effect of propranolol on the membrane has been shown using different techniques like ESR (Singer, 1977), fluorescence (Akiyama and Igish, 1979) and differential scanning colorimetry (DSC) (Herbette et al., 1983). Most of the investigations showed that propranolol depressed T_t from the gel to liquid crystalline state, indicating the impartment of a high mobility to the hydrocarbon chain of lipids (Srivastava et al., 1983). This effect was suggested to account for the inhibition of ion channels by propranolol (Lee, 1977). In our ESR experiment a number of beta-blockers and Ca^{+2} blockers were found to increase the membrane fluidity (Figure 20 and Table 5). One doubt about the practical significance of this effect is the high drug concentration requirement (Figure 25) which was also reported elsewhere. For instance, Phadek et al. (1981) used 0.1 M propranolol in their TEMPO label measurement. This problem is partially due to the relative nonsensitivity of ESR (Lee, 1982) and the small proportion of the drugs (less than 20% for propranolol) incorporating into liposomes (Srivastava et al., 1983). Because spin level ESR usually needs a high concentration of a drug, so that the dose-response relation in this experiment does not mean that less than 0.01 M of verapamil has no liquifying effect. In DSC (Herbette et al., 1983) and fluorescence experiment (Lee, 1977) millimolar concentration of propranolol induced decrease of a few degrees in T_t of DPPC liposome. The concentration of millimoles, of course, is still too high as compared with the usual plasma drug level. But the latter is not always likely to be a good indicator of the local drug concentration in the target tissues (Kerry et al., 1984).

Verapamil (McIlhenny, 1971) and propranolol (Weskler et al., 1977) were found to accumulate to a marked extent in their target tissues.

Not all researchers agree that the fluidizing effect mimics the real function of propranolol on the living membranes since the experimental data are conflicted and the drug-induced decreases in fluidity was sometimes observed. One criticism is that most of the investigations have been performed by measuring T_t on the liposomes composed of a single type of synthetic phospholipid like dipolmitoyl PC (DPPC) and dimyristoyl PC (DMPC) which have characteristic by distinct phase transitions. On these model membrane systems a depression of T_t may be associated with an increase in fluidity. However, such model membranes do not represent well the biological membranes, which have coexistent liquid or liquid-solid functional states (Engelman, 1970) and undergo a phase transition over broad temperature ranges, which cannot be distinguished in many cases, because of the extreme diversity in constructural compositions. There have been few experiments done on such membranes (which exclude the application of DSC, the most commonly used technique) and thus it is not clear how the heart drugs influence the fluidity of the membranes which are already in the liquid or liquid-solid coexistent state. Phadek et al. (1981) reported an opposite effect of propranolol on the membranes of different states: while liquifying the gel-state DPPC liposomes, the drug stiffened egg PC, and egg PC plus cholesterol liposomes which were known to be in the a liquid crystalline state. Surewicz and Leyko (1981) reported that propranolol exerted a marked ordering effect on liquid-state bilayer prepared from the certain phospholipids and thus they proposed that some drugs may affect the membrane order and fluidity quite differently or even

oppositely, depending on the original physical state of the membranes. A decrease in T_t on the liquid-crystalline membrane does not imply further disordering of the lipid membrane. In this model, propranolol acts as a modulator, like cholesterol and some intrinsic membrane proteins, adjusting the membrane fluidity to a critical point or range necessary for the membrane function. They liquify the membrane if the fluidity is below the point, and they impart rigidity to the fluid state of the hydrocarbon chains above it.

The interior of BLM is liquid-like owing to the existence of a small amount of organic solvent (Krasne et al., 1971). According to the "modulator hypothesis", propranolol and other heart drugs should increase the degree of lipid order. However, our ESR experiment showed that in all the liposomes tested, regardless of their physical state, the membrane fluidity was always uniformly increased by the drugs. The T_t of DMPC liposomes was decreased 10°C by 0.1 M verapamil (Figure 21). The natural lecithin, which was the very one used in the ionophore transport experiment, and heart lipid liposomes were shown to be in the coexisted liquid-solid state and their fluidity was increased by the drugs over a wide temperature range (Figures 22, 23 and 24). It seems that the drugs liquify these types of membrane lipids, irrespective of the original state. The ESR results corroborate the idea that the inhibition of the carrier-mediated cation translocation in Part II is mainly caused by the electrical effect rather than the fluidity effect of the drugs. Theoretically, the fluidizing of the membrane by these drugs may influence the transport process, but it was found to be negligible since the diffusion of the negative species $H(DNP)_2^-$ and neutral species $Ca(A23187)_2$ were not accelerated by it.

