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Voltage-Dependent Orientation of Membrane Proteins

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In order to study the influence of electrostatic forces on the disposition of proteins in membranes, we have examined the interaction of a receptor protein and of a membrane-active peptide with black lipid membranes. In the first study we show that the hepatic asialoglycoprotein receptor can insert spontaneously into lipid bilayers from the aqueous medium. Under the influence of a trans-positive membrane potential, the receptor, a negatively charged protein, appears to change its disposition with respect to the membrane. In the second study we consider melittin, an amphipathic peptide containing a generally hydrophobic stretch of 19 amino acids followed by a cluster of four positively charged residues at the carboxy terminus. The hydrophobic region contains two positively charged residues. In response to trans-negative electrical potential, melittin appears to assume a transbilayer position.

These findings indicate that electrostatic forces can influence the disposition, and perhaps the orientation, of membrane proteins. Given the inside-negative potential of most or all cells, we would expect transmembrane proteins to have clusters of positively charged residues adjacent to the cytoplasmic ends of their hydrophobic transmembrane segments, and clusters of negatively charged residues just to the extracytoplasmic side. This expectation has been borne out by examination of the few transmembrane proteins for which there is sufficient information on both sequence and orientation. Surface and dipole potentials may similarly affect the orientation of membrane proteins.

Key words: melittin, membrane potential, asialoglycoprotein receptor, surface charge, dipole potential, charge clusters, phospholipid vesicles, black lipid membrane (BLM)

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Although insertion of proteins into membranes is probably determined by hydrophobic forces and by protein folding, studies in our laboratory suggest that electrostatic forces may influence the exact disposition of the inserted protein [1-3]. In order to study the influence of electrostatic forces on the disposition of proteins in the membrane we have used lipid model membranes. We examine the insertion of the protein or peptide into a preformed bilayer and study the forces (eg, the membrane potential) that affect changes in the protein and/or in the lipid. Those model systems focus attention on questions about the physical nature and energetics of protein insertion: What is the effect of protein folding upon its ability to insert into a membrane? What structural changes in the lipid bilayer accompany protein insertion? What are the effects of the membrane potential? What are the effects of surface charge on the lipid? What are the effects of charge clusters in the protein? How do leader sequences affect lipid bilayer structure, protein folding or translocation?

We have studied a protein, the asialoglycoprotein receptor, and a membrane-active peptide, melittin. Our studies with the asialoglycoprotein receptor were initially motivated by studies done in Aswell's laboratory [4]. Those studies indicated that in hepatocytes the ligand, serum asialoglycoprotein, is degraded in about 20 min, whereas the receptor is free to recycle. The protein has a half-life of 90 hr. Studies by Tanabe et al [5] indicated that the receptor escapes lysosomal degradation by changing its topology in the lysosomal membrane. Subsequent experiments, however, have shown that this receptor never reaches the lysosome [6]; its separation from ligand [7,7a] takes place in a compartment prior to lysosomal entry. The ligand is then targeted to the lysosome, whereas the receptor recycles back to the plasma membrane.

We showed evidence of topological changes of the purified receptor protein in a lipid bilayer, induced by voltage, ligand and millimolar Ca^{2+} [1]. Although the physiological significance of these observations is still unclear, they have directed our attention toward the possible role of the membrane potential in regulating the disposition and function of membrane proteins.

The asialoglycoprotein receptor is a complex molecule of unknown primary, secondary, and tertiary structure. In order to pursue our initial observations on voltage-dependent orientation, we switched to a molecule of known structure, the bee venom melittin [2]. Its primary, secondary, and tertiary structure were known, the crystal structure having been determined by Terwilliger et al [8].

Our findings on the role of the membrane potential in changing protein disposition in artificial membranes leads to specific predictions about the placement of charged amino acids in biosynthetically inserted proteins [3]. The analysis involves identification of charge clusters on either side of a hydrophobic membrane-spanning segment and the interaction of those charges with various components of the membrane potential.

