PHOSPHOLIPID HEADGROUP SUPERLATTICE MODULATION OF CARDIAC CALCIUM CHANNEL ACTIVITY

by

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CHAPTER I

INTRODUCTION

The plasma membrane of cells consists of phospholipids, sterols, and proteins and maintains a steady composition through a regulatory mechanism that remains largely unknown. The interactions that occur among the membrane constituents are complex and are involved in compositional control. Among the proteins embedded is a class of transmembrane proteins called ion channels, specifically, the ryanodine receptors located in the sarcoplasmic reticulum of myocytes. These channels are responsible for the release of Ca^{2+} ions, necessary for the activation of myofilaments to produce contractions. The receptors, through a process known as gating, undergo changes in conformation during the transition between open and closed states. Since proteins are surrounded by lipids and change conformations, the lipid-protein interactions must influence the protein state by hydrophobic coupling or on the headgroup level. By reconstituting the channels in planar lipid bilayers composed of two phospholipids with differing headgroup sizes, POPE and POPC and varying the ratio of the two lipids that form the bilayer, the channel functioned as sensors to determine how bilayer changes affect the channel. A peak in channel activity was observed over a narrow region of high PE:PC bilayers, indicating that lipid-protein interactions do have an important role in channel function. The results are interpreted as reflecting lateral organization by the lipids on the headgroup level in order to minimize the stress across the bilayer due to deformation, curvature, and packing, providing a favorable condition for the channel to enter its preferred conformation.

CHAPTER II

RYANODINE RECEPTOR: STRUCTURE AND FUNCTION

Ion channels are transmemebrane proteins that control the flow of ions across the plasma membrane. In nerve and muscle cells, the channels are responsible for the rapid changes in the membrane potential that are associated with action potentials and the influx and outflux of Ca^{2+} ions necessary for the activation of proteins and enzymes. Three properties common to all ion channels are i) conduction of ions ii) recognition and selection of specific ions and iii) opening and closing in response to electrical, mechanical or chemical stimuli (Berne 1993).

Ion selectivity is achieved through physical-chemical interactions between the ion and various amino acid residues that line the wall of the channel. The channel structure is thought to be shaped like an inverted teepee with a selectivity filter at the end of the channel and a large, water-filled cavity near the middle of the channel pore. At the selectivity filter, the ion must shed its water of hydration and form weak bonds with the polar amino acid residues that line the filter wall. An ion is capable of entering the filter only if the energy of interaction with the residues can compensate for the loss of the waters of hydration and is of an appropriate size. The ion is propelled through the filter by an electrochemical gradient and also by electrostatic repulsion due to other ions entering the filter (Doyle 1998).

Channels have at least one open state and one closed state. The transition of a channel between the open and closed states is called gating and involves conformational changes in the protein structure. High resolution crystal structures have shown that

distinct conformational changes occur through twisting and tilting of the subunits that form the channel structure (Doyle 1998, Toyoshima 2000).

Located within the plasma membrane of the sarcoplasmic reticulum (SR) of cardiac myocytes, there are Ca^{2+} channels called ryanodine receptors (RyR) that have a key role in the control of cardiac contractility. Located in the surface membrane and T-tubules are dihydropyridine receptors (DHPR) which are voltage activated Ca^{2+} channels and when opened, allow the entry of Ca^{2+} to the closely located RyRs in the SR (Figure 1). This leads to the activation of the RyR and subsequent release of massive amounts SR Ca^{2+} that will activate the contractile proteins, myofilaments. This process is referred to as calcium-induced calcium release (CICR) (Fabiato 1985).

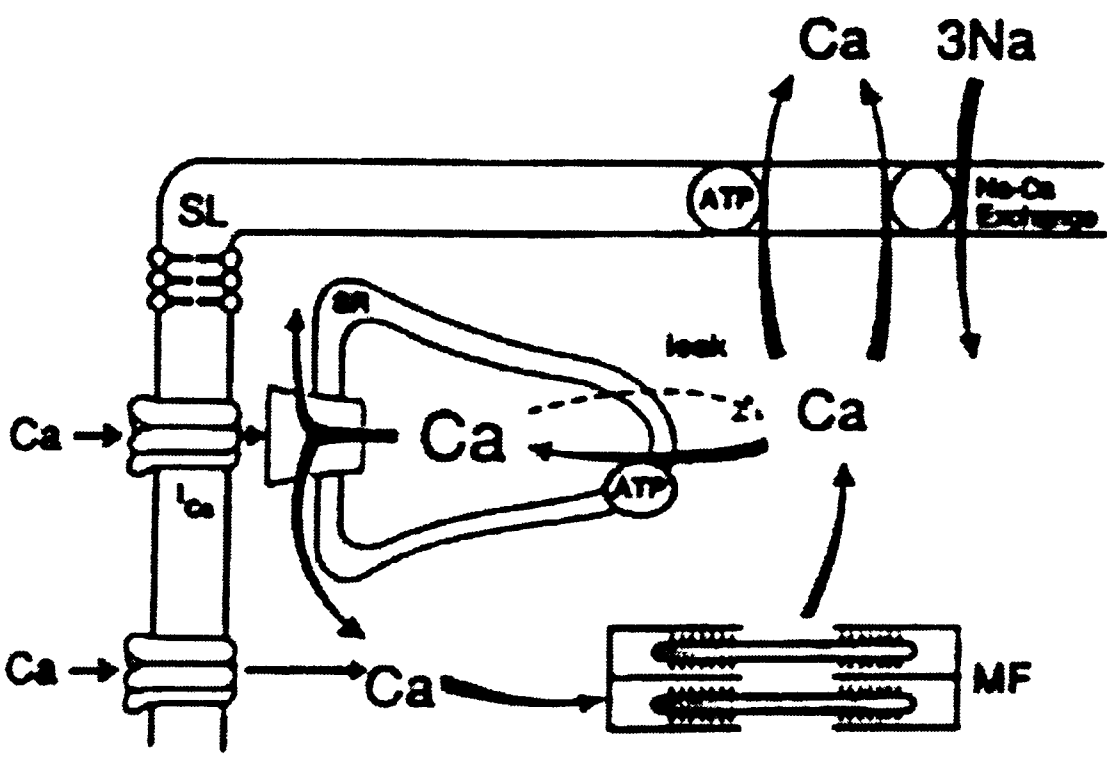


Figure 1. CICR Mechanism (Bers 1991).

The RyRs are the largest known ion channels with a net molecular weight of 2.3 MDa. These channels exist as homotetramers. Figure 2 shows surface representations of the RyR in its open and closed states. The two states are very similar in their overall features. There are two main regions, the cytoplasmic assembly and the transmembrane

assembly. The cytoplasmic assembly constitutes 80% of the total mass of the receptor and has a square shape with dimensions of $270\,\mathring{A} \times 270\,\mathring{A} \times 110\,\mathring{A}$. During the transition from the open to closed state, the cytoplasmic assembly decreases in height while there is no change in the lateral dimensions. The transmembrane assembly, the channel pore, undergoes a significant change in its conformation during gating. In the open state, the four subunts are elongated and have rotated 4° clockwise with respect to the closed state to form an opening of $20\,\mathring{A}$ (Orlova 1998). The RyRs are cation-selective channels that have a larger ion conductance for monovalent cations (460pS for Cs^{+}) than for divalent ions (150pS for Ca^{2+}) (Meissner 1998).

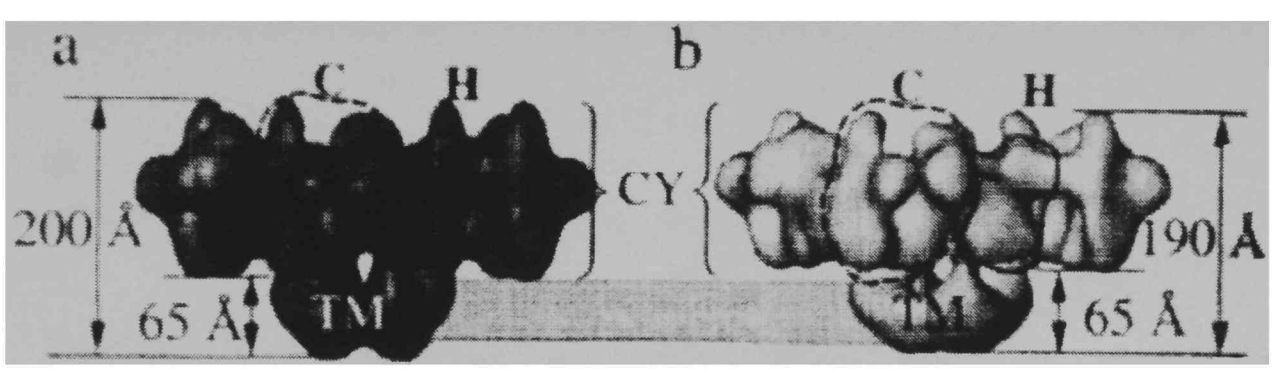


Figure 2. Surface representations of a) Open state and b) Closed state for RyR (Orlova 1998).

CHAPTER III

PHOSPHOLIPID PROPERTIES

The lipid bilayer is composed of phospholipids, which are amphiphilic molecules that consist of a polar headgroup and two nonpolar acyl chains. The amphiphilic nature of the lipids provides the mechanism for the self-assembly of well-defined structures like lipid bilayers in an aqueous environment with the acyl chains forming a hydrophobic core, minimizing the thermodynamically unfavorable interactions with water and ordering of water through the hydrophobic effect (Nossal 1992). The phospholipids used were 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) (Figure 3).