The fluidity change induced by the heart drugs does not contribute much to the blockade of the carrier transport in BLM, but this does not exclude the possibility of this mechanism is acting in the living membranes in vivo. Thayer et al. (1985) reported that verapamil and D600 impaired the binding function of their receptors through the perturbation of the membrane fluidity. Earlier, Godin et al. (1976) found that propranolol, through an alteration in the structural state of amine phospholipids, impaired the activity of Mg^{+2}-stimulated nitrophynel phosphatase on the erythrocyte membrane. This enzyme is influenced by the fluidity of the phospholipids which controled the affinity of the enzyme to Mg^{+2} (Goldman and Albert, 1973). There has been no evidence whether or not, and how the fluidizing effect of beta-blockers and Ca^{+2} blockers involves their pharmacological functions, e.g., the blockade of Ca^{+2} uptake. A prevailing viewpoint is that a high membrane fluidity is usually associated with the activation of membrane proteins, and how a drug blocks the protein channels by liquifying constitutive lipids. One hypothesis about it is the annular transition model proposed by Lee (1976) for interpreting the cation of local anesthetics. In this theory, the Na channel is presumed to be a complex form of the pore protein and annulus lipids. The latter are normally in gel state and the rigidity keeps the channel open. The addition of drugs triggers a change in lipid from the gel to a fluid state, causing the channel to relax into its most stable state with consequent closing of the pore. Can a similar model be applied to the blockade of Ca^{+2} channels by the heart drugs? We do not know it because of the lack of knowledge about the structure and properties of the channels and about the interaction between lipids and proteins. For

instance, we have no idea whether the channel exists as protein-lipid complex, in which a partically functional state of the lipids is required for a channel's opening.

PART IV

RECONSTITUTION OF SARCOLEMMA K^+ CHANNEL INTO BLM

METHODS AND RESULTS

In the second part of the investigation, we want to study the action of the heart drugs on the native protein channels. While not successful in reconstituting calcium and sodium channels, we have incorporated voltage-dependent potassium channels into BLM. The preparation of the protein channels was from the fragmented cardiac sarcolemma which is the action site of the heart drugs and also is a good source for various ionic channels. The sarcolemmal vesicles were prepared from rat hearts according to the differential centrifugation method used by Bukoski (1983), simply:

1) Blend and homogenize fresh rat hearts in the physiological salt solution buffered with 1 mM HEPES (pH 7.4).

2) Centrifuge the tissue homogenate at 1800 r.p.m. for 5 minutes and discard the pellet.

3) Centrifuge the supernatant obtained in 2) at 8,000 g for 10 minutes and discard the pellet.

4) Centrifugate the supernatant obtained in 3) at 100,000 g for 1 hour and discard the supernatant. The pellet was dissolved in 1 mM HEPES buffer (pH 7.4).

The procedure was performed at a low temperature (< 4^{o} C). The resulting preparation consisted mainly of sarcolemma, but was contaminated with sarcoplasmic reticulum (SR) (Jones et al., 1980). The sarcolemmal vesicle solution was stored at -40^{o} C and an small aliquot was thawed for each day of work. The protein concentration of the preparation was determined by spectrophotometry (Bradfold, 1976) on a Beckmann spectrometer using bovine serum albumin (BSA) as the standard, and a dye-binding solution as the analytical reagent (both were from Bio-Rad Laboratory). The electrical measurement on BLM was the same as described previously except that a pair of glass electrodes were used.

1. **Stepwise Increases of BLM K^{+} Conductance**

The incorporation of channels was achieved by adding sarcolemmal vesicle directly to the bulk solution in the cis side (Krueger et al., 1983). A small quantity of sarcolemmal preparation in 0.1 M KCl buffer (1 mM HEPES, pH 6.0) greatly changed the conductance of BLM (PC + C + PE + PS, or natural lecithin + cholesterol). With up to 10 μg/ml of protein in bathing solution, the membrane conductance increased 2 orders in a few seconds. In the log-log plot (Figure 27), the steady-state conductance showed a first order dependence of the protein concentration (slope = 0.78), suggesting that the channel has a monomeric structure in the conducting state (Mueller and Rudin, 1968).

