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Voltage-Gated Cation Conductance Channel from Fragmented Sarcoplasmic Reticulum: Steady-State Electrical Properties

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Summary. The interaction of fragmented sarcoplasmic reticulum (SR) with an artificial planar phospholipid membrane under conditions known to induce fusion of phospholipid vesicles raises the conductance of the planar bilayer by several orders of magnitude. Measurements of steady-state electrical properties of bilayers thus modified by SR show that two types of conductance pathways are present. One is a voltage-independent pathway which may be somewhat anion-selective. The other is a voltage-gated ionophore showing selectivity to small monovalent cations. This latter ionophore is fully oriented within the artificial bilayer and is inhibited asymmetrically by divalent cations. It is also inhibited below pH 6. The ionophore displays single-channel conductance fluctuations between two states, "open" and "closed", with an open-state conductance of 1.4×10^{-10} mho in 0.1 M K^+. The physiological function of this ionophore is unknown.

The central event of excitation-contraction coupling in vertebrate skeletal muscle is the release of Ca^{++} ion stored in the sarcoplasmic reticulum (SR) space (for reviews, *see* Ebashi, 1976; Endo, 1977). This release is the result of a transient rise in the Ca^{++} permeability of the SR membrane by a factor of 100 to 1000 (DeBoland, Jilka & Martonosi, 1975). Although it is known that this increase in Ca^{++} permeability is triggered by the depolarization of the surface and transverse tubule membranes of the muscle cell, the mechanism by which this signal is transmitted to the SR membrane is completely unknown. Furthermore, the process directly causing the rise in SR membrane permeability is not understood.

Among the numerous hypotheses advanced to explain the Ca^{++} release phenomenon, perhaps the best supported experimentally is the

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direct electrical mechanism. This proposal states that upon depolarization of the transverse tubule membrane, the voltage across the SR membrane also changes (intra-SR side becoming more negative with respect to the myoplasmic side), and that it is this change in electrical potential which activates a voltage-gated Ca^{++} permeability pathway (Kasai & Miyamoto, 1973, 1976*a–b*; Endo, 1977). Support for this hypothesis comes mainly from work on the skinned muscle fiber preparation (Constantin & Podolsky, 1967; Thorens & Endo, 1975; Stephenson & Podolsky, 1977), as well as from studies on fragmented SR vesicles (Inesi & Malan, 1976; Kasai & Miyamoto, 1976*a–b*). Both systems have been claimed to release stored Ca^{++} upon electrical depolarization induced chemically by exposing the SR membrane to appropriate ion gradients. The validity of these experiments has been disputed recently (Meissner & McKinley, 1976), and the hypothesis must be considered tentative. A major problem with experiments using chemically-induced depolarization is that the membrane potential can be neither measured nor controlled; only the direction of the voltage change can be known. More satisfactory would be a method allowing the control of membrane potential and direct measurement of membrane conductance, i.e., the voltage-clamp technique. This approach is not possible for the SR *in situ* because of the small dimensions involved.

Recently, methods have been introduced for direct electrical study of permeability pathways derived from the SR membrane (Sergeyeva, Poglazov & Vladimirov, 1975; Miller & Racker, 1976). Isolated SR vesicles can be made to fuse with an artificial planar bilayer membrane, the "black lipid membrane" of Mueller and Rudin (1969). By measuring the electrical conductance of the artificial membrane, Miller and Racker (1976) were able to assay the incorporation of permeability pathways from the SR membrane into the planar bilayer; in a typical experiment, SR vesicles caused the membrane conductance to increase by 3–5 orders of magnitude. The main concern with this previous study was the *process* by which fragmented SR vesicles cause the increase in artificial membrane conductance. The purpose of the present report is to describe some of the steady-state electrical properties of artificial membranes modified by fusion with SR vesicles. The data will show that ionophores of at least two types are present—one type which is voltage-independent and possibly anion-specific, and one which is voltage-gated, cation-specific, channel-like, and asymmetrically oriented within the planar bilayer. While it is too early to assign a biological function to this latter ionophore, the voltage-dependence suggests a possible involvement in excitation-contraction coupling.

Materials and Methods

Biochemical

Fragmented SR vesicles were prepared from rabbit white dorsal and leg muscle by the method of MacLennan (1970) or of Meissner (1975). The vesicles were stored in small aliquots in 0.3 M sucrose-5 mM HEPES[1]-Tris (pH 7.4) at −70°. The vesicles could be stored at least six months without loss of ionophoric activity.