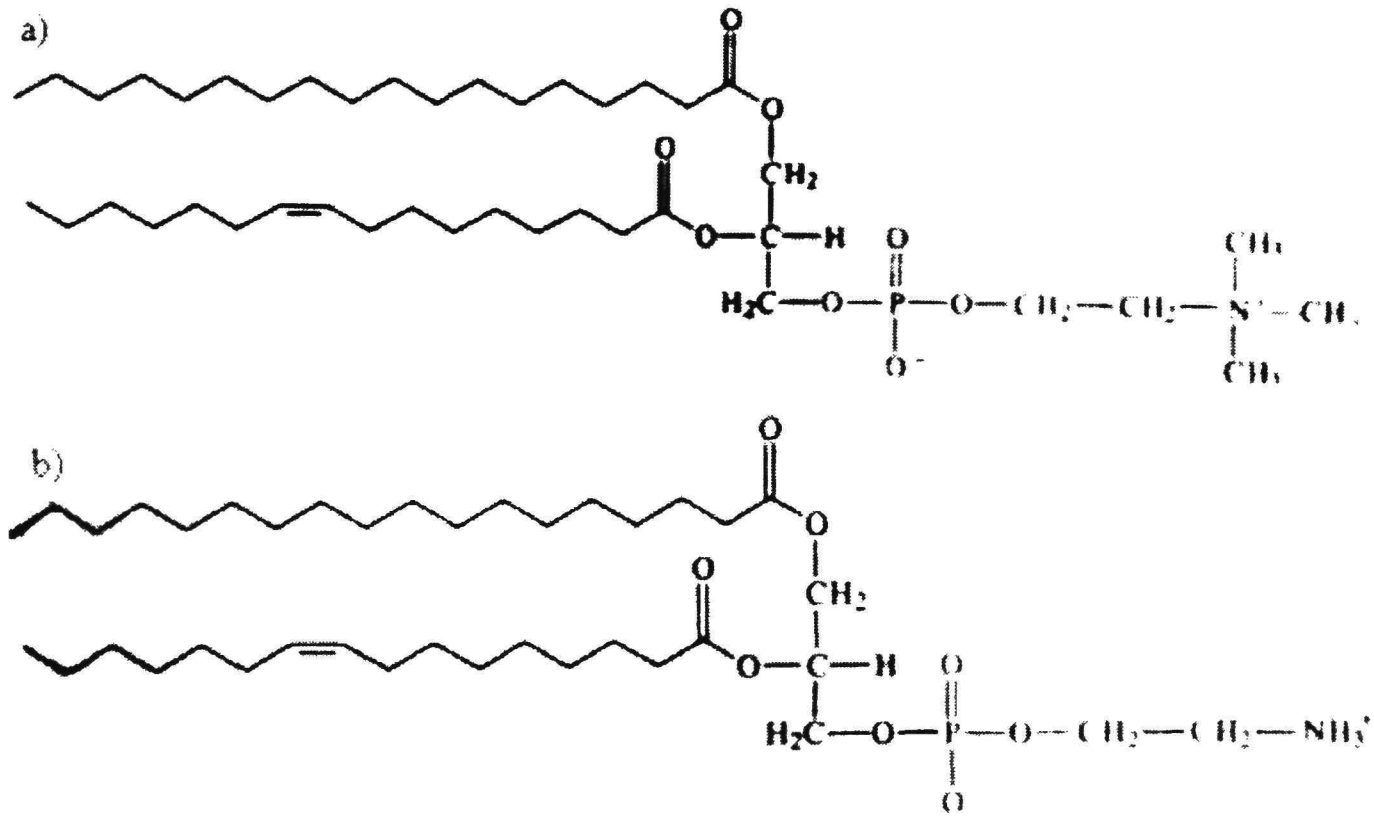


Figure 3. Diagrams for a) POPC and b) POPE (Voet 1997).

POPC has a larger headgroup than POPE due to the choline moiety, which has three methyl groups bonded to the nitrogen, allowing the formation of more hydrogen

bonds and greater hydration. The POPE headgroup only has three hydrogens covalently bonded to the nitrogen. The difference in the areas of the headgroup and the acyl chains has allowed POPE and POPC to have their shapes defined geometrically through the use of the critical packing parameter, $v/a_o l_c$. The critical packing parameter defines v as the hydrocarbon volume, a_o for the optimal headgroup size, and l_c as the length of the full extension of the acyl chains. PC has a packing parameter of 0.5 – 1, which corresponds to a truncated cone and PE has a packing parameter that is greater than 1, which has the shape of an inverted truncated cone (Israelachivili 1992). By examining these geometries, POPE and POPC can be identified as having complementary shapes (Figure 4). Bilayers composed of pure PC will have considerable crowding on the headgroup level as a consequence of the large headgroup size (Figure 5a). Also, the PC

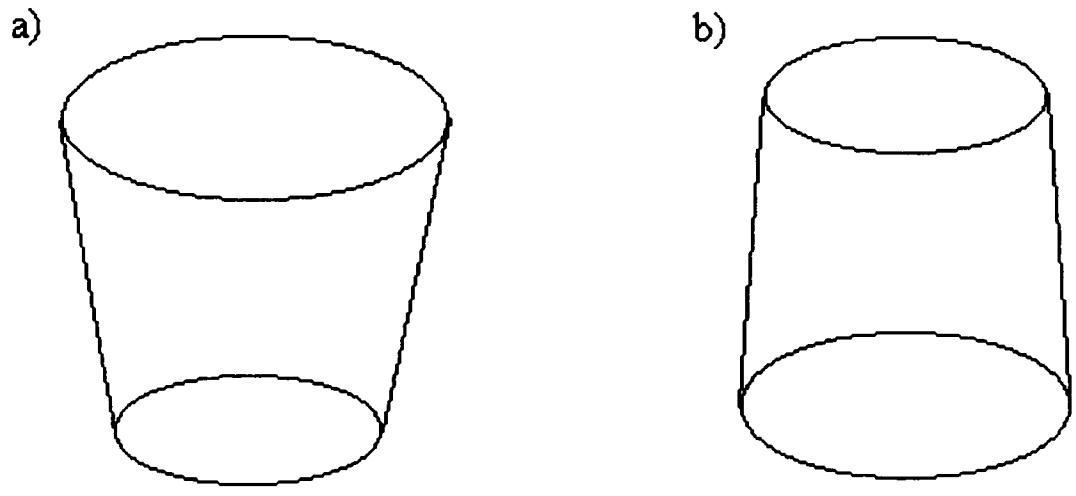


Figure 4. Packing geometries for a) POPC and b) POPE

headgroups will tilt toward the hydrocarbons, dipole moments will be created that will create electrostatic repulsion (Dill 1988). For pure PE bilayers, there will be packing strain on the acyl chain level from the small headgoup size. At temperatures above 27° C, PE monolayers have the tendency to form the more favorable inverted hexagonal II phase that comes from the headgroups curling to form water-filled cavities and the acyl

chains in a more relaxed state on the exterior (Figure 5b) (Israelachvili 1992).

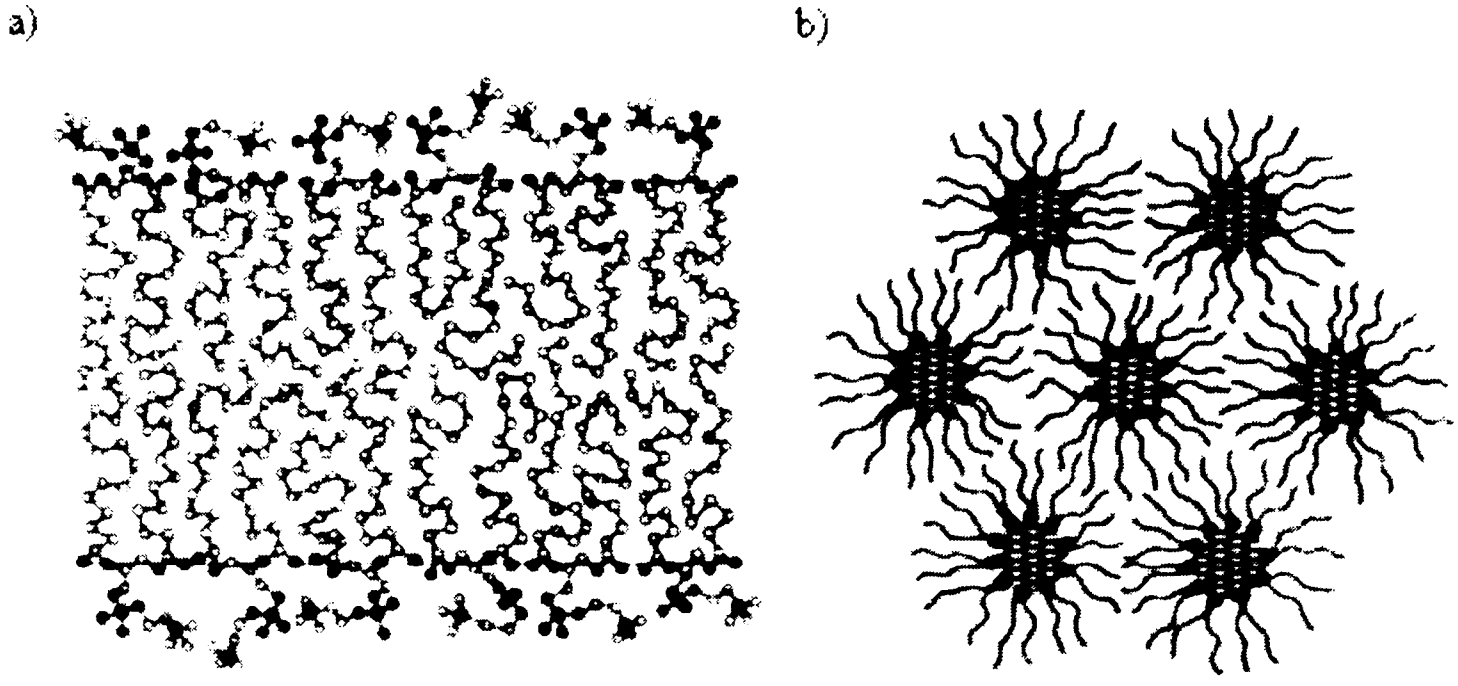


Figure 5. Naturally occurring states for a) Bilayer state for POPC b) H_{II} phase for POPE (Israelachivili 1992).

CHAPTER IV

LIPID BILAYER MODELS

The mechanisms that are responsible for the organization and composition of the cell membrane remain unresolved. It is unknown how the cell maintains precise control of the ratio of the various phospholipids and sterols present, as well as the differences in the headgroups and acyl chains. Four main platforms have been developed to explain various features of the membrane and various characteristics of the interactions between individual components such as lipid-lipid and lipid-protein, all drawing on the others, while maintaining a unique perception.

The intrinsic curvature hypothesis by Gruner considers the amount of intrinsic curvature due to nonbilayer-forming lipids such as PE to be an important feature in membrane regulation. The nonbilayer-forming lipids have a small R_o, which is the spontaneous radius of curvature and is measured as the radius of the water cavity in the H_{II} phase. This small R_o means that the these lipids when arranged into monolayers and uninhibited, have the tendency to curl into the H_{II} phase to minimize their elastic free energy that is given as

$$\mu_E = \frac{1}{2} k \left(\frac{1}{R} - \frac{1}{R_o} \right)^2 \tag{1}$$

where k is the modulus of elasticity and R is the radius of the current structure. The curvature is defined as 1/R. For lamellar structures, $R = \infty$, so nonbilayer lipids that are placed into a bilayer have an unfavorable elastic free energy. The bilayer-forming lipids such as PC have large R_o so that their μ_E is minimized in bilayer form. When lipids of

differing curvature, PE and PC, are mixed, the intrinsic curvature, C, of the resulting bilayer would be expressed as

$$C = X_{PE}C_{PE} + (1-X_{PE})C_{PC} \quad (2)$$

The level of curvature present in the bilayer would indicate the amount of strain, in that increasing the ratio of nonbilayer lipids to bilayer lipids would cause greater strain. At a certain mixture of two lipids, like PE and PC, there could occur a critical edge of stability such that the introduction of a protein could destabilize the bilayer. Ultimately this theory postulates that the membranes will homeostatically control their intrinsic curvature to be in some optimum range (Gruner 1985).

