

# Phospholipid-Cholesterol Membrane Model

## *Control of resistance by ions or current flow*

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**ABSTRACT** A cephalin-cholesterol membrane model is described whose electrical resistance can be reversibly raised by  $\text{CaCl}_2$  or lowered by KCl or NaCl whether these ions are added to the membrane by mechanical immersion or are driven in electrically. Either KCl or NaCl acts antagonistically to  $\text{CaCl}_2$ . Experiments with controlled pH indicate that the above effects depend somehow on combination of the cations with the phospholipid acidic groups. Also, they are correlated with decreased membrane hydration in  $\text{CaCl}_2$  solutions, and increased hydration in KCl or NaCl solutions. It is conjectured that cells may regulate their transsurface ion pathways and fluxes by K-Ca competition for negatively charged binding sites on plasma membrane phospholipid. It is regarded as a corollary to say that a fundamental event in excitation is displacement of membrane Ca from such a site by catelectrotonically propelled K.

It has been suggested by one of us (1,2) that a fundamental event in excitation is displacement by potassium of calcium from binding to a negative site on a structural molecule in the cell surface, and that a good candidate for the negative site is the phosphate oxygen in the phospholipid of a phospholipoprotein complex in the cell membrane. We now supply evidence, in accord with this hypothesis, that the resistance of a cephalin-cholesterol membrane model can be controlled by the ambient Ca, K, or Na concentration, or by ionic current flow, both being suggestive of living cell responses.

### METHODS

A lipid barrier is made by dipping a Millipore filter disc (a cellulose nitrate-cellulose acetate material, Millipore Corporation, Bedford, Massachusetts, pore size  $100 \pm 20$  Å, 0.15 mm thick) in benzene containing lipid, allowing 2 minutes between dips and time at the end for evaporation of benzene. Usually 1.0 to 1.8 mg of lipid are deposited per sq cm, but the distribution is not accurately known. The barrier is then clamped between two 15 ml plastic vessels which contain experimental solutions and

electrodes for measuring resistance, capacity, and potential. Only resistance data are reported here.

The chemicals used were Eastman best grade cholesterol, Nutritional Biochemicals Corporation "animal cephalin" (phosphatidyl serine *ca.* 60 to 85 per cent, phosphatidyl ethanolamine *ca.* 15 per cent, traces of inositol and sphingomyelin), and Merck, terpene-free benzene. Reagent grade salt solutions were made in distilled water passed through an ion exchanger and redistilled from glass.

Resistance and capacity were measured at 5000 cps through two bright Pt electrodes, 1 cm square, connected to a standard Wheatstone bridge, using a tektronix type 502 oscilloscope as balance detector.

TABLE I  
RESISTANCE OF BARRIER AS A FUNCTION OF SALT IN THE MEDIUM

Material on Millipore		Ambient salt		Resistance*	
Name	mg cm <sup>-2</sup>	Name	Meq/liter	No. of different preparations	Ohm cm <sup>-2</sup>
Cephalin‡ alone	1.2	KCl	100	2	12
	0.9	NaCl	100	2	9
	1.1	CaCl <sub>2</sub>	100	3	853
Equimolar cephalin‡ and cholesterol	1.6	KCl	100	5	21
	1.4	NaCl	100	5	26
	1.5	CaCl <sub>2</sub>	100	7	1075
Equimolar cephalin‡ and cholesterol	1.7	MgCl <sub>2</sub>	100	1	425
	1.5	BaCl <sub>2</sub>	100	1	260
	—	AlCl <sub>3</sub>	100	2	1290

\* Resistance is the difference between the resistance of the cell with the barrier intact and with the barrier removed.

‡ Composition of cephalin is given under Methods.

Stirring did not significantly alter the resistance measurements. Absolute ohmic values are not taken very seriously. They were variable among experiments, probably due to differences among the barriers.

#### RESULTS

Unless specified, the same solution has been present on both sides of the barrier. Controls with Millipore alone show it to have none of the properties found after lipid impregnation.