The current changed smoothly at high protein concentration, and at low protein concentration stepwise increases in the membrane current were observed. As shown in Figure 28, the current jump was in a staircase fashion and only appeared at protein concentration below 5 μg/ml. All of steps were directed upward whereas terminating events were rarely recorded. One can note that the increase of each jump remained approxi-

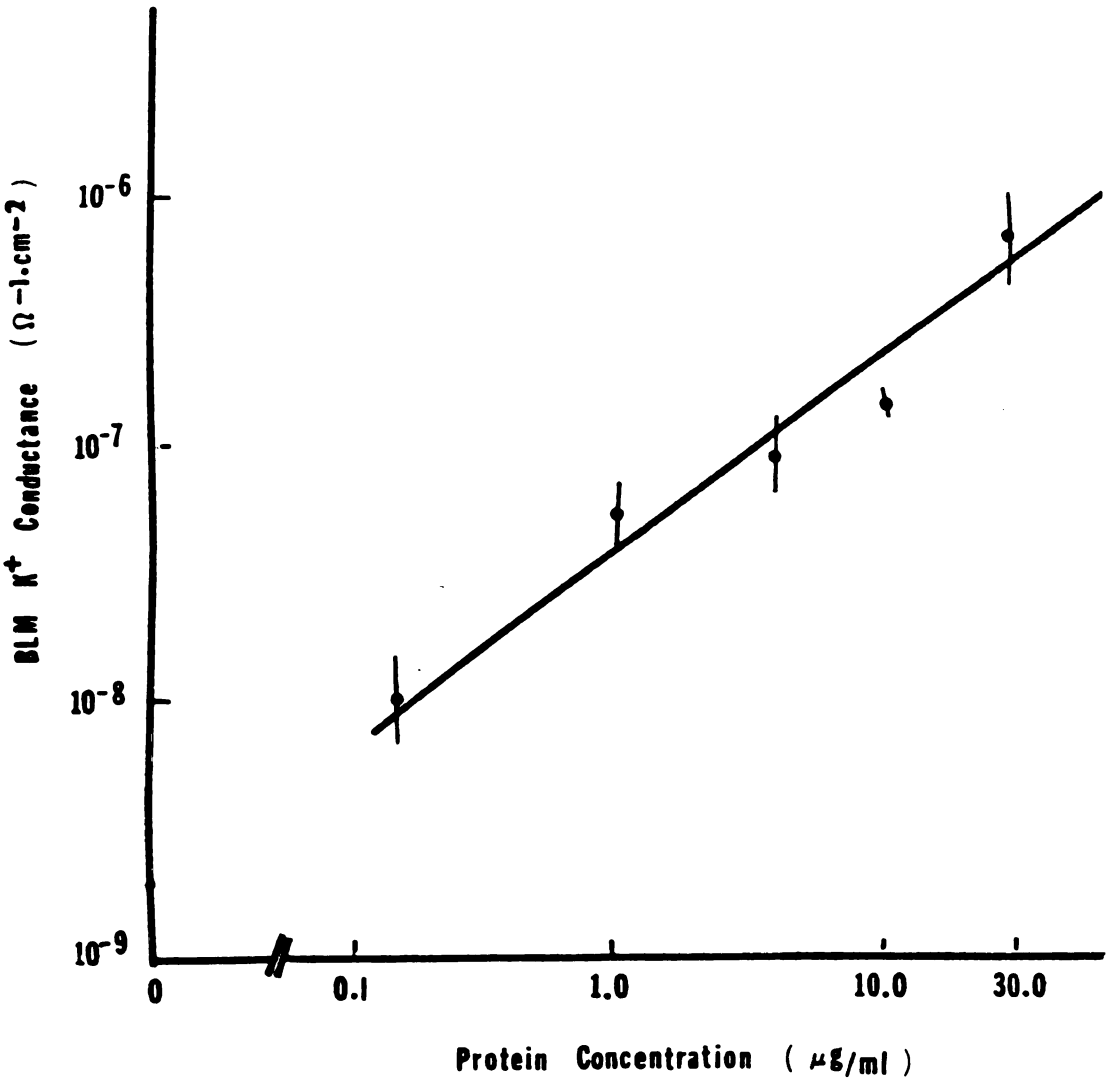


Figure 27. Double logarithmic plot of BLM K^+ conductance vs. protein concentration in sarcolemmal solution. The regression curve has slope of 0.78. BLM used in the measurement was composed of PC + PE + OC + PS (see part 1) and the bathing solution was 0.1MKCl, 1mM HEPES buffer (pH 6.0).