The structural stress hypothesis bases its control of membrane composition on lateral stress and curvature stress. The lateral stress depends on the modulus of compressibility, K, such that for larger headgroups, the lateral stress will be less because of the lower K. From the intrinsic curvature model, the curvature stress arises from the spontaneous radius of curvature and that as the curvature increases, the free energy increases accordingly, causing greater stress. Bilayers consisting of bilayer-forming lipids will have less curvature-dependent stress. Proteins would have a greater propensity for entering their preferred conformation in bilayers with lower structural stress since the protein would encounter a smaller reactive force from the surrounding lipids during conformational changes than if the surrounding bilayer medium had a higher K. This model relies upon decreases in structural stress to being favorable toward protein activity (Chang 1995).

Using molecular level interactions to describe the properties of lipid mixtures has resulted in the umbrella model, based on the ability of the phospholipid headgroups

to effectively cover the hydrocarbon region. By considering cholesterol multibody interactions and cholesterol-water interactions as energetically unfavorable, there exists a maximum solubility limit at which the headgroup can no longer shield cholesterol from water exposure and the subsequent formation of monohydrate crystals occurs. The effective cross-sectional area of the headgroup and the ability to reorient itself to increase coverage seems to be the most important factor in determining the maximum solubility values for different phospholipids. There are not any chemical associations between the phospholipids and the cholesterols in determining the maximum solubility. As the cholesterol content increases toward its solubility limit, the packing on the hydrocarbon is expected to increase because of the limited coverage and that as the packing increases, the cholesterols may have the tendecy to distribute themselves regularly to minimize the multibody interactions. A mechanism in the composition control of the membrane may be to ensure a certain level of shielding on the headgroup level and that cholesterol-cholesterol interactions are limited (Huang 1999).

Evidence has been accumulating which indicates that membrane lipids such as phospholipids and cholesterol have the tendency to distribute into a regular pattern. The superlattice (SL) model describes the lateral organization of the lipids and how cells may maintain the boundaries between distinct organelles and regulate the lipid composition of the membranes. The two main driving forces are attractive and repulsive interactions. Attractive lipid-lipid and lipid-protein interactions are the result of hydrogen bonding, Coulombic attraction, and van der Waals interactions. Repulsive interactions involve steric strain, dipolar interactions, and Coulombic repulsion and have more long-range effect than the attractive interactions. Pyrene lipids have been

shown to be regularly distributed in liquid-crystalline PC bilayers as has cholesterol in phospholipid bilayers (Somerharju 1985, Virtanen 1995). The SL model explains the distribution by proposing that the substitution of an alkyl chain with a bulkier guest like a sterol induces a steric strain and increases lateral pressure, which can only be minimized by the guests adopting a regular distribution. For molecules with a large headgroup cross-sectional area compared to the acyl chains like PC, the addition of complementary shaped molecules, like PE, can relieve the packing frustration by decreasing the crowding on the headgroup level and allowing the acyl chains to become more ordered. Differences in the acyl chain lengths of lipid are another source of steric strain and may possibly cause domains to form that are predominantly of the same lipid. The introduction of integral membrane proteins to a bilayer with mismatched acyl chains can cause the exclusion of the lipids from the protein-lipid boundary that do not have the same length as the membrane spanning helices of the protein. A lipid can also be excluded from the protein-lipid boundary if it is too inflexible to conform to the protein contour. Membrane spanning helices of multi-helix proteins, such as ion channels, are often tilted with respect to the membrane surface, which may cause lipids with a smaller headgroup, such as PE, to be partitioned to the boundary region.

The SL model proposes that different phospholipids tend to adopt regular lateral distributions, but there exist only a limited number of critical concentrations that result in the superlattice formation. There are three important ideas to the model. First, superlattices are considered to be the most energetically favorable packing of the membrane components. Second, the whole membrane is not covered by a single superlattice, but is in a dynamic equilibrium with randomly arranged domains as

superlattices of varying composition. Third, the superlattices are soft, in that there is no long range order, but a high degree of local order. The softness and dynamic nature of the superlattices would allow membrane bending, lateral diffusion, and conformational changes in membrane proteins. Thermodynamically, the formation of superlattices is driven by enthalpic and entropic factors. Although the loss of entropy through the ordering of the lipids would be unfavorable, the gain due to the increase in the rotational freedom of the bulkier headgroup would be greater. The enthalpic effect originates from the acyl chains being allowed to be in their preferred conformations when the packing frustration is lessened.

Since nucleated cells are continually synthesizing and degrading the membrane lipids, there exists some regulatory mechanism since the lipid content remains constant. The formation of superlattices may serve as the primary signal for the regulation of the membrane lipid composition. The basis is that the membrane lipid composition would prefer a low energy state, such as a superlattice, and that any fluctuations in the membrane composition would be sensed by regulatory enzymes through an aggregation/disaggregation mechanism. The superlattice model predicts that the membrane is tightly packed when it is laterally organized, forcing the exclusion and aggregation of enzymes, and other impurities, into aggregates that would lead the enzymes into being in an inactive state until the critical composition and organization is disrupted at which point the enzymes would be reactivated (Somerharju 1999).

CHAPTER V

EXPERIMENTAL BACKGROUND

Previous experiments involving the effect of the lipid bilayer membrane composition on single calcium channel kinetics used varying concentrations of the neutral lipid phosphatidylethanolamine (PE) and the negatively-charged lipid phosphatidylserine (PS). There was no observed correlation between the surface potential and the channel activity, although the channel kinetics were seen to be altered with increasing PS concentration (Coronado 1987).

The intrinsic curvature hypothesis proposes that lipid bilayer behavior depends upon the mixing of bilayer lipids (PC) and nonbilayer lipids (PE) and that the curvature serves as a parameter for biomembrane composition. Single alamethicin channels were used to show that as the curvature of the lipid bilayer increased the probability of higher conductance levels increased. The conductance of the different levels was independent of the bilayer composition, indicating that the basic functioning was not affected by lipid-protein interactions. The curvature of the bilayer was increased by increasing the molar fraction of DOPE in the total lipid mixture (DOPE + DOPC) because of the nonlamellar tendency of DOPE. The alamethicin concentration required for incorporation into DOPE bilayers was ten times greater than that needed for insertion into DOPC bilayers (Keller 1993). Further studies have shown that as the ratio the large to small headgroup size of the lipids was decreased, the ratio of alamethicin to lipid was increased to insert the channel, although the lipid mixtures that contained PE never attained full insertion (Heller 1997). Increasing the mole fraction of DOPE in mixtures

of DOPC/DOPE causes a linear increase in the binding free energy and a decrease in the binding of alamethicin. The increase in binding free energy was found to be linearly dependent on the spontaneous curvature of the bilayer (Lewis 1999).

The incorporation of potassium channels in bilayers composed entirely of N-meth-DOPE, DOPE, or DOPC caused a significant change in the channel kinetics. The mean channel opentime was shown to increase by a factor of two when the bilayers had PC instead of PE as the headgroup of the lipids and with the same acyl chains. There was no significant change in the probability of the channel being in an open state. Using the same headgroup and changing the order of the acyl chains was shown to have no significant effects on the channel properties, indicating that the bilayer thickness does not readily affect the protein function. The structural stress hypothesis was used to explain the results by proposing that the increase in the mean channel opentime in the DOPC bilayer was due to a decrease in the structural stress due to smaller K, thereby imparting a smaller reactive force to close the channel. Increasing the order of the acyl chain should cause an increase in the spontaneous radius of curvature and a reduction in the curvature stress of the bilayer, seeming to favor channel activity, but there was no apparent change when performed. Similar to the alamethicin experiments, there was no change in the conductance and amplitude of the channels (Chang 1995). The presence of cholesterol was shown to change the conductance of the channels. The insertion of cholesterol into the bilayers caused a decrease in the activity of the potassium channels. Cholesterol increases the modulus of compressibility leading to structural stress through the resulting lateral stress. Also, cholesterol has been found to promote the formation of H_{II} structures and additional stress through the increased curvature (Chang 1995).

Gramicidin channels, which are dimers, have been shown to have their activity decreased by the insertion of cholesterol in DOPC bilayers. The results are based on increases in membrane stiffness and membrane deformation energy modulating the channel function by increasing the free energy difference between open and closed protein conformations. The relation between the free energy difference between conformations can be expressed as the sum of the intrinsic free energy change in the protein and the bilayer deformation energy

$$\Delta G^o = \Delta G^o_{prot} + \Delta G^o_{def} \tag{3}$$

(Lundbaek 1996). Further altering of gramicidin channel activity occurred through the incorporation of the channels into bilayers consisting of DOPC/DOPS, DOPE/DOPS or DOPS. This showed that the electrostatic energy of the bilayer affected the membrane protein through interactions among the headgroups of the bilayer and that the difference in headgroup size of the neutral lipid caused the channel duration to increase when the larger headgroup, PC, was included in the bilayer, as opposed to PE (Lundbaek 1997). Sarcoplasmic reticulum Ca-ATPase was shown to have its uptake of Ca^{2+} increase with decreasing mole fraction of PC in PE/PC mixtures (Cheng 1985).

Fluorescent techniques have been provided evidence that PE lipids destabilize lipid-protein contacts in DMPC/DMPE bilayers because of either the small size as compared to the PC headgroup or because of competition from PE-PE interactions. This was observed through the increased rotational motion of the gramicidin indoles with increasing PE content (Scarlata 1997).

Fluorescent studies of POPE/POPC mixtures have shown the presence of kinks at the lipid ratios deemed critical as for the formation of superlattices (Cheng 1997).

The presence of superlattices in biological membranes has been applied to the phospholipid composition of mammalian red blood cells. The phospholipid content is very near the critical composition predicted by the SL model, indicating that the conditions are present for the regular distribution in both membrane leaflets (Virtanen 1998). A signal transduction control enzyme, protein kinase C, was found to have itsactivity peak at a moderately high DOPE:POPE (Slater 1994). Phospholipase, a regulatory enzyme, was found to have activity minima at several cholesterol concentrations defined as critical by the SL model (Liu 1999).

CHAPTER VI

PROCEDURE

Lipid Preparation

The lipids, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) (10 mg/ml in chloroform, purchased from Avanti Polar Lipids) were used to prepare the mixtures for the planar lipid membranes. The lipids have the same acyl chains, but differ in headgroup size in order to observe the affect of headgroup interactions on the ion channel activity and minimize any acyl chain effects such as bilayer thickness and packing. The headgroup cross-sectional area of POPC is 63 $\mathring{A}$ and POPE is 40 $\mathring{A}$. The molar fraction of POPE of the total lipid mixture (POPE + POPC) was increased from .30 to 1.00 so that the fraction of the larger headgroup decreased. The lipids were mixed and then dried using a stream of N_2 gas to remove the chloroform. Decane was added to redissolve dried lipids. Heavy sarcoplasmic reticulum microsomes were prepared from rat ventricular muscle and stored at -80°C.