##### *Resistance of the Barrier as a Function of Single Salts in the Medium*

Cholesterol alone on Millipore, whether in CaCl<sub>2</sub>, KCl, or NaCl, 100 meq per liter, has a very high resistance, over 100,000 ohms per sq cm, and is essentially non-wettable by the salt solutions. In contrast, however, if barriers of the animal cephalin alone are used the resistance ranges from a few ohms in KCl or NaCl to several hundred ohms in CaCl<sub>2</sub> (Table I).

If the barrier now is made of a mixture of equimolar cholesterol and phospholipid (cholesterol:phosphatidyl-serine:phosphatidyl-ethanolamine :: 1.0:0.86:0.16), then the resistances in the same salts resemble those obtained with phosphatidyl serine-phosphatidyl ethanolamine alone. The steady state resistance in  $\text{CaCl}_2$  is high and reached relatively slowly, whereas in  $\text{KCl}$  or  $\text{NaCl}$  it is low and reached almost immediately (Table I, Fig. 1). Also, after exposure to  $\text{CaCl}_2$ , the resistance in  $\text{KCl}$  or  $\text{NaCl}$  does not fall to the initial levels for those salts unless the barrier is washed once or twice, some of the Ca probably being held on it.

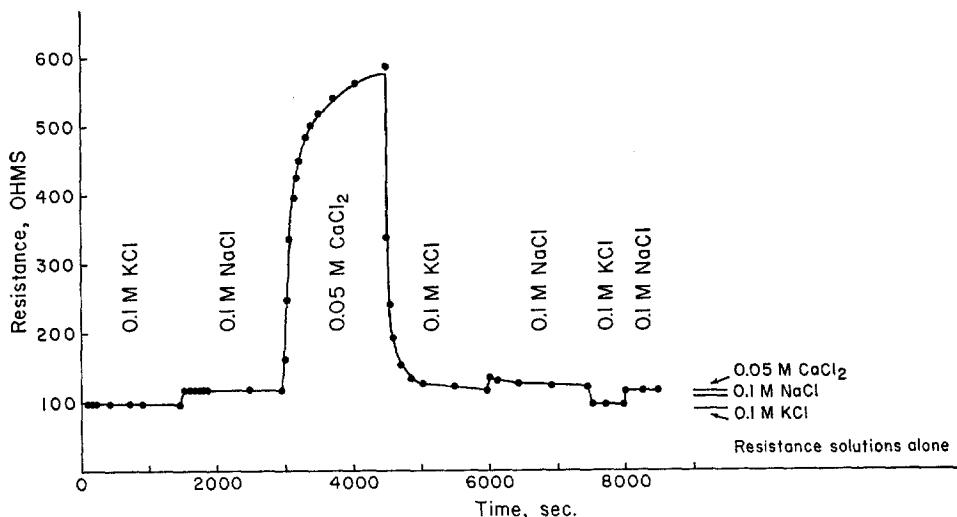


FIGURE 1. Resistance of barrier, ohms per square centimeter, in equiequivalent solutions of  $\text{KCl}$ ,  $\text{NaCl}$ , or  $\text{CaCl}_2$ . The lines on the right indicate resistance values for the solutions in the chamber without the barrier. When these latter are subtracted from the plotted values one obtains resistance of the barrier corrected for resistance of the medium, electrodes, etc. The cycles are repeatable.

Three comments are relevant: (a) The biologically interesting effects found so far seem to depend on the phospholipid. Nonetheless cholesterol has been left in to establish a baseline of data for such a mixture which is in the same phospholipid-cholesterol ratio as is found in cells (3). (b) It may be conjectured that a major role of phospholipid in the cell surface is to provide a path of finite and modifiable ion mobility. Protein incorporation has not yet been tried. (c) Magnesium chloride,  $\text{BaCl}_2$ , or  $\text{AlCl}_3$  behave more like  $\text{CaCl}_2$  than like  $\text{KCl}$  or  $\text{NaCl}$ .