MATERIALS AND METHODS

Black lipid membranes (BLM) were formed by spreading 2% oxidized cholesterol in decane (Lipopure, Applied Science Laboratories, Inc) across a circular hole (1-mm diameter) in a Teflon cup, according to the procedure of Mueller et al [9]. Each chamber contained 3 ml of an aqueous solution of KCl(6mM), NaCl (139 mM), and HEPES (10mM) at pH 7.4. Oxidized cholesterol was produced following the

procedure described by Tien et al [10]. Rabbit hepatic binding protein, isolated according to published procedure [5a], was kindly provided by K. Bridges. Melittin (Sigma) solutions (10 μg/ml) in HEPES buffer at pH 7.4 were always prepared fresh. Measurements were performed in a voltage clamp BLM apparatus. Voltage was applied from a DC battery switched at arbitrary times by hand. Current was passed through the BLM by means of Ag/AgCl electrodes and fed into a current transducer that converted membrane current into a voltage signal. The quality of the membranes was tested by conductance and capacitance measurements. Normal values were in the range 10 nSeimens (nS = 1/ohms)/cm^2 and 0.4 μF/cm^2, respectively.

RESULTS

The purified asialoglycoprotein receptor is homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [5a] (with two bands of molecular weights 40,000 and 48,000). After precipitation in ethanol it can be solubilized in aqueous solution (almost) free of detergent. In this form the receptor does not bind ligand (asialo-orosomucoid). After adding back detergent, the ligand-binding capacity is restored. In this sense the asialoglycoprotein receptor is similar in behaviour to the phosphomannose receptor.We can reconstitute the asialoglycoprotein receptor by mixing it in aqueous solution with phospholidylcholine vesicles [11]. The spontaneous reconstitution restores ligand-binding activity, and it is stable in high salt and ethylenediamine tetracetic acid (EDTA). Membrane proteins are usually reconstituted by detergent removal [13]. However, there are some proteins (eg, cytochrome b5 [12]), which can be reconstituted in phospholipid vesicles by spontaneous association as can the asialoglycoprotein receptor.

The reconstitution reveals some interesting features about the interaction between the asialoglycoprotein receptor and lipids: (1) circular dichoism shows a change in secondary structure of the receptor in the bilayer; the amount of β-structure is enhanced. This would appear to violate the principle that in order to intercalate in a membrane, a protein should assume an α-helical structure to satisfy the internal hydrogen bonding requirement of the polypeptide backbone [27]. However, we do not know whether the enhanced β-structure is in the aqueous or membrane-intercalated domain of the protein. (2)Fluorescence quenching indicates reduced accessibility of the receptor's tryptophans to aqueous collisional quenchers upon interaction with the bilayer. Moreover, ligand binding decreases this accessibility further, indicating that the ligand induces a change in the disposition of the receptor in the bilayer.

Given that we could reconstitute the receptor with vesicles in such a simple fashion, we examined its effect on black lipid membranes (BLM). BLM can be formed by painting a solution of lipid in organic solvent across a circular hole in a Teflon septum. Electron microscopy, capacitance measurements, and water permeability measurements suggest that they are bilayers. The conductance is very low, typically 10–100 pS. Small changes in conductance, induced by carriers, channels, or agents which perturb lipid bilayer structure [13], can readily be measured. The particular advantages of the BLM are that the voltage can be manipulated and fixed; therefore voltage-dependent changes in the protein's disposition can easily be detected. Moreover, both sides of the membrane are accessible to reagents, and effects on the trans side can readily be assessed when reagent is added to the cis side.

When we add the purified asialoglycoprotein receptor to one side (cis) of the BLM, we observe a voltage-dependent conductance increase [1] (Fig. 1A). When the membrane potential is trans-negative, the conductance is equal to that of an unmodified membrane. With a trans-positive potential, however, there is an increase in conductance. The direction of the voltage dependence makes sense; the receptor is negatively charged at neutral pH and it appears to be "electrophoresed" to the electrically positive pole. The irregularity in conductance stepsize indicates that the receptor is perturbating lipid bilayer structure [13], rather than forming channels. The time course (not shown) of conductance change is asymmetrical; there is a slow increase as the protein penetrates the membrane, and a fast decrease with the trans-positive voltage. The rate of increase rises sharply above about 20–30 mV. The voltage-dependent penetration of BLM is matched by an apparently similar phenomenon in vesicles. With an inside-positive potential across the vesicle membrane (imposed by high K^+ inside and valinomycin) we observe a decrease in tryptophan quenching by the aqueous collisional quencher acrylamide. This observation is consistent with deeper penetration by the protein in response to an inside-positive potential, causing a burial of the tryptophan groups in the bilayer.