Ion Channel Reconstitution

To reconstitute single SR Ca^{2+} channels, a Delrin partition with a hole was used to separate two chambers, the cis and trans (Figure 6). Initially, the cis and trans chamber are each filled with 20mM $CsCH_3SO_3$ and HEPES with pH 7.4. One end of a glass electrode, treated in an agar/3M KCl solution, was placed in each chamber for voltage clamping and current detection. The other end of each electrode was placed in

separate chambers containing 3M KCl and an Ag/AgCl pellet that is connected to the headstage and the acquisition system.

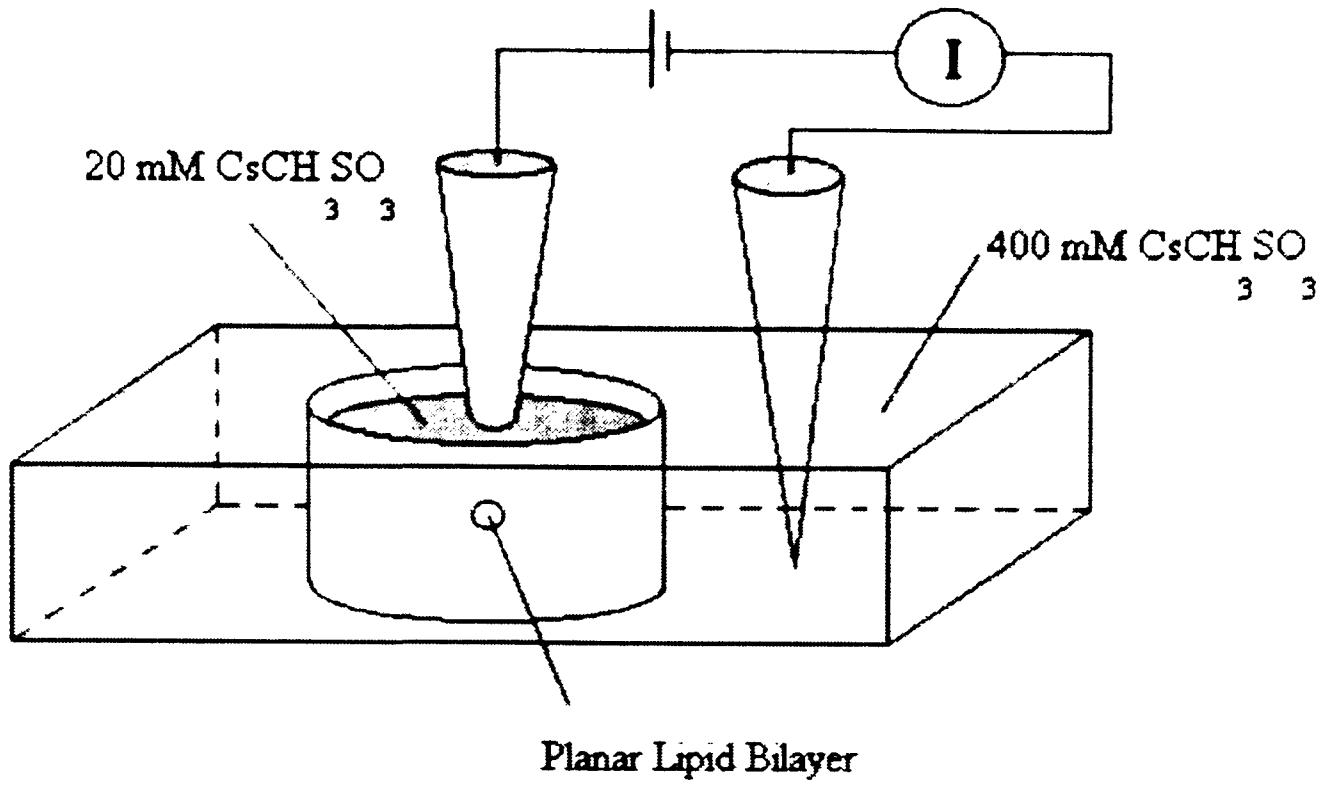


Figure 6. Bilayer experiment setup

Prior to painting the lipid onto the opening in the Delrin partition, a ramp protocol was applied across opening that changes the voltage from +50 to –50 mV. The capacitance is recorded during this protocol and has a value of 400 pF. This voltage ramping is continued while the lipid mixture is painted across the opening and when the capacitance is lowered to less than 50 pF, a workable bilayer has been formed and the ramping is stopped. The channels are reconstituted on the cis side of the partition. After a suitable bilayer membrane has been formed, 4M $CsCH_3SO_3$ and HEPES with pH 7.4 was added to the cis chamber to increase the cis concentration to 400mM $CsCH_3SO_3$ and create a concentration gradient from the cis to trans chambers. The heavy SR microsomes were added by pipette to the cis chamber to incorporate the channels by fusing to the membrane. To monitor the onset of channel activity, the incorporation

protocol was used, which consisted of applying voltage steps of +50mV and –50mV at 1sec intervals and measuring the current. During the incorporation protocol, the current should be near 0 pA, until there is channel activity. The appearance of sudden deflections from the baseline current indicates channel activity. To prevent further fusion by the microsomes, the gradient is removed by equilibrating the trans chamber concentration to the cis concentration. The steady state activity of the channels was recorded by measuring the current response to a +40 mV potential. The experiments were performed on bilayers that contained single channel activity only and the signals were filtered at 3 kHz using an 8 pole Bessel filter. After each trial, the Delrin partition was cleaned. All trials were at constant room temperature (Gyorke 1998).

Analysis

Single channel analysis was performed using pClamp 6 software. The open probability was defined as

$$P_o = \frac{T_{open}}{T_{total}} \tag{4}$$

The mean channel open time is

$$t_o = \frac{T_{open}(ms)}{N} \tag{5}$$

The open state events were summed and binned into lifetime histograms for each bilayer composition. The open lifetimes were determined by fitting the open time histograms with a second order exponentially decaying function

$$F(t) = P_1 e^{-t/\tau_1} + P_2 e^{-t/\tau_2} \tag{6}$$

CHAPTER VII

RESULTS

By measuring the current across the lipid bilayer membrane, the activity of the Ca^{2+} channels could be determined based on the number of events recorded, the total record time, and the open time of the channels. Since the lipid bilayer was a binary mixture of POPE and POPC with varying ratios of the phospholipids, the activity of the channel was measured as a function of the molar fraction of POPE, X_{PE}. The headgroup of POPE is smaller than the headgroup of POPC, so that changes in channel activity are directly dependent on the lipid-protein interactions on the headgroup level with the lipids having the same acyl chain lengths.

Figure 7 shows typical traces of the channel activity recorded for a) $X_{PE} = 0.41$ b) $X_{PE} = 0.81$ and c) $X_{PE} = 1.00$, where the only variable in the conditions was the bilayer composition. The upward deflections indicate the channel has entered the open state conformation. As can be observed from the trace, the channel differs in activity in terms of both the number of events and the length of the events when the lipid bilayer is altered. The activity of the channel in bilayers composed of $X_{PE} = 0.41$ and 1.00 appear to have similar activity, although the composition is very different. For the $X_{PE} = 0.80$ traces, the channel has longer open states and the occurrence of openings is greater and more intense. For each X_{PE}, the results were summed to form bin time histograms to determine the lifetime of the channels and shown are the histograms corresponding to the traces (Figure 8). For the three histograms shown, the shorter states are more

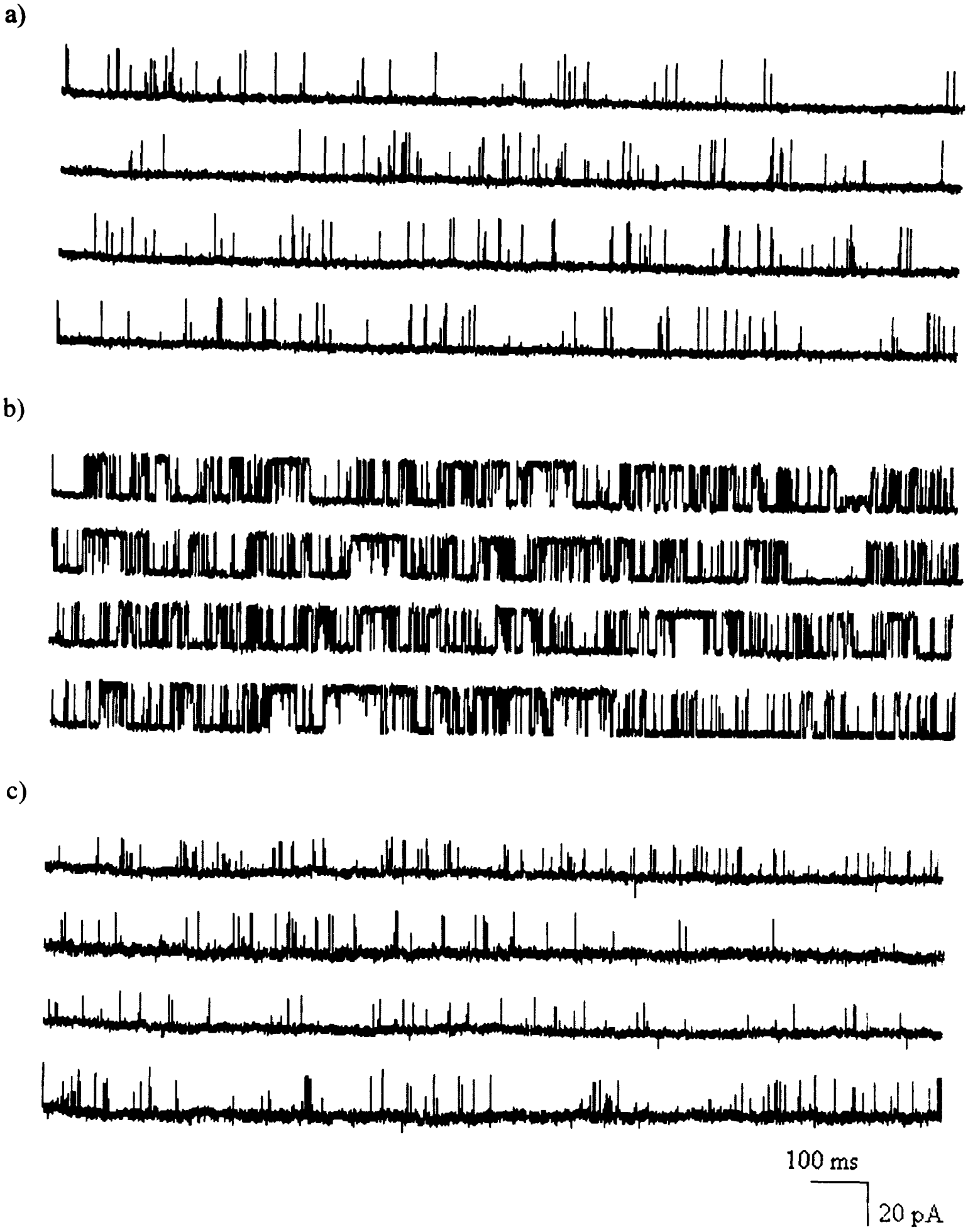
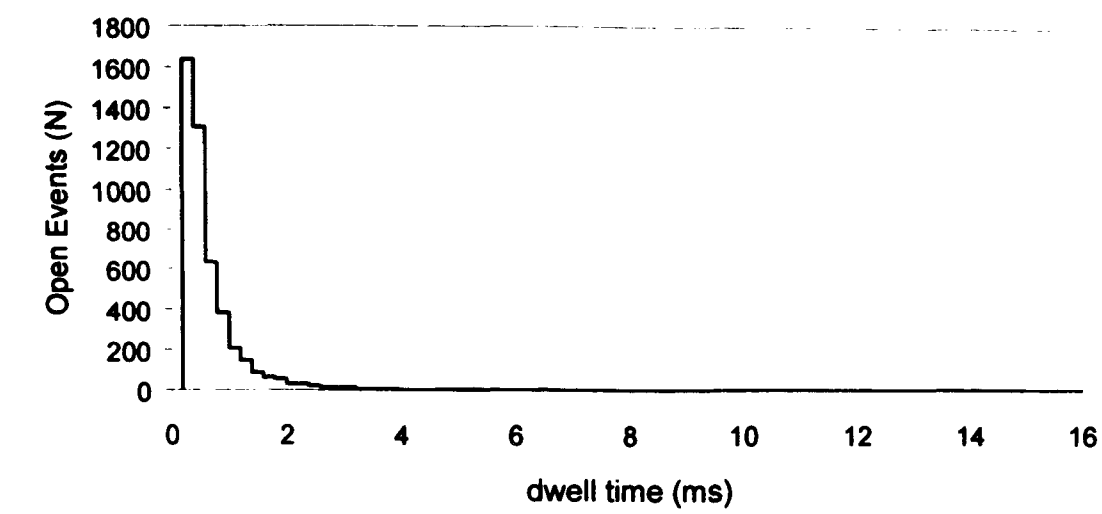