Figure 28. Staircase-fashioned stepwise increases in BLM K^+ conductance induced by sarcolemma solutions at various protein concentrations indicated in μg/ml on figure. The aqueous solution (pH 5.5) contained 0.1MKCl, 1mM HEPES. BLM was formed from a solution of 5% natural lecithin plus 1% OC in n-octane. The early part of the conductance was monitored after addition (at arrows) of sarcolemma stock. The applied voltage was +30mv. The full scale of current measurement range was 3×10^{-10}A.

mately a unit value or multiples of the unit, while the frequency of the jumps increased rapidly with the protein concentration. The appearance of the stepwise current increase is thought to result from the fusion of the discrete quanta of the conductive pathway into BLM (Miller and Racker, 1976). This quantization phenomenon, especially the protein concentration-independence of the jump size and protein concentration-dependence of the jump frequency, provides the convincing evidence for the incorporation and formation of ionic channels on BLM (Cohen et al., 1980). This can be clearly seen from the transition from the discrete to continuous increase. At 4-5 g/ml protein, the average size of each jump became larger, implying the more chances for several vesicles to fuse into BLM simultaneously in the time scale used. The further increase in protein level led to more vesicles to fuse simultaneously and the "quantum" phenomenon disappeared. A single conductance jump usually is not equivalent to a single channel event unless each package only contains 1 or 0 channel protein average. Latorre et al. (1982) reported a stepwise increase in K^+ conductance corresponding to the single channel opening. This might also be the case in our experiment because only unit size jump was observed at very low protein concentration (0.15 μ g/ml).

If the sarcolemmal solution was pretreated by incubating it in 70° C water bath for 5 minutes, neither the stepwise nor smooth current increases happened. The addition of protein denaturers, 1 mM $HgCl_2$ or 5 mM pronase, also eliminated the electrical changes. These inhibitory experiments proved the protein identity of the ionophore.

2. Features of the Sarcolemmal Channel

One interesting thing is that there were two types of current jump that occurred, probably representing two populations of K^+ channels. There was another current rising after the addition of 10 mM $CaCl_2$ into KCl bulk solution. This current increase was characteristic of the more complex time course: each jump being composed of a spike followed by relaxation to a lower steady-state level (Figure 29). It appeared that this conductance jump did not correspond to Ca^{+2} channel and may represent Ca^{+2}-dependent K^+ channel because it was not induced by Ca^{+2} alone. More work is needed for exploring the conducting selectivity of this channel. The staircase jump channel is simple and easily analyzed and thus was chosen for the further study which revealed the following features of this channel.

1) It is K^+-specific. When KCl in bathing solution was displaced with NaCl or $CaCl_2$, the discrete current increase was never observed. Very high sarcolemmal vesicle concentration in NaCl or $CaCl_2$ solution only had very small effect on the macroconductance of BLMs. Thus the channel is not conductive for Na^+, Ca^{+2} or Cl^{-1}.

2) The channel has a 330 PS single conductance jump, the same order as that (200 PS) of K^+ channels in SR vesicle reported by Latoree and coworkers (1982).

3) This K^+ channels is voltage-dependent. As shown in the G-V curve in Figure 30, the conductance at negative voltage remained at a low level and then it increased in a non-linear manner. No saturated conductance was discerned up to +50 mV. The applied voltage did not influence the size of single conductance jump within the measuremental error: 10 mV: 340 PS, 20 mv: 310 PS, 30 mV: 330 PS (Figure 31), but it

Figure 29. Ca^{+2}-dependent stepwise increases in BLM conductance induced by sarcolemma solution. Notice the complex fashion of single current jump: a spike followed by relaxation to a lower steady-state level. The aqueous solution (pH 5.5) contained 0.1MKCl, 0.01M$CaCl_2$ and 1mM. The protein concentration in sarcolemma solution was 3 μg/ml. The applied voltage was +30mv and +50mv. The full scale of current measurement range was 3 x 10^{-10}A.