The asymmetrical pattern of conductance increase seen in Figure 1A can be changed by addition of ligand (Fig. 1B): When the receptor and ligand, asialo-orosomucoid (ASOR), are both added to the cis side we observe a symmetrical conductance (Fig. 1B). ASOR alone has no effect, and neither does the nonbinding ligand analogue agalacto-orosomucoid in the presence of the receptor. High Ca^{2+} (Fig. 1C) mimicks the symmetrization effect of ASOR, albeit with a slower rate of conductance increase.

It appears that the symmetrization might represent translocation of the receptor molecule toward the trans side of the membrane, whence it could return towards the cis side upon reversal of the applied potential. This interpretation is consistent with our observation of a symmetrical conductance when the receptor protein is added to both sides of the BLM. We find that conductance, induced by adding receptor and ligand to the cis side of a BLM, becomes sensitive to pronase added to the trans side (not shown). (We will show a similar experiment when we discuss melittin-induced conductance.) Moreover, ligand, added to the trans side, enhances conductance of a Ca^{2+}-symmetrized membrane (not shown). In summary, receptor-bilayer interaction-involves the following steps:

(1) Spontaneous hydrophobic association of the asialoglycoprotein receptor with the bilayer, independent of the membrane potential. We demonstrated this with lipid vesicles.

(2) Reversible promotion of the protein into a conducting state under the influence of a trans-positive field. This involves perturbation of the bilayer to permit passage of ions.

(3) Ligand confers pronase-sensitivity and high Ca^{2+} confers ligand sensitivity on the trans side to the receptor protein.

We feel that movement of the asialoglycoprotein receptor into the bilayer involves aggregation (protein-protein interaction), since we can not rationalize partitioning of the charged and hydrophilic moieties through the bilayer. Charge delocalization and neutralization, by apposition of charged residues, could lower the barrier.

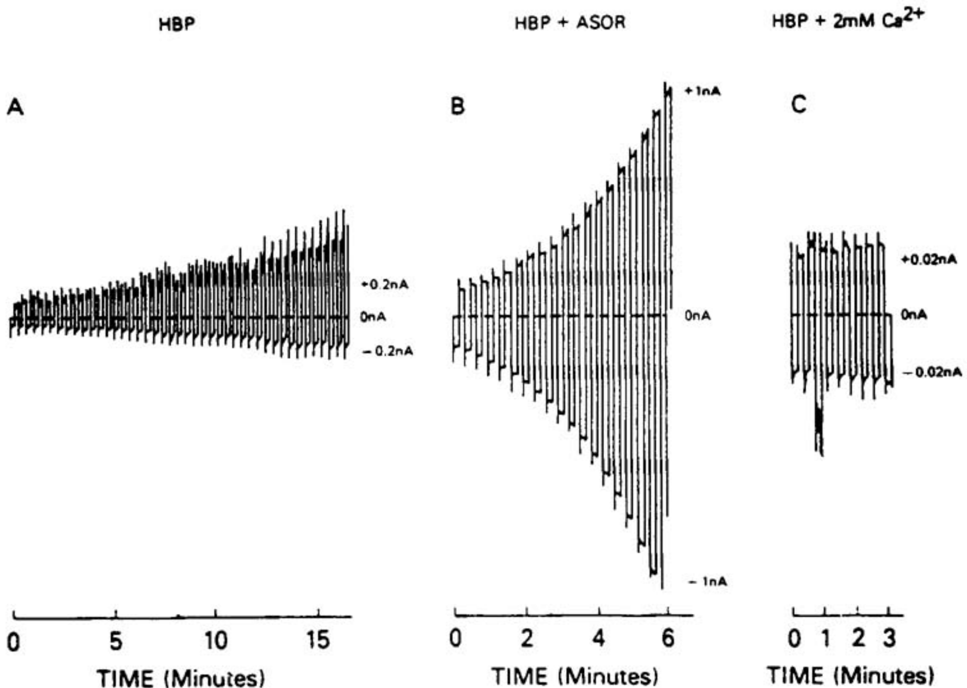

Fig. 1. Conductance change in BLMs induced by the asialoglycoprotein receptor. The BLM was formed from a solution of oxidized cholesterol (2% in n-decane) as described in Materials and Methods. Both compartments contained 13g mM NaCl, 6 mM KCl, and 10 mM Hepes at pH = 7.4. The asialoglycoprotein receptor was added to one compartment (cis) at a final concentration of 10 μg/ml. A. Receptor alone (10 μg/ml). B. In the presence of specific ligand asialo-orosomucoid (10 μg/ml in the cis compartment. C. With Ca^{2+} (2mM) in both compartments. Membrane potential is defined as $\psi_{trans}-\psi_{cis}$.