Phosphatidylethanolamine (PE) from bovine heart mitochondria and phosphatidylserine (PS) from bovine brain were prepared as described (Miller & Racker, 1976). Phosphatidic acid (PA) prepared from egg yolk lipids, was purchased from Sigma, and diphosphatidylglycerol (DPG) from bovine heart was purchased from Avanti Biochemicals; these lipids were used without further purification. In some experiments, mixed soybean phospholipids (asolectin), obtained from Sigma and washed according to Kagawa and Racker (1971), were used.

Electrical

Artificial bilayer membranes were formed by the method of Mueller and Rudin (1969). The cell consisted of two cylindrical Teflon chambers (5 ml volume) separated by a Teflon disc partition (0.5 mm thick) with a 1.2-mm diameter hole drilled in it. The chambers were inserted into a plastic sleeve fitted with a compression screw for tight sealing. The sleeve rested in a brass block. The assembly contained appropriate windows and openings for viewing the membrane formation and for access of electrodes and pipettes. Membranes were formed by applying a drop of phospholipid solution (15–20 mM in *n*-decane) to the hole in the partition with a Teflon rod. Formation was monitored visually in reflected light. After thinning, membranes displayed conductances lower than 5×10^{-9} mho/cm^2 and capacitances in the range 0.3–0.5 μF/cm^2.

The aqueous phase of the artificial membrane system (hereafter designated "buffer") normally contained 50–100 mM of the appropriate salt, 5 mM HEPES, and 0.1 mM EDTA, adjusted to pH 7.3 with tris base. When Ca^{++} was added to the buffer, its concentration in excess of the EDTA is reported. Each aqueous chamber was connected to a calomel electrode by means of a plastic salt bridge filled with the experimental buffer in series with a saturated KCl salt bridge.

Membrane conductance was measured under voltage clamp conditions by a circuit described by Montal and Mueller (1972), except that the operational amplifier was Analog Devices 41 L. Open-circuit voltage could also be measured by switching to current-clamp conditions. Voltage- or current-clamp command was supplied by a Wavetek function generator (model 184) or by the amplifier power supply stepped down through a variable voltage divider circuit. The amplifier output was recorded on a chart recorder or storage oscilloscope. Since measured currents were never greater than 10^{-7} amperes, the above two-electrode system without compensated feedback was entirely adequate for voltage-clamping.

Fusion of SR with the Planar Bilayer

SR vesicles were incorporated into the planar bilayer by a previously described Ca^{++}-induced fusion process which is dependent upon anionic lipid in the artificial

1 *Abbreviations used:* EDTA, (Ethylenedinitrilo)tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane.

membrane and an osmotic gradient across the SR vesicle membrane (Miller & Racker, 1976). After complete thinning of the planar membrane, Ca^{++} (as the glucuronate salt) was added to the "back" chamber with constant stirring, to a final concentration of 0.1–1.0 mM. SR vesicles (10–100 μgm/ml final concentration) were then added to the same chamber, and conductance was monitored between +25 and −25 mV. (The "front" chamber, i.e., the chamber to which SR is *not* added, is defined as zero potential.) Experiments were done at room temperature, 20°–23°, unless otherwise noted.

For measurement of steady-state conductance properties of planar bilayers thus modified by SR vesicles, fusion was stopped by addition of EDTA (tris salt) in excess of the Ca^{++} in the back chamber or by merely allowing enough time to pass (10–15 min) so that the SR vesicles would dissipate their osmotic gradient, which has been shown to be an absolute requirement for the fusion process (Miller & Racker, 1976). In most experiments, membrane conductance increased over three orders of magnitude before data were collected.

The steady-state conductance-voltage relation (g-V curve) was determined either by recording the current response to a low-frequency (0.005 hz) sawtooth voltage command, or by recording the steady-state current following application of a voltage step. The latter method was found to be more convenient for accurate measurement of the g-V curve, and was used for most of the data here. While this report is not concerned with the kinetics of the conductance response to a voltage-step, it is worthwhile mentioning that these kinetics are highly temperature-dependent; under conditions used here, the steady-state conductance was attained in 1–5 sec after the voltage step.

In the experiments to be described, it was desirable to measure the conductance of either a cation (e.g., K^+) or an anion (e.g., Cl^-). In order to do this, counterions had to be chosen which did not contribute appreciably to the conductance. For cation conductance, the glucuronate salt was used, while for anion conductance, the tris or choline salt was used. Control experiments using potential measurements in the presence of salt gradients or direct conductance measurements in tris-glucuronate buffers insured that the counterion did not contribute more than 20% of the total conductance near zero millivolts, and for cation conductance at positive voltages, the contribution was much less than this.