Figure 7. Typical current traces of single Ca^{2+} channel activity in POPE/POPC bilayers for X_{PE} values of a) 0.41 b) 0.81 c) 1.00.

a)



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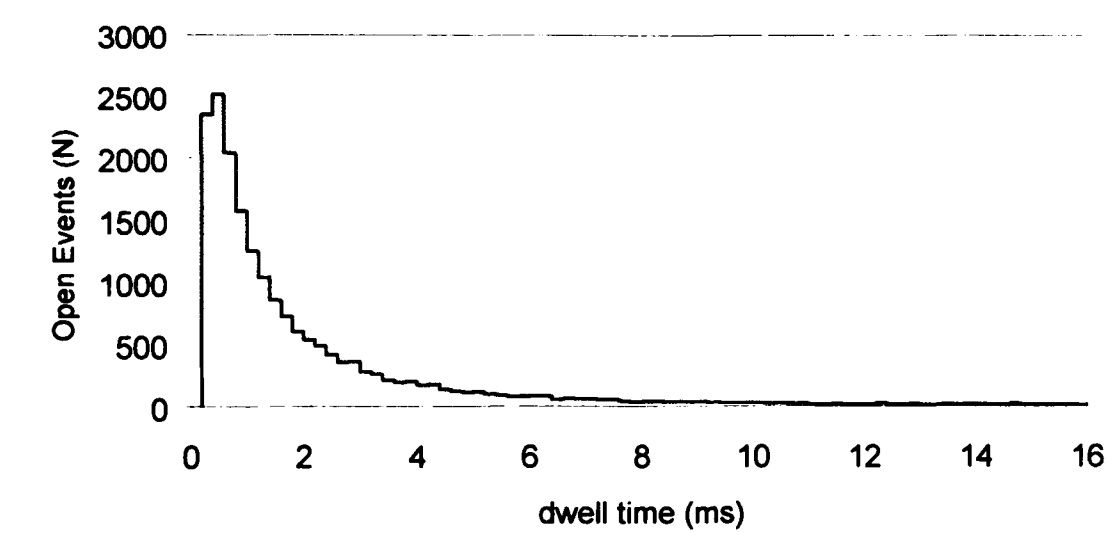


c)

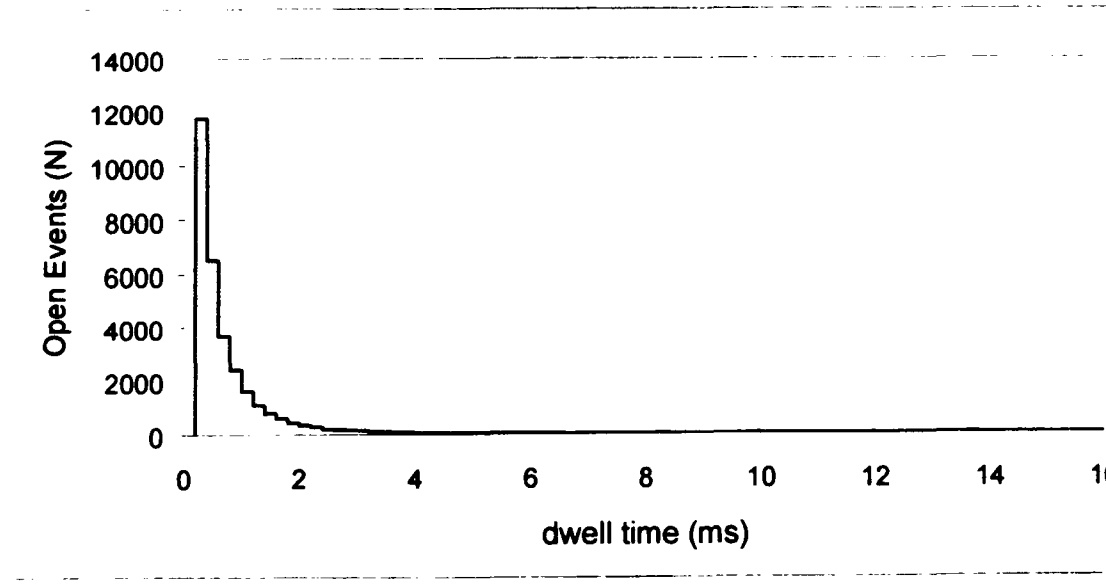


Figure 8. Open time histograms in bilayers for X_{PE} values of a) 0.41 b) 0.81 and c) 1.00.

numerous, but the ability of the channel to have longer open states can be seen at X_{PE} = 0.81 with the decay of the number of states at each time being much slower. As could be observed from the traces, the channel behaves similarly in bilayers with X_{PE} = 0.41 and 1.00 with very few states of open time beyond 3.00ms. There was no change in the current amplitude for the channel entering the open state in any of the different lipid bilayer compositions indicating that there were no changes in the structure function aspect of the Ca^{2+} channels.

The changes in the open probability (P_o) of the channel over the increaing PE molar fraction show the existence of a distinct region where there is a significantly higher probability that the channel will be in the open state (Figure 9). The open probability in the regions with $0.30 < X_{PE} < 0.75$ and $0.82<X_{PE}<0.90$ is consistently between 0.03 and 0.08. The open probability is highest in two areas: i) X_{PE} = 0.79 - 0.81 and P_o = 0.12 to 0.18 with the peak value P_o = 0.18 at X_{PE} = 0.81 and ii) X_{PE} = 1.00 and

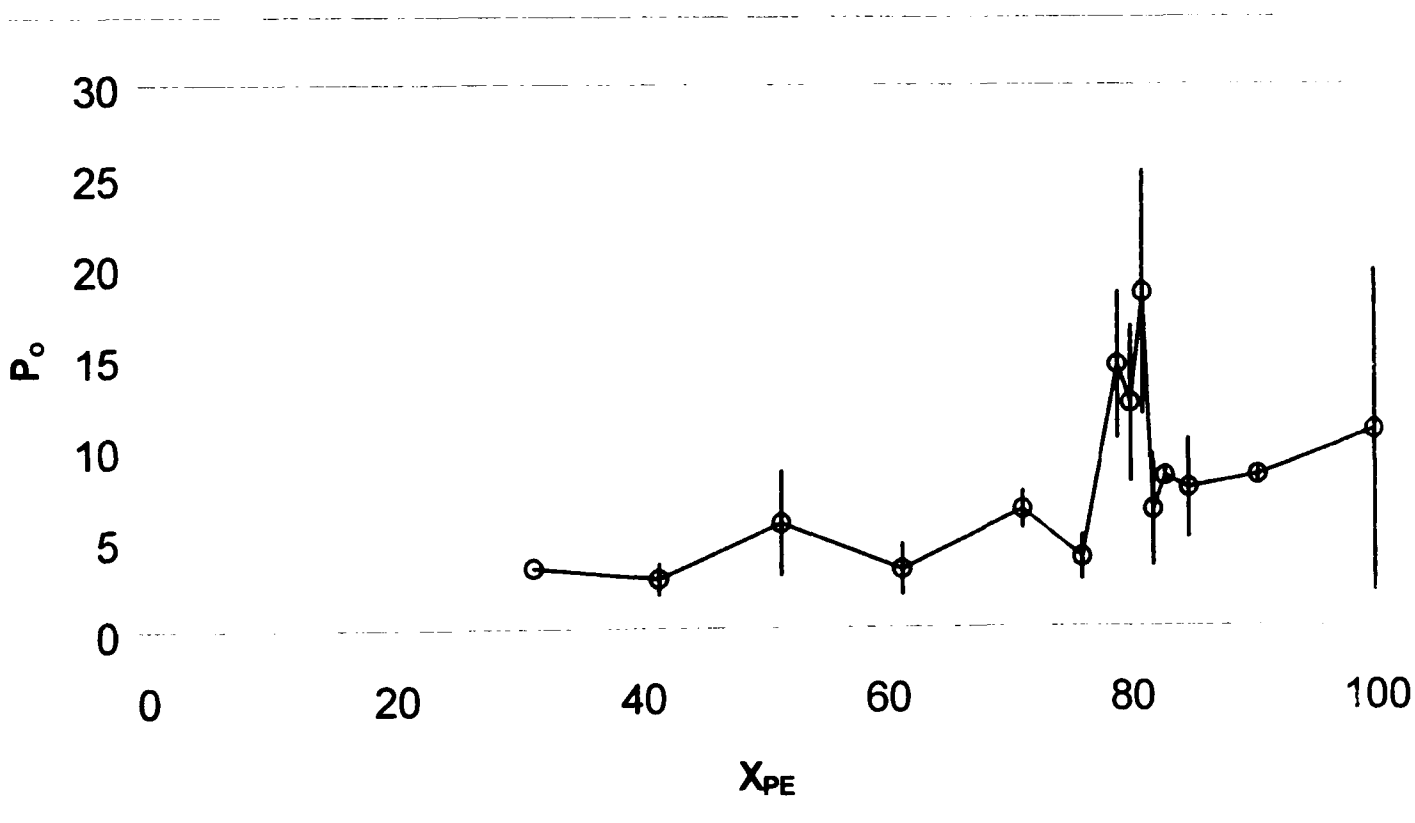


Figure 9. Open probability of Ca2+ channel in bilayers of increasing PE concentration.