Melittin

We wished to relate our findings on voltage-dependent translocation to specific charge residues and other structural features of the receptor. However, neither the amino acid sequence nor any higher-order structure of the receptor protein is known. We therefore turned to a simpler molecule. Melittin, a component of the bee venom, is a 26-amino acid peptide known to interact strongly with lipid bilayers [14–17]. Its sequence contains a largely hydrophobic stretch of 19 amino acids, followed by a cluster of four positively charged residues at the COOH terminus [18]. According to one model for melittin's disposition in the membrane, its chain doubles over to form a hydrophobic "wedge" penetrating the membrane [15]. When we add melittin (to a final concentration of 17 ng/ml) to the aqueous solution on one side of the BLM, a voltage-dependent conductance appears (Fig. 2). There is a steep increase in conductance with trans-negative potential; a trans-positive potential produces little change. This asymmetry of the conductance with respect to applied voltage reflects the

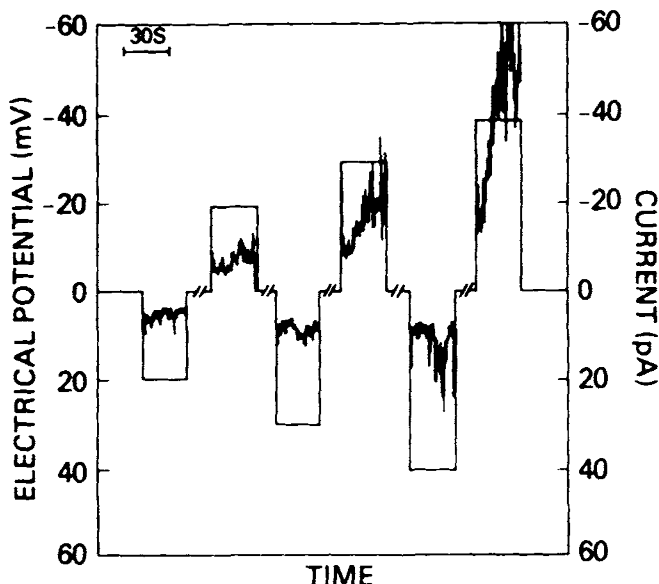

Fig. 2. Melittin-induced conductance changes. The BLM was formed as in Figure 1. Melittin was added to one compartment (cis) at a final concentration of 17 ng/ml. The "shaggy" lines represent current; the square pulses, voltage.

asymmetric association of the peptide with the bilayer; this is demonstrated by adding melittin to both sides of the bilayer and finding symmetrical conductance. The distribution of conductance fluctuations indicates that the conductance is induced by lipid perturbation rather than by formation of pores [13]. In a different BLM system, discrete pores formed by melittin have been observed [19]. However, it is also known that melittin can dramatically affect lipid bilayer structure [14,17]. We chose oxidized cholesterol since this was a convenient and stable bilayer-forming solution. However, it was a serendipitous choice, since even the most purified melittin preparations have some phospholipase activity [17], and it cannot be ruled out that changes observed in phospholipid bilayers are due to this activity.

Figure 3a shows that melittin-induced conductance is abolished by adding pronase to the cis side. Likewise, adding pronase to the trans side of such a membrane in the presence of a trans-negative conductance results in the elimination of melittin-induced conductance (Fig. 3B). On the other hand, when pronase is added to the trans side in the absence of a membrane potential and subsequently removed, melittin-induced conductance is not abolished. These studies with pronase indicate movement of some portion of melittin to the trans side in response to a trans-negative potential; in the absence of a trans-negative voltage no proteolytic digestion of the peptide takes place.