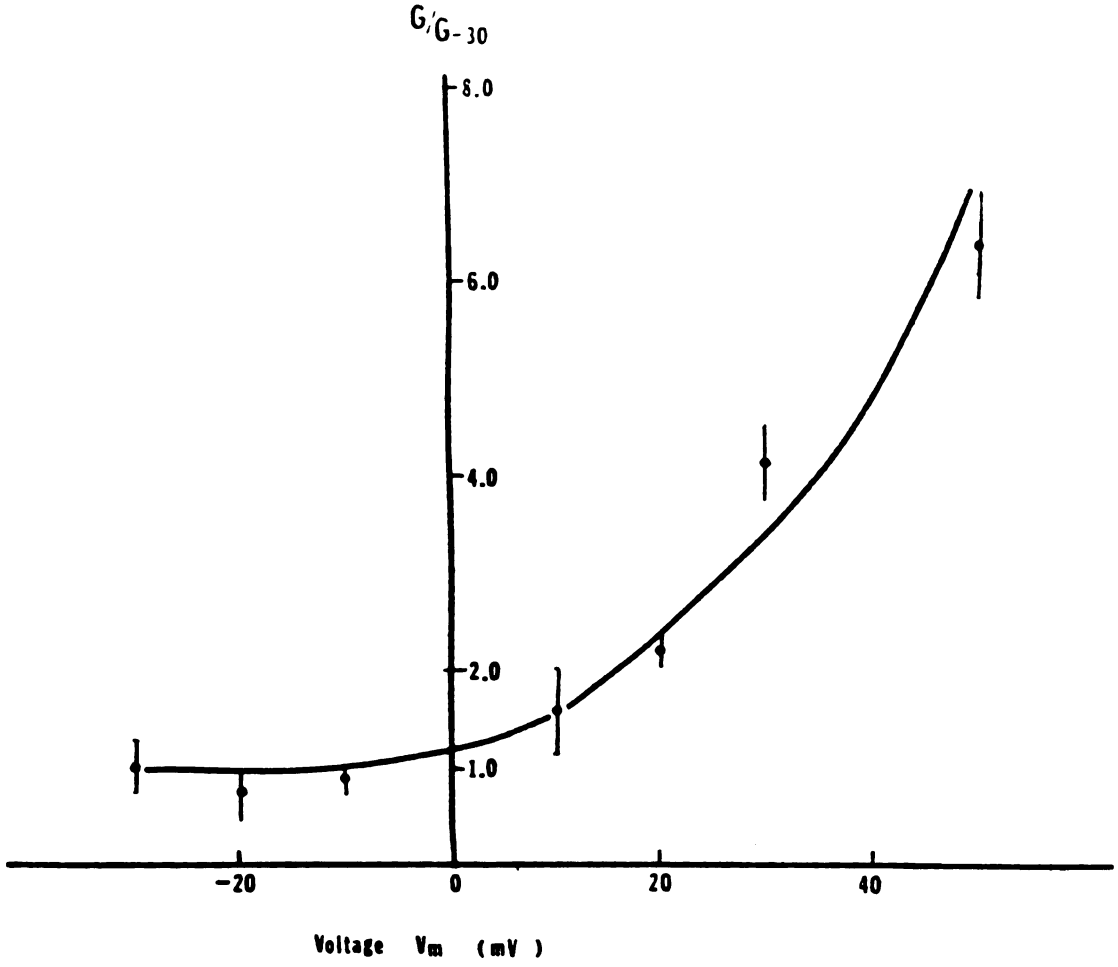
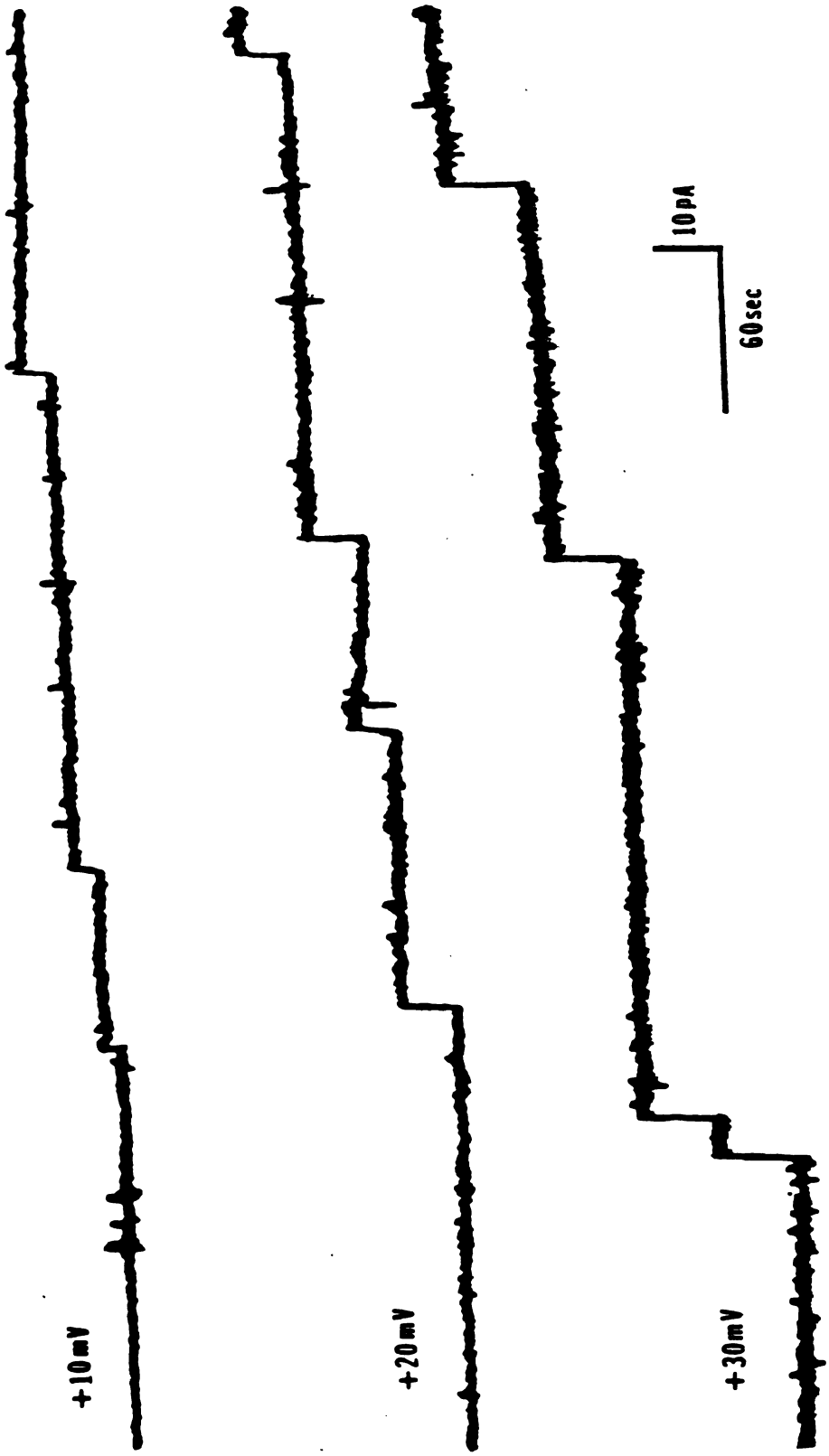