P_o = 0.11. An interesting feature is that the open probability seems to increase monotonically with the increasing X_{PE}, excluding the narrow region of high activity.

The mean channel open time (t_o) reveals a pattern that is similar to the open probability with the occurrence of a region where the open time per event is markedly longer (Figure 10). In the region from $0.30 < X_{PE} < 0.75$, the open time has the smallest duration with 0.56 ms < t_o< 0.88 ms. The longest open times occur in the nearly the same region as the highest P_o with X_{PE} = 0.78 to 0.83 and having the value range 1.03 ms < t_o< 3.70 ms and the largest value occurring at X_{PE} = 0.80 with t_o= 3.70 ms. In the region where PE comprised the highest percentage of the lipid bilayer with $0.85 < X_{PE} <$ 1.00, the mean channel open times returned to smaller durations with t_o between 0.70 and 1.07ms. There does not appear to be any general trend that characterizes the channel open time except the occurrence of the localized region of longer open times.

By fitting the open time histograms with the double exponential function eq 6,

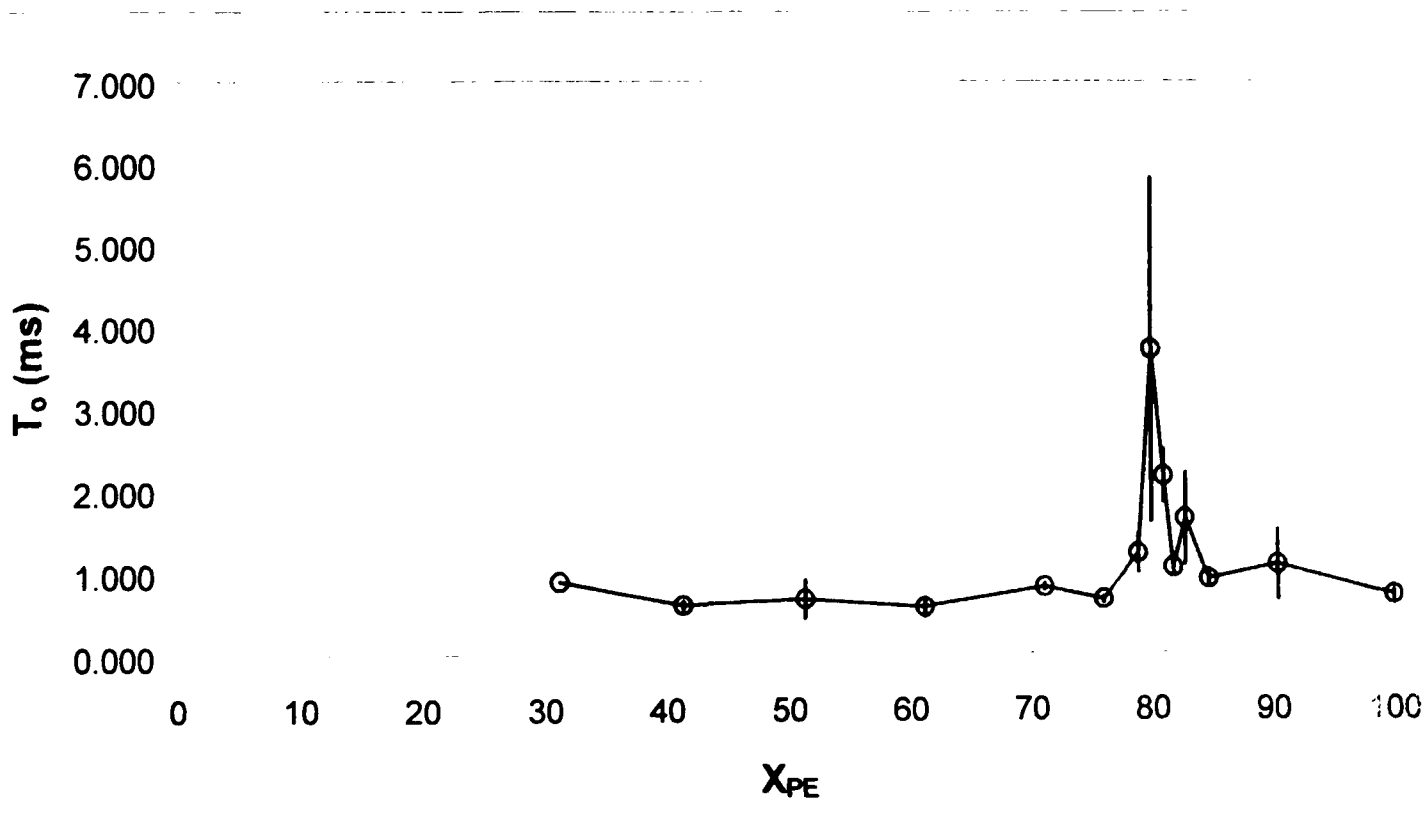
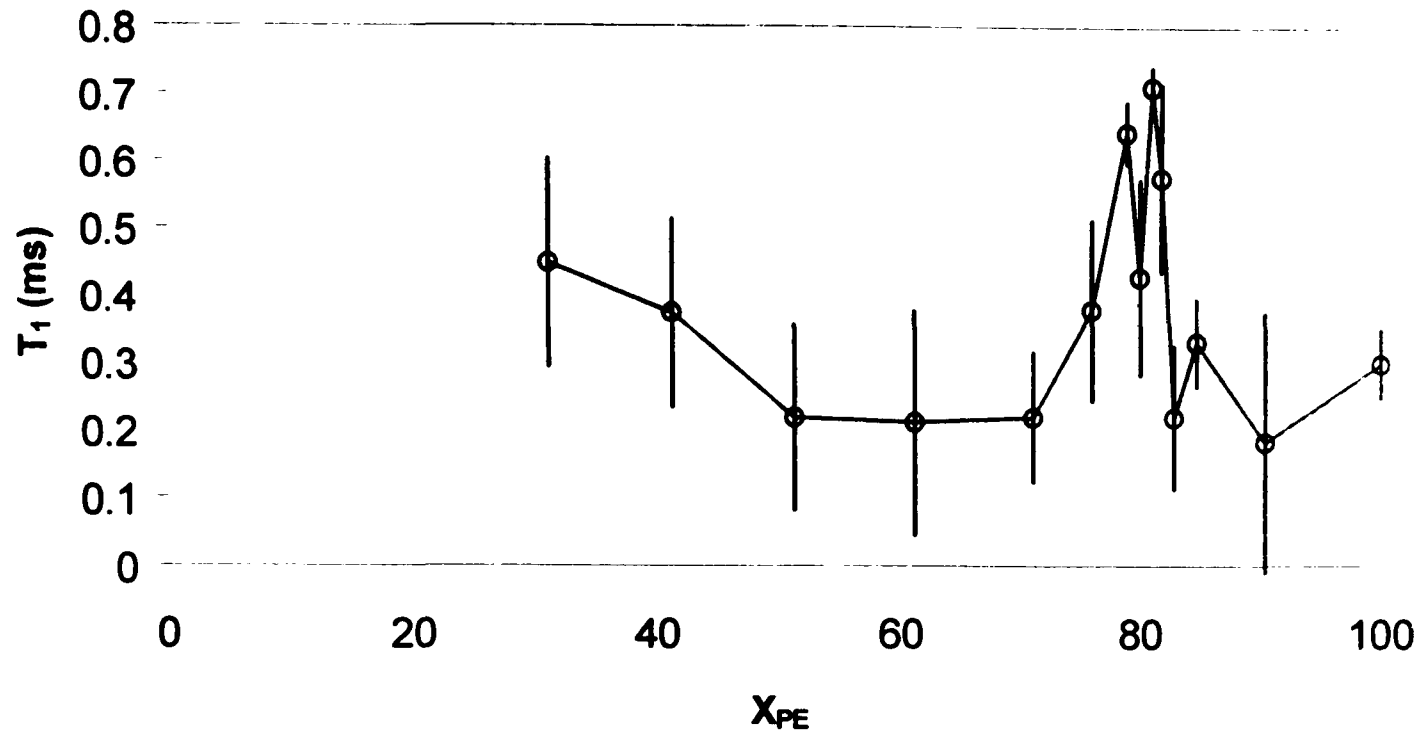


Figure 10. Mean channel open time of channels in bilayers of increasing X_{PE}.

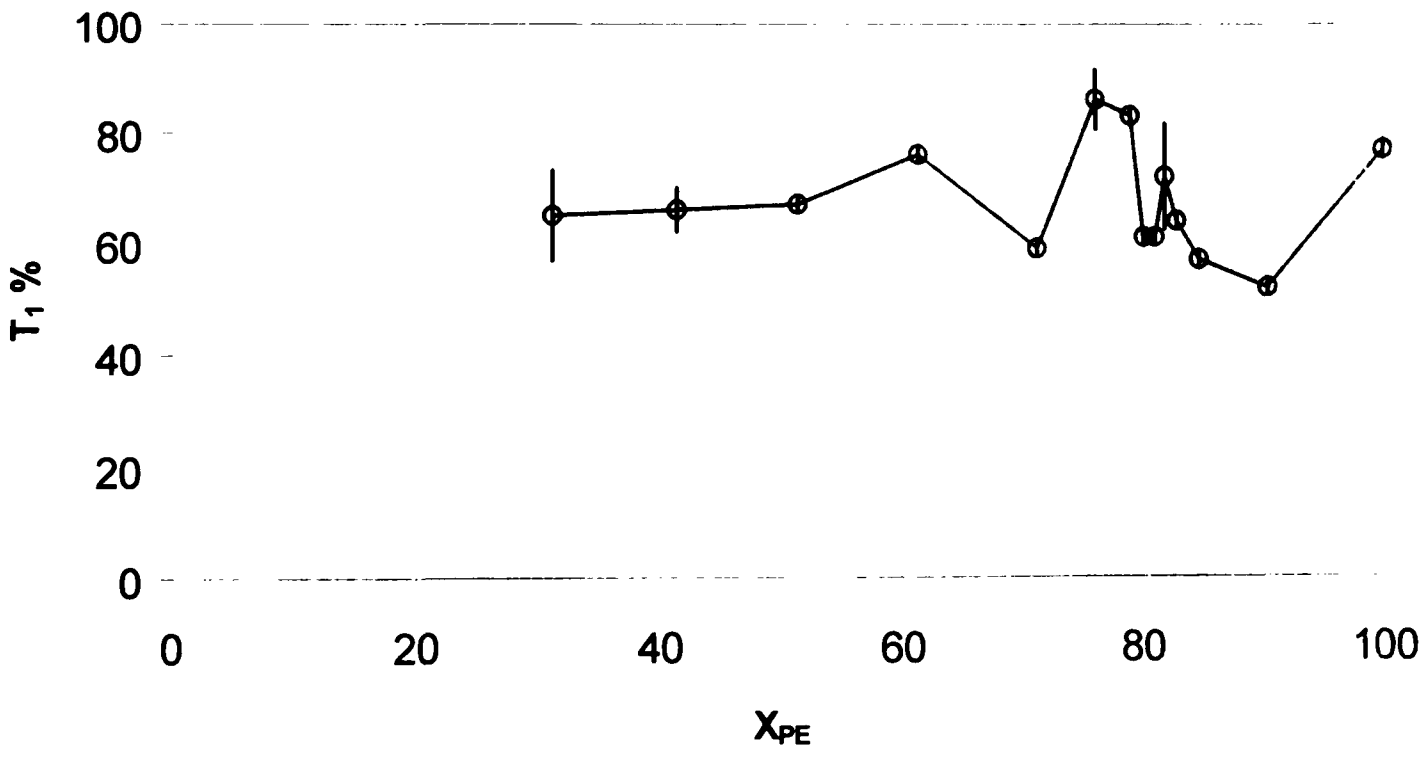
the channel activity can be further quantified by determining τ_1 and τ_2, which correspond to the lifetimes of the short and long openings and the percentage of number of open channel events in the short and long open time states. Figure 11 shows the changes in τ_1 as the molar fraction of PE increases in the lipid bilayer. The most significant changes in τ_1 occur at i) $X_{PE} < 0.60$ where the magnitude of the short event time increases linearly from 0.22ms to 0.45 ms and ii) $0.76 < X_{PE} < 0.82$ where τ_1 has its largest magnitude with short event duration up to 0.71ms. The shortest τ_1's were recorded for lipid bilayers that consisted of at least 50% PE excluding the optimum region $0.76 < X_{PE} < 0.82$. For all of the lipid bilayers, the percentage of the events that correspond to τ_1 is greater than 52%, so that the shorter lifetime is the predominant open state (Figure 11b). There is a higher percentage of the number of channel events with a shorter lifetime for the X_{PE}'s leading to and surpassing $X_{PE} = 0.80$ which has the largest mean open time and for the bilayer composed entirely of PE at $X_{PE} = 1.00$. These fluctuations may indicate some incomplete change occurring in the bilayer organization.