The relocation of melittin established by a trans-negative voltage is reversible. The build-up of conductance during a trans-negative pulse is much lower than that for the decrease in conductance during a trans-positive pulse. This finding is consis-

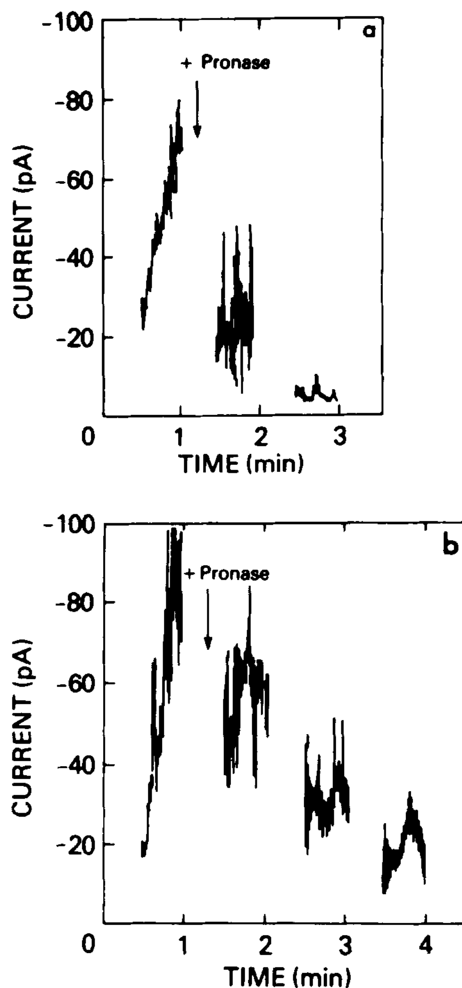

Fig. 3. Effect of pronase on current through a melittin-doped BLM. Conditions as in Figure 1a. A 80-mV trans-negative potential was applied. After the conductance increase was observed, 10 μg/ml of pronase were added to the cis (a) and trans (b) sides of the BLM. In the intervals between current traces, the membrane potential was switched to 0 mV, and the low level of current is not shown.

tent with the notion that the activation energy for moving (part of) the melittin into the bilayer is greater than that for moving the peptide out.

When we plot the logarithm of the rate of conductance increase as a function of voltage, we obtain a slope consistent with movement of two charages through the bilayer. This could either be due to a single peptide with two charges forming the conducting unit or with two peptide molecules with one charge each. Movement of lysine 7 and N-terminal glycine could account for this observation. We have recently studied a melittin derivative whose N-terminal glycine is acetylated. This molecule yields a voltage-dependent conductance with a slope of 1 — consistent with movement of one charge — perhaps lysine 7 [Blumenthal, Maulet and Kempf, unpublished

observations]. This result also indicates that the monomer is the predominant conducting unit in our system. Studies by Hider et al [20] on melittin-induced lysis of red cells also indicate that the monomer is the lytic unit. It is still unclear what structural features determine the conducting unit, and we are continuing to examine this question.

Membrane Proteins

The possible role of the membrane potential in protein orientation leads to specific predictions about charged amino acids in membrane proteins [3]: Assuming the cytoplasm to be electrically negative with respect to the extracellular space, we expect clusters of positive charge (lysine, arginine) to be located near the hydrophobic segment on its cytoplasmic end and negative charge (glutamic, aspartic) near the extracytoplasmic end. When we examined the few trans-membrane proteins for which the requisite information on trans-membrane disposition and amino acid sequence was available, the prediction was largely borne out (see Fig. 4). Glycophorin has a cluster of six negative residues (with one positive) on the electrically positive outer side and five positive residues (with one negative) on the cytoplasmic side [21]. When assembled into the membrane of Escherichia coli, the M13 coat protein has five negative and two positive charges external to the bilayer, with four positive and one negative on the cytoplasmic side [22]. The heavy chain of mouse surface immunoglobulin μ