#### *Resistance of the Barrier as a Function of Mixed Salts in the Medium*

Data for  $\text{KCl}$  and  $\text{CaCl}_2$  are shown in Fig. 2. Those for mixtures of  $\text{NaCl}$  and  $\text{CaCl}_2$  are similar and so are omitted. The following emerges: Adding  $\text{CaCl}_2$

(10 to 100 meq per liter) to KCl or NaCl of fixed concentration (100 meq per liter) lowers resistance of the solution as total salt concentration increases, but raises resistance of the barrier; *i.e.*, exposure of the barrier to CaCl<sub>2</sub> increases its resistance to all these ions. Contrariwise, adding KCl or NaCl (10 to 100 meq per liter) to CaCl<sub>2</sub> (100 meq per liter) lowers resistance of the barrier.

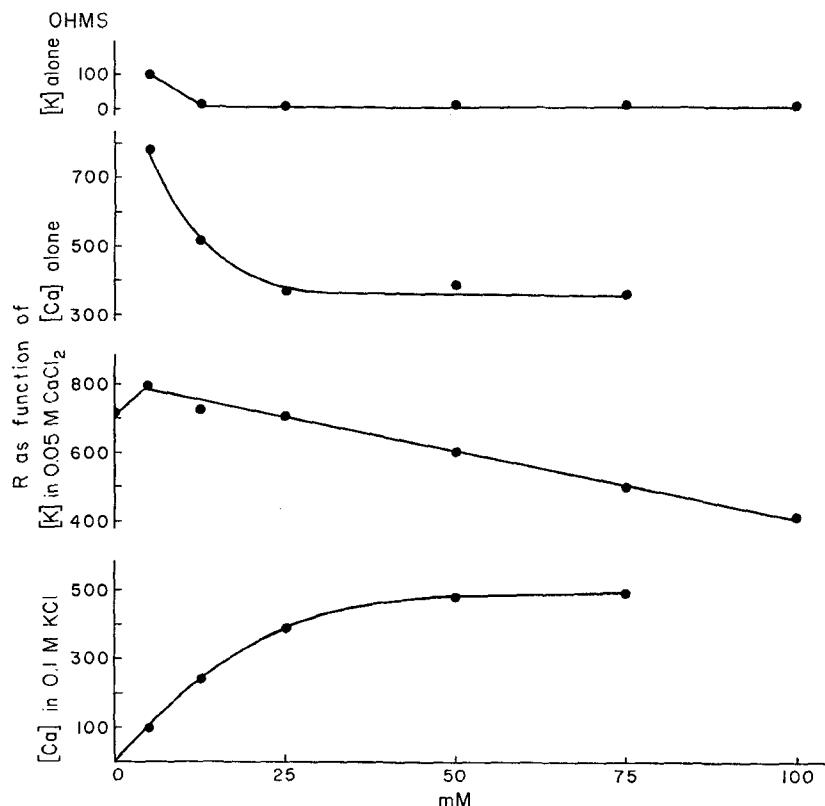


FIGURE 2. Resistance of barrier, ohms per square centimeter, already corrected for solution, electrode, etc. resistances. These curves are made with different barriers. Curve shapes and orders of magnitude are important, not the absolute values.

Clearly then, the resistance of a phospholipid-cholesterol barrier whose components almost certainly are also part of the plasma membrane, can be controlled by the ionic environment, and this is not simply a matter of number of available ions. There is a qualitative effect such that CaCl<sub>2</sub> raises the resistance, and KCl or NaCl lowers it.

In the experiment illustrated by Table II, barrier resistance was measured in solutions containing CaCl<sub>2</sub> and KCl in different total concentrations but in constant ratio to each other. The resistance of the barrier was, under these conditions, a linear function of the resistance of the medium, the pro-

portionality constant being about 4.6 in this case. It is the physical meaning of this constancy which is of some interest, but we cannot, at the moment, interpret it rigorously.