The long event open lifetime, τ_2, provides better insight into the open lifetimes of the channel and the affect of the lipid bilayer composition (Figure 12). For $X_{PE} = 0.80$, the τ_2 has its peak value of 3.60ms. The compositions $X_{PE} = 0.79$ to 0.82 also have larger long event times than those with greater or lesser PE molar fraction. As noted for the τ_1 percentages, the only regions which differ from a steady value are at $X_{PE} = 0.76$, 0.79 and 1.00. The ability of the channel to remains in its open state for longer lifetimes appears to be the dominant action in the increased mean channel open time since the peak times occur over a very narrow region of X_{PE}.

a)



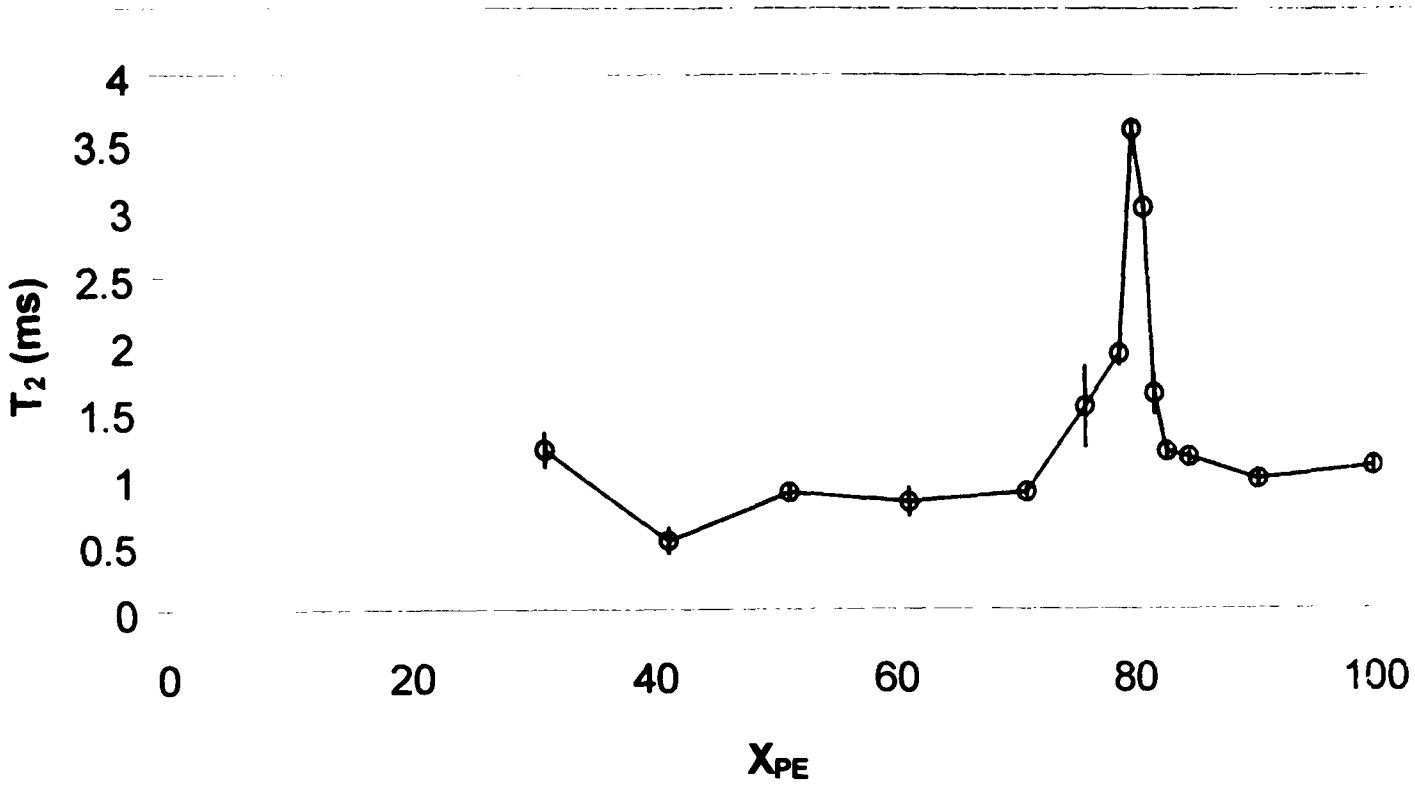
b)



c

Figure 11. Short lifetime event characteristics for different X_{PE} values show a) lifetime trend and b) changes in percentage of openings with short lifetime.

a)



b)

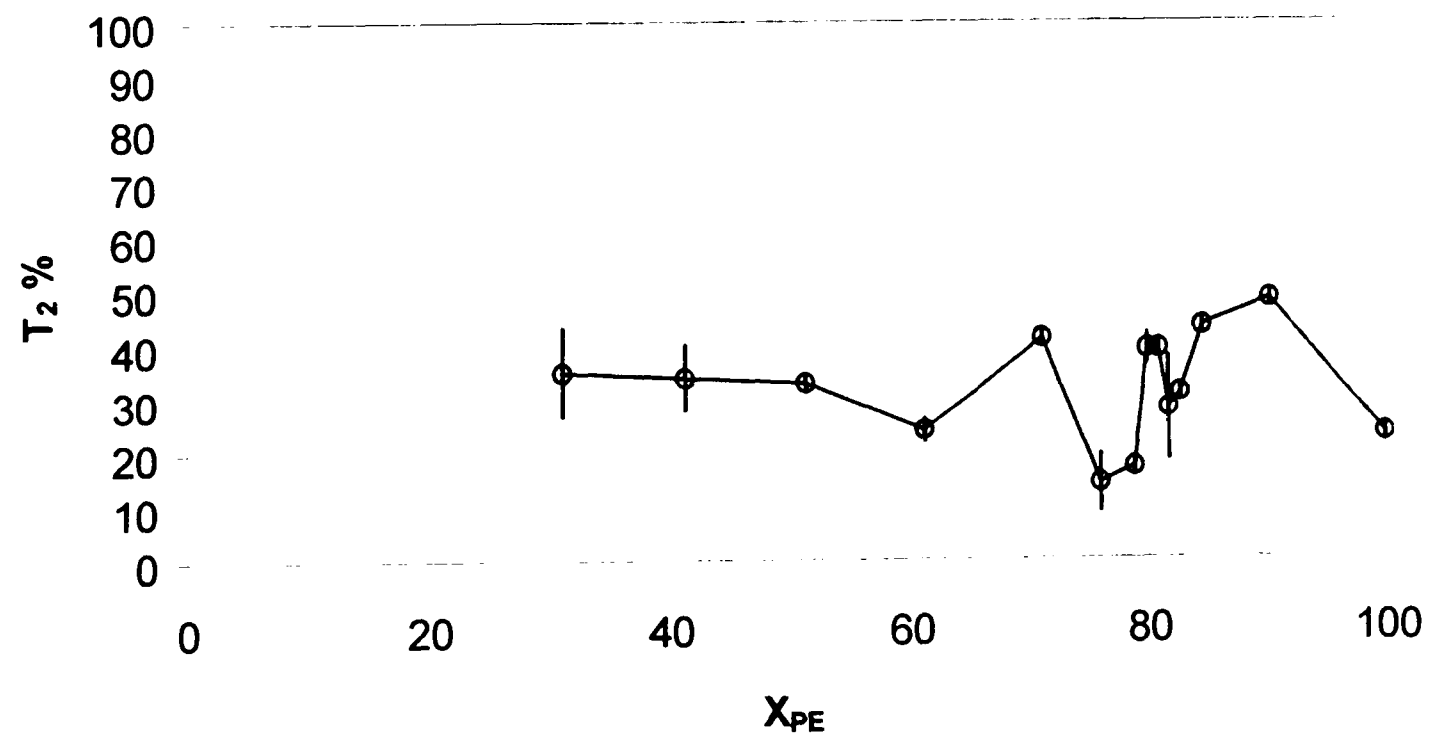


Figure 12. Long lifetime event characteristics for different X_{PE} values show a) lifetime trend and b) changes in percentage of openings with long lifetime.

CHAPTER VIII

DISCUSSION

To determine how lipid-lipid and lipid-protein interactions influence the activity of the Ca^{2+} channel, ryanodine receptor, the lipid bilayer membrane was constructed from a binary mixture in which the two lipids had the same acyl chains, but differing headgroup sizes, PE and PC. Previous work has shown that decreasing the headgroup size does change the channel activity and that greater percentages of PE enhance some characteristic of various membrane features such as enzyme activity, probability of higher conductance states, or Ca^{2+} uptake (Slater 1994, Keller 1993, Cheng 1985). The results of this experiment in terms of the open probability and open time show that there is an optimum lipid bilayer composition at X_{PE} = 0.78 to 0.82 such that the channel tends to be more active. The optimum region is very narrow and its occurence at a very high PE concentration means that the smaller headgroup size is more favorable to the channel. Also, the region appears to dependent on the presence of the larger PC headgroup and small changes in the fraction of PC in the total lipid content cause distinct differences in the channel activity.