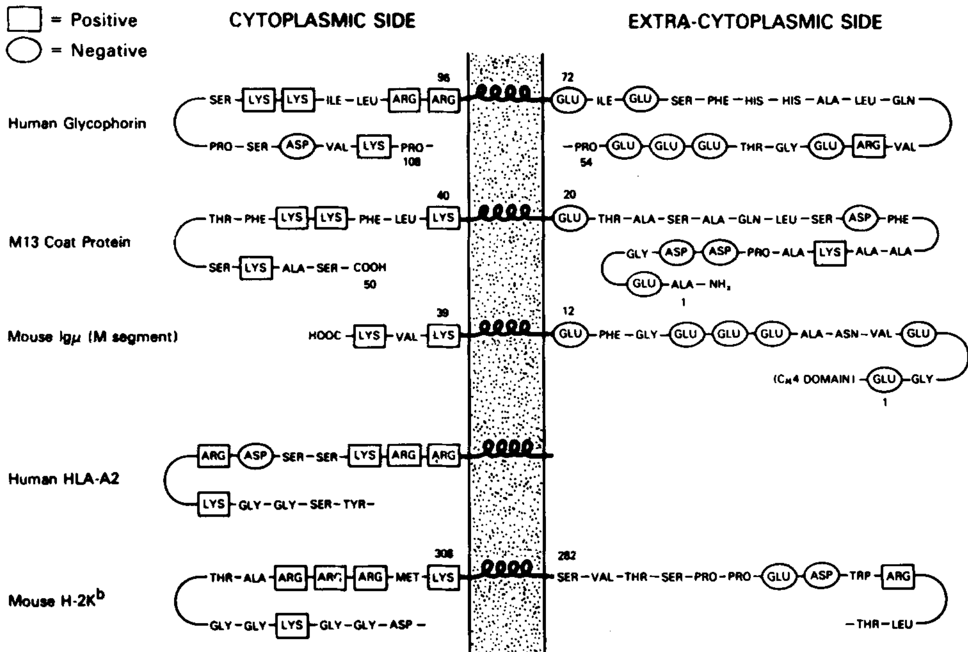

Fig. 4. Clusters of charged residues in transmembrane proteins. Each protein has a predominantly positive cluster on the cytoplasmic side and a predominantly negative cluster on the extracytoplasmic side.

has six negative charges on the extra cytoplasmic side and two positive plus the C-terminus on the extracytoplasmic side [23]. The human histocompatibility antigens HLA-A2 and HLA-B7 (the latter not shown) each have the predicted positive clusters on the cytoplasmic side [24]. H-2K^b antigen has the positive charge cluster on the cytoplasmic side, but only two negative with one positive on the opposite side [25].

Our predictions seem only to hold for those simple membrane proteins that loop just once through the membrane. The peptide chain of bacteriorhodopsin loops seven times through the membrane and no consistent pattern of charge asymmetry is evident in the structural model proposed by Engelman et al [26]. Transport proteins would not be expected necessarily to follow the pattern insofar as they had hydrophilic interiors, or developed such regions upon aggregation to form functional units.

In our analysis of the influence of electrostatic forces on correct protein orientation we are not considering the effect of hydrophobic forces. Rather we reason that if a protein must be oriented in one way or another in the membrane, it might more easily locate itself with a positive cluster on the cytoplasmic surface and a negative cluster on the extracytoplasmic side. We consider a single extrusion through the membrane and examine charge clusters next to the hydrophobic regions. The basis of our analysis is thermodynamic, although kinetic considerations might also come into effect. We assume that the orientation in the endoplasmic reticulum determines eventual orientation in the plasma membrane: hydrophilic segments in the cisternae are destined for the extracellular space, and cytoplasmic segments remain cytoplasmic.

To insert a protein into a membrane an energy barrier must be overcome. The free energy difference between an uninserted and inserted protein is

$$\Delta G = \Delta G_h + zFV. \qquad (1)$$

ΔG_h is the free energy due to hydrophobic forces, calculated by Engelman and Steitz to be about 40 kcal/mol for a helical hairpin [27]. The second term on the right hand side of Equation 1 is the electrostatic contribution; V, the membrane potential; F, the Faraday constant; and z, the number of charges in the cluster. For 60 mV and one charge zFV equals 2.4 kcal/mol, not large compared with the hydrophobic contribution to the free energy. If, however, we consider the free energy difference between a correctly and incorrectly inserted protein we get

$$\delta\Delta G = 2zFV. \qquad (2)$$

We have calculated the probability of incorrect transmembrane orientation: For instance, a protein with clusters of three positive charges on the cytoplasmic side and three negative charges on the extracytoplasmic side would have only a 0.17% chance of incorrect orientation at equilibrium for a membrane potential of −60 mV (negative inside). The bias for correct orientation according to this analysis is quite compelling.