Barrier resistance in  $\text{CaCl}_2$  alone falls, but at a decreasing rate, to a steady but relatively high value as  $\text{CaCl}_2$  concentration rises from zero (Fig. 2). However, in KCl or NaCl alone barrier resistance falls rapidly to a few ohms as concentration increases. At first sight this resistance drop in  $\text{CaCl}_2$  alone might seem at variance with the fact that a similar Ca increase in a KCl or NaCl solution raises barrier resistance. Taken together these data mean that either  $\text{CaCl}_2$ , KCl, or NaCl can, simply by making ions available, lower resistance which is high for lack of current carriers. However,  $\text{CaCl}_2$ , unlike KCl or NaCl, simultaneously raises barrier resistance to ions in general. Therefore the

TABLE II  
RESISTANCE OF BARRIER IN KCl- $\text{CaCl}_2$  SOLUTIONS OF DIFFERENT CONCENTRATIONS BUT CONSTANT RATIO

Medium	Resistance of medium plus barrier, ohms (I)	Resistance of medium alone, ohms (II)	Resistance ratio $\frac{(I - II)}{II}$
KCl, 200 meq per liter plus $\text{CaCl}_2$ , 100 meq per liter	216	37.1	4.8
KCl, 100 meq per liter plus $\text{CaCl}_2$ , 50 meq per liter	378	69.6	4.4
KCl, 50 meq per liter plus $\text{CaCl}_2$ , 25 meq per liter	712	131.2	4.5

conductance rise due to  $\text{CaCl}_2$  supplying ions is opposed by its specific property of raising barrier resistance, and when the two balance the resultant levels off at a relatively high value. Resistance in pure KCl or NaCl falls rapidly to a low level as concentration rises, because there is no similar action on the barrier to increase its resistance. Since the anion has been chloride throughout the effects observed seem due to a difference between Ca and K or Na.

It should be mentioned that although it is small compared to the effect with Ca, still the lipid barrier also has a significant resistance for K and Na, about 10 per cent greater than that of free solution.

#### *Mechanism*

I. Fig. 3 shows how resistance of a barrier exposed to a fixed concentration of  $\text{CaCl}_2$ -KCl (0.05 M and 0.1 M) varies with pH adjusted by adding small amounts of HCl or KOH. Above the resistance curve is a titration curve for phosphatidyl serine as reconstructed from literature pK values for serine and phosphoric acid. There are uncertainties about the titration curve, but a

comparison is interesting. Thus, at high pH where there are fewer H<sup>+</sup> ions to displace the Ca from the acidic groups the resistance should rise and it does. At low pH where there are more H<sup>+</sup> ions to displace the Ca the resistance should fall and it does. At intermediate values where pH has little effect on dissociation of the acidic groups there should be little effect on resistance, and this too is the case. Also, the inflections in the two curves come at about the same pH values. Without CaCl<sub>2</sub> pH has no such effects.

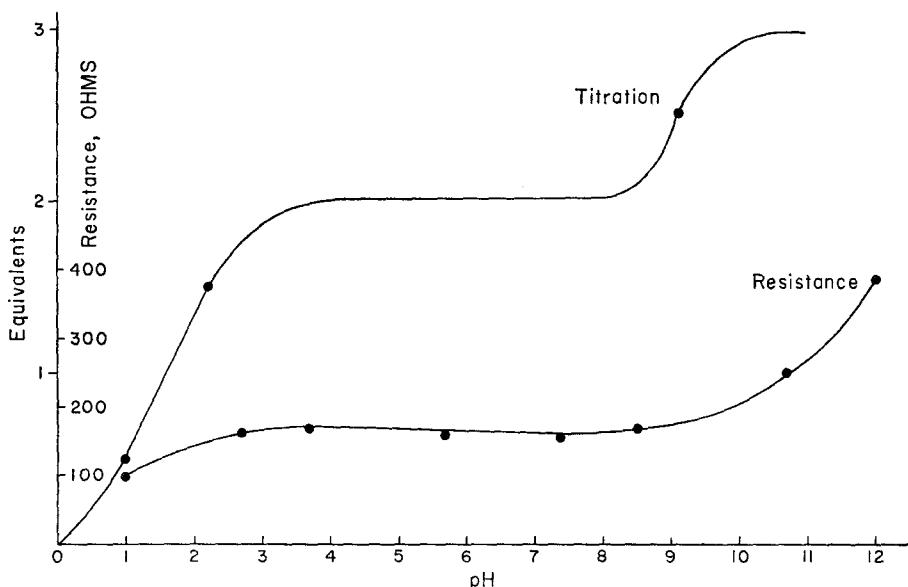


FIGURE 3. Lower curve shows resistance of barrier, ohms per square centimeter, in CaCl<sub>2</sub> 100 meq plus KCl 100 meq, plotted as a function of pH. Upper curve is titration curve for phosphatidyl serine plotted from literature pK values.