Channel incorporation was facilitated with greater ease in this optimum range and the channel seemed to have greater stability. PE, a nonbilayer lipid, has the tendency to form the H_{II} phase as a monolayer because of the smaller headgroup size, so when forced into a planar lipid bilayer, there should be a large amount of negative curvature stress. The negative curvature of the PE bilayer may cause a headgroup spacing effect that could allow an increased level of interstitial hydrogen-bonded water.

It has been speculated that the degree of insertion of the hydrophobic region of intrinsic proteins would be aided by wider headgroup spacing (Slater 1993). During channel incorporation, this would seem to give PE bilayers a distinct advantage over PC bilayers, which have considerable crowding on the headgroup level. Such headgroup crowding should make protein insertion more difficult since it would require greater disruption of the lipid bilayer surface, explaining why channels were more easily incorporated into the higher X_{PE} bilayers. Neat POPC bilayers should have considerable packing strain on the headgroup level with its headgroup cross-sectional area (63 $\mathring{A}^2$) being larger than that for the two acyl chains (40 $\mathring{A}^2$) resulting in crowding. The insertion of the bilayer lipid, PC, with a larger headgroup, with zero or positive curvature may tend to alleviate the some of the stress across PE bilayers, creating a more stable planar bilayer.

Modulation of the protein function by membrane lipids may occur through changes in the hydrophobic coupling between transmembrane proteins and the lipids (Mouritsen 1984). Extending protein modulation to the bilayer curvature hypothesis according to Gruner, the amount of stress across the bilayer could influence the preferred state of the protein conformation, such as higher conductance states in alamethicin, a monomer, in bilayers with increasing curvature (Keller 1993). The Ca^{2+} channel seemed to favor a lipid bilayer membrane with a high level of curvature, but the results show clearly that curvature is not the only factor influencing the activity. Otherwise, the activity would have continued to increase monotonically to $X_{PE} = 1.00$. Although there does appear to be an overall trend of increasing open probability with

increasing X_{PE} , the occurrence of such a narrow, heightened activity region cannot be readily explained on curvature alone, though the possibility of there being an optimum amount cannot be discounted. There must be some lipid-lipid interactions on the headgroup level that affect the changes in protein conformation since the bilayer thickness does not change and the acyl chains are the same length so that hydrophobic mismatching should be negligible.

The structural stress hypothesis of Chang proposes that the increase in mean channel open time occurs through a decrease in the curvature stress and the lateral stress. The decrease in lateral stress by lowering of the compressibility is thought to be the most important feature, in that the increase in headgroup size of the lipid lowers K. From the results of this experiment, the inclusion of a larger headgroup is important for the channel to undergo an increase in open time, but there is no trend that indicates that increasing the amount of PC and thus lowering K, causes an increase in the open time. Although there did appear to be an increase in the length of the short lifetime with increasing PC content, measuring limitations and error make the observation questionable. As for the role of curvature stress, the protein open state prefers increased intrinsic curvature implying increased stress. A decrease in the compressibilty of the bilayer seems to be a favorable condition for the channel to open, but its occurrence may not reflect an overall increase in headgroup size, but through a tighter arrangement off the headgroups.

The rapid increase in the channel activity begins at $X_{PE} = 0.79$. The range of X_{PE} values at which the peak in activity occurs closely coincides with one of the critical concentrations for superlattice formation, $X_{PE} = 0.80$, predicted by the SL model. This

would indicate that the compositional conditions are present for the formation of a rectangularly arranged superlattice (Figure 13).

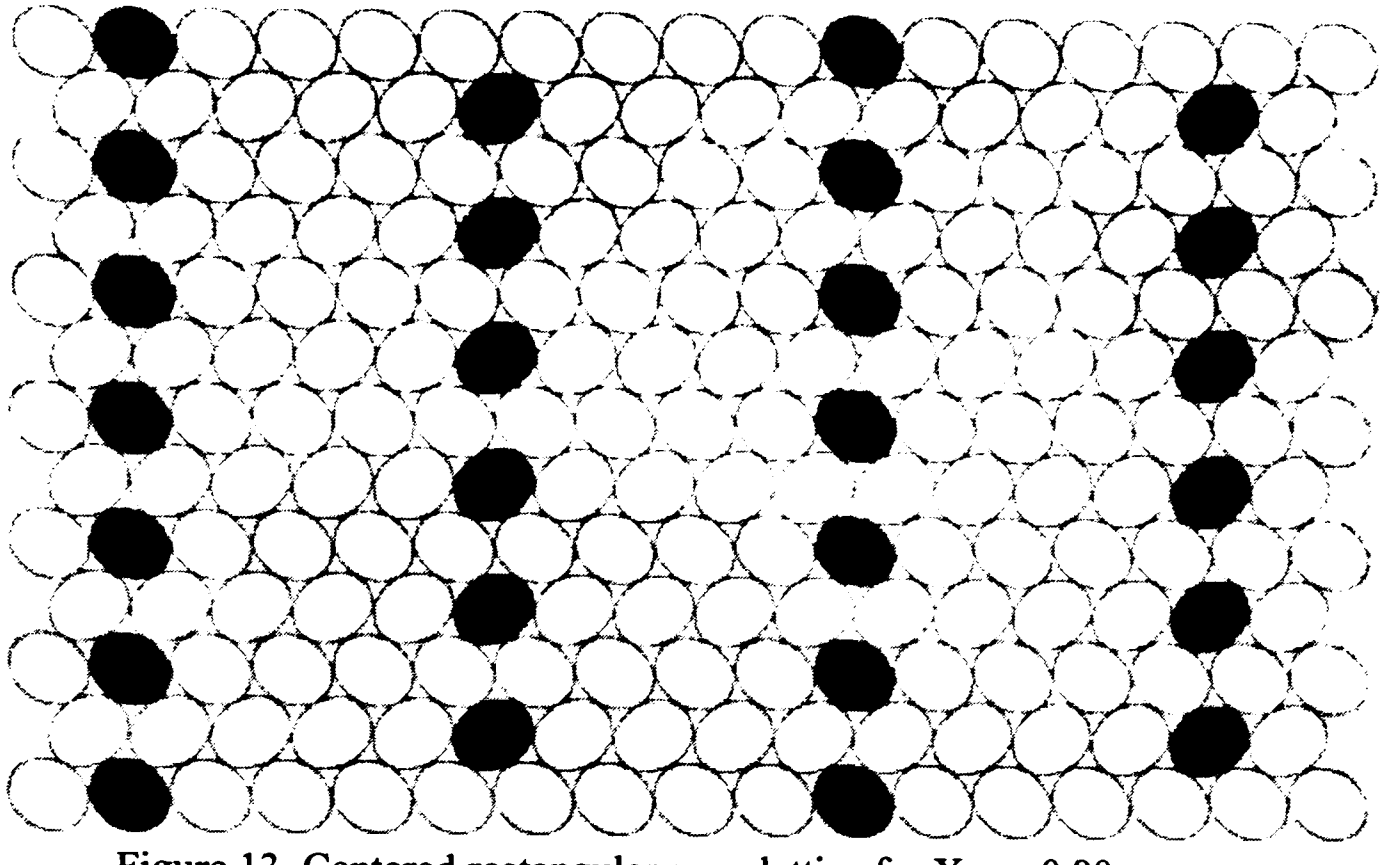


Figure 13. Centered rectangular superlattice for $X_{PE} = 0.80$.

According to the SL model, such an arrangement is defined by the following for $X_{PE} > 0.50$

$$X_{R,PE} = 1 - \frac{1}{ab + b^2} \qquad \text{where a = 4 and b = 1} \tag{7}$$

and the unit cell size by

$$P = 2ab + b^2 \tag{8}$$

The superlattices domains formed would be in dynamic equilibrium with randomly arranged domains and other superlattices. The superlattice, representing the most tightly packed and lowest energy configuration for the lipids, may be coupled to the Ca^{2+} channel (Virtanen 1999). If this were true, then changes in the conformation of the channel, as in the transitioning between the open and closed states, would depend on the

interactions between the superlattices and the channel with coupling occurring between the channel and the domains.

The question that remains is that how would the formation of superlattice domains around the channel lead to the optimum activity of the channel. For a reduction in the lateral stress in the bilayer to explain the higher probability and longer open time would require that over a very narrow range of the bilayer composition, there must be some event occurring that changes the organization of the bilayer. The tight packing that would occur through the superlattice formation could be the necessary event to significantly lower compressibility, which would tend the channel toward a longer open state. This event if it were superlattice driven would have increased lateral organization of the bilayer allowing the lipids to be in their most relaxed state with headgroup crowding and acyl chain packing strains markedly reduced at this critical composition. The superlattice explanation would seem to agree with the affect the membrane deformation has on the protein conformation in that the deformation energy should be at a minimum when the lipids become organized thereby making that bilayer state more favorable for the protein to enter its preferred conformation than a disorganized bilayer. This would lower the free energy difference between the protein states.

The presence of the PE lipids at the protein may promote channel activity since PE has been shown to destabilize lipid-protein contacts, increasing the rotational freedom of the protein (Scarlata 1997). The tilt of membrane-spanning helices could lead to the selective partitioning of lipids with a small head group, like PE, to the protein boundary (Virtanen 1998). The superlattice domains formed at $X_{PE} = 0.80$ would have PE lipids at the protein-lipid boundary. The ryanodine receptor is composed

of four identical subunits much like the K^+ channel. The each subunit of the K^+ channel is composed of 3 helices, an inner an outer, and part of the selectivity filter. The four outer helices of the K^+ channel are tilted by approximately 25° from the normal to the bilayer plane (Doyle 1998). For a transmembrane structure like an ion channel with four outer helices, this could lead to the formation of a tilt domains corresponding to each helix. There is the possibility that the helices could force the proximal acyl chains into a tilted orientation that could propagate over several orders of acyl chains from the helix boundary to form tilt domains. The tilt domains could possibly adopt a regular pattern of tiling, which could further encourage the ordering within the bilayer (Virtanen 1998). Since, the subunits that form the transmembrane assembly undergo an increased tilt with a 4° rotation when entering the open state, there must be some rearrangement that occurs on the hydrocarbon level to accomodate and promote such movement.

By discrete changes in the headgroup composition of the lipid bilayer, the ryanodine receptor was found to have optimum activity at a very narrow region of high concentrations of POPE in POPE/POPC bilayers. The occurrence of the narrow region coincides with a predicted critical concentration of the SL model for the formation of superlattices. The X_{PE} value indicates the exogenous content of the bilayer. Since the proteins do contain some endogenous lipids, locally, the exact content of the bilayer at the protein may not coincide with the prepared mixture, skewing slightly, the actual value the maximum may occur. The overall affect the lipid bilayer has on the protein behavior should remain unaltered. The results indicate that the ryanodine receptor is sensitive to its medium and that this sensitivity must have implications on its function in the cell membrane. The regulatory mechanisms that control the cell membrane content

must receive some signal from the proteins as was shown in this experiment that slight changes in the protein environment do result in significant changes in activity.

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