Components of the Membrane Potential

The potential measured by electrodes is the macroscopic transmembrane potential (Fig. 5a). In biological membranes V is usually associated with diffusion potentials set up by the presence of selective channels or electrogenic pumps. In membranes with surface charge there is also a surface potential. The surface charge on all natural

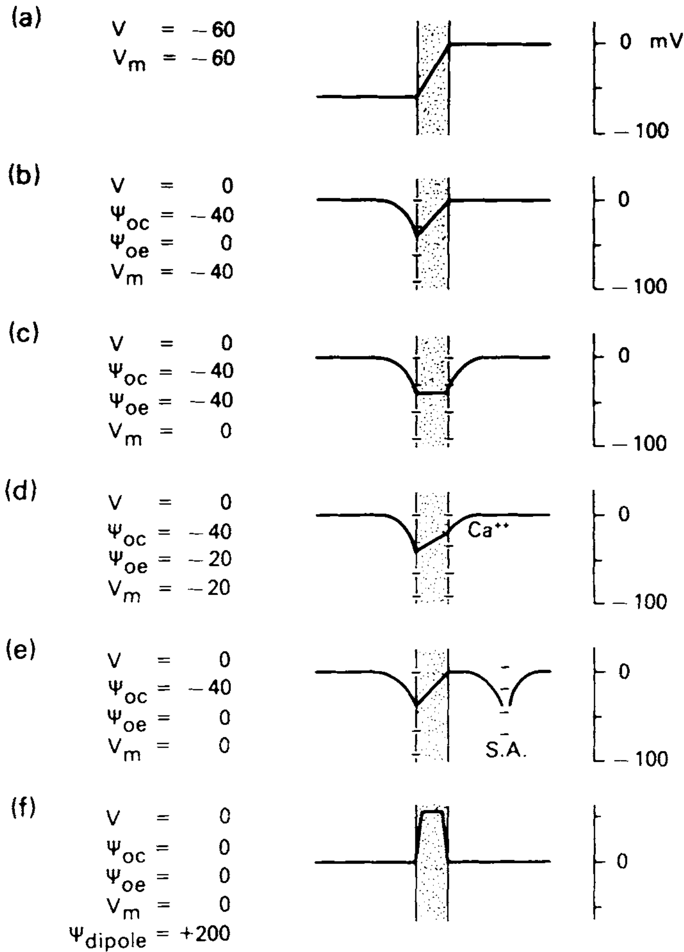

Fig. 5. Components of the membrane potential. V = macroscopic potential, measured by electrodes; ψ_{oc} and ψ_{oe} are surface potentials on the cytoplasmic and extracytoplasmic surfaces respectively; $V_m = \Psi_{oc} - \Psi_{oe}$; Ψ_{dipole} is the dipole potential (see text); S.A. is sialic acid.

membranes as far as we know is negative, due to phospholipid headgroups such as phosphatidylserine, phosphatidic acid, phosphatidylglycerol, and phosphatidylinositol. Free cations from the medium accumulate in the interfacial region and partially "screen" the charges. The distribution of these cations can be described by the classical Gouy-Chapman formulation for the diffuse double layer [28]. A balance is struck between electrostatic attraction of the cations to the negative charges at the membrane surface and the entropic tendency of the cations to spread uniformly throughout the medium. At physiological ionic strength, surface potential falls off nearly exponentially from the membrane surface with a characteristic distance of about 9 Å. It is negligible beyond about 30 Å from the membrane and therefore cannot be detected by electrodes. However, the surface potential is reflected experimentally in the potential-dependence of membrane enzyme function [29], the gating

components of axons [30], and in the fluorescence signals of potential-sensitive dyes [31].