Thus, the resistance rise due to Ca seems dependent on its combination with acidic groups in the phospholipid.

II. The next question is how such ion combinations with acidic groups in the phospholipid can determine over-all resistance. We shall mention two mechanisms, but have evidence bearing only on the first.

1. High resistance due to Ca may result from decreased membrane hydration with a simple diminution of transsurface waterways available as ion conduits. There are two pieces of evidence:

(a) If Ca reduces membrane hydration it may also make it more hydrophobic whereas K may favor hydrophilicity.

Pieces of barrier, soaked for 1500 seconds in one or another salt solution, were lifted into mineral oil floating on the salt solution. When released the fragments settled down to the oil-salt solution interface and there the pieces pretreated with CaCl<sub>2</sub> or with CaCl<sub>2</sub> plus KCl remained in the oil, whereas

those treated with KCl or NaCl descended into the aqueous phase (Table III). Secondary soaking in the opposite solutions reversed these preferences. Thus, high electrical resistance is correlated with preference for the oil phase. This probably is not simply a matter of density difference, since the oil is

TABLE III  
WETTABILITY OF BARRIER AS A FUNCTION  
OF SALT PRETREATMENT

Solution <sup>1</sup> in which barrier is presoaked 1500 sec.	After presoaking it is better wetted by mineral oil or aqueous salt solution
CaCl <sub>2</sub> , 100 meq per liter	Oil
KCl, 100 meq per liter	Aqueous KCl
NaCl, 100 meq per liter	Aqueous NaCl
KCl, 100 meq per liter plus CaCl <sub>2</sub> , 100 meq per liter	Oil

TABLE IV  
WATER CONTENT OF BARRIER AS A FUNCTION  
OF EXPOSURE TO KCl OR CaCl<sub>2</sub>

Water Content in arbitrary figures	
After exposure to 100 meq KCl	After exposure to 100 meq CaCl <sub>2</sub>
100	33
100	60
100	60
100	72
100	37

1. Horizontal pairs of data are from the two halves of a single barrier in each case.
2. Because of difficulty in freeing KCl-soaked barriers of grossly adherent water, as by blotting, without removing fragments of barrier (see text), a number of blotting, shaking, etc. techniques were used in getting wet weight; the same, of course, also being used for the corresponding CaCl<sub>2</sub> sample. Therefore, the extent of hydration in KCl is in each case expressed as 100, and the extent of hydration in CaCl<sub>2</sub> as a measured fraction of 100.

seen to cling to the CaCl<sub>2</sub>-treated barrier and not to that soaked in KCl or NaCl.

(b) If Ca reduces and K increases barrier hydration the difference should be measurable. However, since KCl makes the lipid mixture soft and spongy it is difficult to remove gross water for wet and dry weight determination. Therefore, precision has not been achieved. Nonetheless, water in the barrier, though variable among experiments, has always been higher after exposure to KCl than after CaCl<sub>2</sub> (Table IV). The qualitative observation that KCl

makes the barrier soft and gelatinous, whereas  $\text{CaCl}_2$  leaves it hard and compact also demonstrates the hydration difference.

These findings then favor the hypothesis that  $\text{CaCl}_2$  raises barrier resistance over that reached in KCl by somehow decreasing hydrophilicity and water content.