Figure 30. Conductance-voltage (G-V) curve—the voltage dependence of the steady-state conductance; G-30 the conductance at -30mv applied voltage. The local protein concentration in the bulk solution was 10 μg/ml.

Figure 31. Stepwise increases in BLM K^+ conductance induced by cardiac sarcolemma solution at various applied voltage: the voltage independence of the jump size. The protein concentration of sarcolemma solution is 0.15 ug/ml. The applied voltages is indicated on the figure. The calculated values for single conductance jump are 330 pS at +30 mV, 310 pS at 20 +mV and 340 pS at +10 mV.


+10mV
+20mV
+30mV
10pA
60sec

affected on membrane conductance by altering the jump frequency and probably also the duration of the open state. (This was not measured.)

4) It was reported that the osmotic gradient and Ca^{+2} are two essential factors for the incorporation of many sarcolemmal or SR vesicle into BLM (Miller, 1978). But in our experiment, the fusion of the channel preparation did not require such osmotic gradient across BLM. Ca^{+2} in the bathing solution showed neither positive nor negative effect on K^{+} conductance, which was proved by adding an excess of the Ca^{+2} chelater EGTA. It may be that the K^{+} channel in our experiment is different from Ca^{+2}-dependencent K^{+} channel reported elsewhere (Miller, 1978; Latorre et al., 1982; Coronado and Latorre, 1982).

Kass (1982) reported that certain sarcolemmal K^{+} channels are inhibited by verapamil, but the blockade of K^{+} conductance by the heart drugs has not been observed on the BLM modified with our cardiac sarcolemma preparations. So far we have been not succeeded in reconstituting Ca^{+2} channels. This may be due to the biochemical isolation procedure, but the electric measurement also remains a problem. Generally Ca^{+2} channel currents are more difficult to be detected because of the lower rate. In the most cases it has been studied by measuring single channel current using the voltage clamp technique (which is not available for us at present). More efforts will be made to toward study in the future.

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