The surface potentials on biological membranes, which generally contain about 15% negatively charged phospholipids, have been estimated to range from −15 to approximately −60 mV [28]. To analyze the sensitivity of surface potential to various conditions, we considered a hypothetical membrane composed of phosphatidylserine (negative) and phosphatidylcholine. For a monovalent cation activity of 130 mM on both sides, we calculate 39 mV on each surface. As illustrated in Figure 5c, symmetrical surface potentials would not affect Vm. The surface potential is likely to be asymmetrical (Fig. 5b), because of the intrinsic transmembrane distribution of charged lipids [32]. However, even with symmetrical membranes, the surface potential is likely to be asymmetrical because of asymmetry in the ionic milieu. Ions decrease surface potential by electrostatic screening and also by nonelectrostatic binding (Fig. 5d). We have calculated the effects of this ion asymmetry on the surface potential [3]. The dominant cytoplasmic ions are Mg^{2+} and K^{+}, whereas the dominant extracytoplasmic ions are Ca^{2+} and Na^{+}. Since the binding affinity of Ca^{2+} for phosphatidylserine is higher than Mg^{2+} the effect on the extracytoplasmic side will be greater, as noted in Figure 5d. Negative charge on sialic acid (Fig. 5e) affects electrophoresis of cells, but it resides for the most part well outside of electrostatic interfacial region of the bilayer and thus is not expected to have a large effect.

Another factor facilitating translocation of negative charges is the dipole potential [33]. Zwitterionic phospholipid membranes are generally orders of magnitude more permeable to hydrophobic anions than to equivalent cations. This can be attributed to the dipole potentials (Fig. 5f), which arise either from the orientation of ester linkages or from the interaction of the headgroups with water. If the dipole potentials are symmetrical, they would not alter the thermodynamics of charge cluster orientation. However, in a kinetic sense, they could lower the potential energy barrier for translocation of negative charges across the membrane while increasing the barrier for movement of positively charged residues. This is precisely the bias required to achieve the orientation shown in Figure 4.

The pH might also play a role in protein insertion. Because H^{+} is concentrated in the hydrophilic region of the membrane (according to the Boltzmann distribution), the pH is calculated to be 0.66 units lower than in the bulk cytoplasm for a 39-mV surface potential. This lowering of the pH would facilitate entry of glutamic (pK 4.3) and aspartic (pK 3.8) residues into the membrane against the countervailing tendency of the negative surface charge to exclude anionic residues from the interfacial region. Lowering the pH on the extracytoplasmic surface would have a similar effect as high Ca^{2+} in that external negative surface charges would be titrated.

DISCUSSION

When we first observed voltage-dependent movement of the asialoglycoprotein receptor, we postulated that the membrane potential might play a role in regulation of the disposition and function of membrane proteins [1]. Although, as stated earlier, it seems that the asiologlycoprotein receptor does not cross the membrane in the physiological system, our data are consistent with a role for the transmembrane potential in regulating its disposition. The receptor is involved in endocytosis of

ligand, and therefore must contain in its structure a number of different functional sites, including a site for ligand binding, a site for targeting into a coated pit, and a site for movement back to the plasma membrane after separation from ligand in the endosome. By regulating the disposition of the protein in the membrane, the membrane potential could play a role in the regulation of those processes.

Our data on voltage-dependent orientation directed our thinking toward the role of the membrane potential for biosynthetic insertion of membrane proteins. At that time, Date et al [34] reported that proper insertion and processing of M13 coat protein required the presence of a membrane potential across the inner membrane of E coli. Subsequently, a number of reports appeared on the voltage-dependent secretion of proteins across the E coli membrane and inner mitochondrial membrane. To a first approximation the direction of secretion is consistent with overall charge distribution. E coli are positive outside and secreted proteins are generally negatively charged. Mitochondria are negative inside, and the imported proteins are generally positive.

However, there are a number of problems with voltage-dependent secretion which are not so easily overcome: (1) The "wrong" charge clusters have to be secreted, whereas they remain in place for membrane proteins. (2) The additional energy gained from the field is not large as compared with the activation energy of translocation of proteins across membranes [see Engelman, these proceedings]. With inserted proteins that energy is given and the field only provided a bias for correct orientation. (3) Once translocated, it is not clear how the secreted protein leaves the membrane, since the major portion of the field is across the membrane.

The notion that membrane potential can modulate the position and perhaps the orientation of molecules in membranes has been suggested repeatedly since Hodgkin and Huxley [35] analyzed membrane excitability as in terms of a voltage-dependent conductance charge. Since then a few antibiotic ionophores [36] (eg, alamethicin, monazomycin) and toxins [37,38] have been shown to exhibit voltage dependent conductances. Recently Fox and Richards [39] proposed a molecular model for gating of the alamethicin channel based on its 1.5 Å resolution crystal structure. Perhaps the data and concepts emerging from the studies on voltage-dependent channels will help in attempting to unravel the mechanism of voltage-dependent transport and secretion of proteins.

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