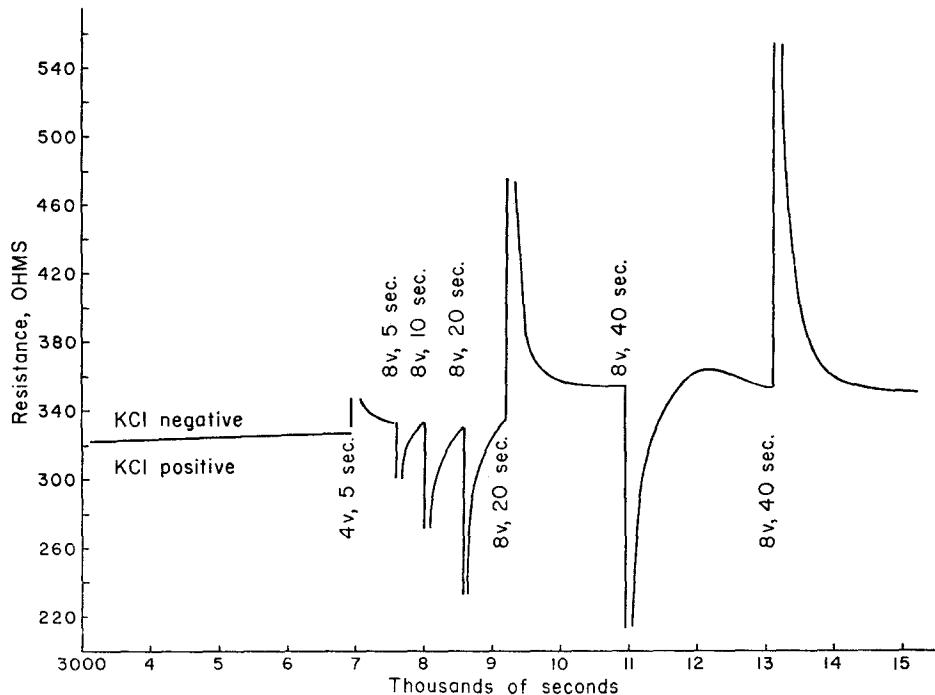


FIGURE 4. Resistance of barrier, ohms per square centimeter, before and after current flow across it, KCl 100 meq on one side,  $\text{CaCl}_2$  100 meq on the other. Curves show resistance changes due to making either the KCl side or the  $\text{CaCl}_2$  side positive for a few seconds.

2. Another mechanism deserves brief mention though we have no evidence bearing on it now.

If K were high it would be the dominant gegenion for the phosphatide acidic groups. Since such K binding is loose, *i.e.* dissociation high, these cations could relatively easily migrate sequentially from one group to the next under the influence of an electromotive force, and if the fixed sites were used as transmembrane stepping stones then resistance should be low. If, however, Ca were high and occupied more sites then, dissociation being small, ease of ion migration from one group to another would be less and resistance would rise.

*Response of the Barrier to Current Flow*

Current flow in a suitably chosen chemical environment should produce reversible changes in barrier resistance. Our methods so far have precluded detecting high speed transients so we describe only events occurring within a few, 3 or more, seconds.

With 0.1 M KCl on one side and 0.05 M CaCl<sub>2</sub> on the other, current was passed through the barrier for a few seconds *via* one pair of Pt electrodes, following which resistance was measured by another pair as a function of time. Fig. 4 shows that with the KCl side positive the barrier resistance fell. This was expected, since such current drives K into the barrier and removes Ca from it. On the contrary with the KCl side negative the resistance rose. This too was expected, since now the current drives Ca into the barrier and removes K from it. Further details about the shapes of the curves and about rectification will be considered at another time.

**DISCUSSION**

We have demonstrated control of the resistance of a cephalin-cholesterol barrier by manipulation of ambient Ca, K, or Na concentration or by current, and we have considered several possible mechanisms to explain these findings. Let us now, briefly and speculatively, fit the material into axonal function.

The axonal counterpart of the first mechanism proposed would have K driven into the axon surface from the axoplasm by a cathode or by cationic current, there to compete with Ca for negative sites as on cephalin. The microlocus involved would increase its hydration and reduce resistance by simply adding aqueous channels for ion flow. In this way the increased ion fluxes of activity would be initiated. Opposite changes would occur anodally and with anelectrotonus. Just how K and Ca produce the hydration changes is not presently known, but such changes have now been demonstrated to occur in the model, and if they also occur in the axon they may be the cause of the light scattering, diameter, rigidity, length, and surface contour changes known to accompany activity (4-12). Höber's (13) discussion of hydration of cell colloids is relevant.

For the second mechanism the barrier is visualized as a perforated solid with channel walls lined by phosphate, carboxyl, and amino groups of phosphatidyl serine. At pH 2.5 to 9 this surface has one mole of positive and two of negative charge per mole of phosphatide. Therefore, at about pH 7, where the present experiments have been done, cations could cross the barrier as gegenions along sequentially arranged negative groups. This hypothesis has elements in common with the fixed charge theory of Teorell (14).

There is a third possibility: In this case potassium and sodium permeability

during activity again depend on Ca and K action on surface phospholipid, but secondary structural changes, permitted when K dominates, underlie the actual ion flux increases. Thus, Ca, bonding pairs of membrane phospholipid molecules together in the resting state, produces an ion-impermeable surface, particularly if there is a cholesterol between adjacent pairs of such phospholipid molecules (15, 16). Now, when K is moved into this structure from the axoplasm by outflowing catelectrotonic current it displaces the Ca. Because the K is monovalent and unable to bind molecules together, the phospholipid pairs separate laterally and increase permeability (1, 2).

Each Ca atom might be only half unbound by this intrusion of K, since very few K ions, 1 to 10 or so (4), are moved into each 100,000 square Angstroms of membrane by catelectrotonic current to reach threshold. Thus, Ca, remaining attached to one of its original phospholipid companions, remains available to reattach to the other when the K diffuses away as during repolarization, accounting perhaps for the relative smallness of increase in Ca exchange with activity. Existing transsurface voltages would help operate this device: At threshold there is still a voltage gradient of some 55,000 to 85,000 volts per cm across the 100 Å membrane, and this voltage would swing the positively charged, partially released calcium phosphatide in towards the axoplasm. However, as the cell interior became less negative, thermal movements would favor replacing the calcium phosphatide in the surface, and if the cell interior became electropositive this voltage too would tend to replace the calcium phosphatide in the surface structure. In this way both the structural disordering which we call excitation and the structural reconstitution which we call recovery take place using only existing voltages to supply energy for the necessary work. Both excitation and recovery are visualized as electrokinetic and needing no immediate metabolic activity, but requiring the kind of Ca and K interaction with phospholipid which is described here.

Protein may be prominent. Thus, detachment of Ca from one phospholipid phosphate by K secondarily relaxes constraints on protein attached to this phospholipid, whereupon residual voltages or intramolecular coulombic forces spatially modify the protein. These reorienting molecules displace phospholipid attached to them thus reducing resistance and producing flux changes. The trigger is catelectrotonic K current, the gear shifting is Ca displaced by K, the motor is protein, the load moved by the protein motor is attached phospholipid, and the result is a change in fluxes. The fact that the geometry of cell surface protein can be changed by detaching its phospholipid companion is relevant (1).

Much information supports the view that fundamentally excitation is produced by unbonding Ca from some structure by K: Effects on threshold, water entry into cells, Na or K entry into axons, salt retention by red cells,

axon membrane resistance, width of axonal surface lamellae, Ca release from muscle or dorsal root ganglia, and axon structural changes due to polarization all depend on K:Ca ratios in a relevant fashion (1, 2, 5, 10, 17-29). Hashimura and Wright have approached a similar formulation (30), and a recent paper by Spyropoulos approximately paraphrases this earlier stated hypothesis (31).

Referring specifically to cephalin, calcium forms insoluble salts with it and condenses cephalin monolayers (32-34). Also, though cation affinities are different for the several acidic lipids, any cation can displace any other depending on concentration (35). In addition (36, 37) interference with cell surface phospholipid reduces impedance and renders the cell inexcitable, but damage to protein need have no such effect.

This study thus supports the hypothesis that acceleration of Na and K fluxes by excitation is initiated by displacement of Ca by K from a negatively charged binding site such as the phosphate oxygen in cephalin. It is as if one has plucked out of the cell surface that component which grades trans-surface ion fluxes. Now one requires a second component which modulates this system and confers ion selectivity, threshold, and triggering properties on it.

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