Results

The basic effect of SR vesicles upon planar bilayer conductance is shown in Fig. 1, for both K^+ and Cl^- conductance. Upon addition of SR vesicles under appropriate conditions (anionic lipid in the artificial membrane, Ca^{++} in the aqueous medium, and an osmotic gradient across the SR membrane), the bilayer conductance rises in discrete steps, each of which Miller and Racker (1976) have suggested results from the fusion of a single SR vesicle with the planar bilayer. The rising phase of each step is complete in at most 2 msec (Fig. 1 *C*), which is the time resolution of the amplifier under these conditions. A comparison of Fig. 1 *A* and *B* shows that the form of the conductance steps is dependent on the type of ion whose conductance is being measured. While the Cl^- conductance rises in a simple staircase fashion, the K^+ conductance

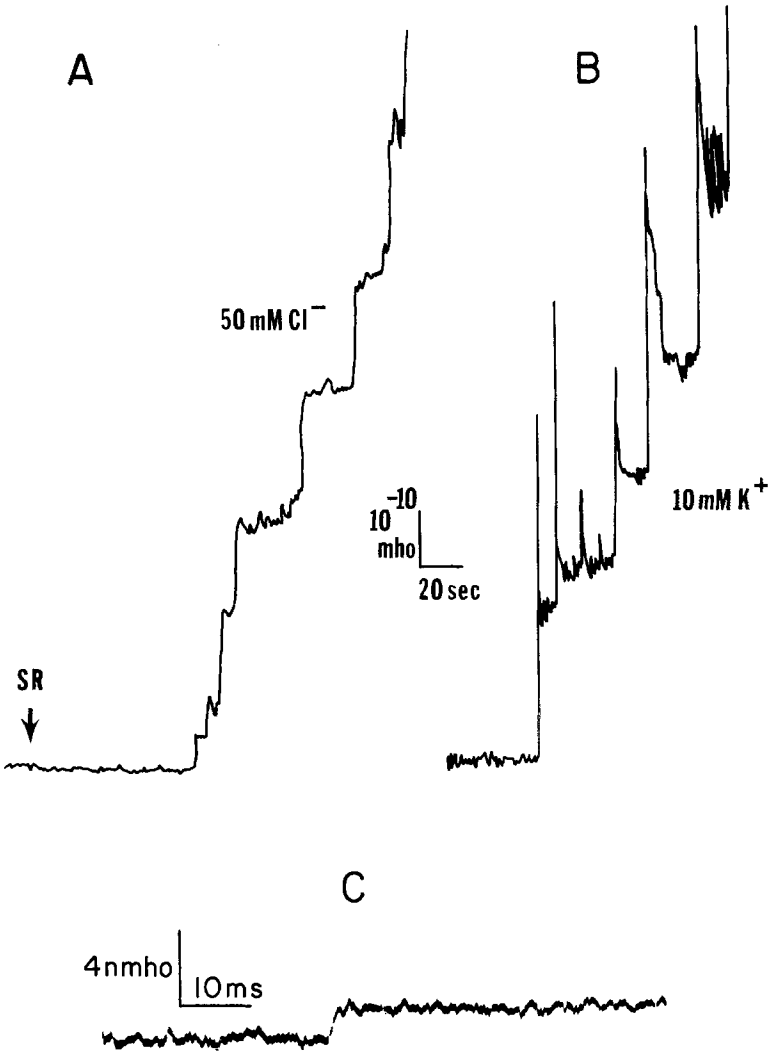

Fig. 1. Fusion of fragmented SR with planar bilayers. Time course of conductance increase of planar bilayers (30% PS–70% PE) after addition of SR vesicles (20 μg/ml) in the presence of Ca^{++} (0.7 mM). Conductance was measured at +25 mV. (*A*): Cl^- conductance, using 50 mM choline Cl^- buffer. (*B*): K^+ conductance, using 10 mM K^+ glucuronate buffer also containing 40 mM choline glucuronate. This lower concentration of K^+ was chosen so that the K^+ and Cl^- conductance traces would be on the same scale. (*C*): A single K^+ conductance step measured with high time resolution, using 100 mM K^+ glucuronate buffer. No relaxation to a lower conductance is seen here because the sweep time scale is much faster than the relaxation time scale. Rising phase of the trace is limited by the time response of the amplifier, which under these conditions is 2 msec

increases with more complex time course, each step being composed of a spike followed by relaxation to a lower steady-state level. In general, ions can be divided into two classes: those which give staircaselike steps and those which give spikelike steps. Among the former class are all anions tested, choline$^+$, tris$^+$, and Li^+; in the latter class are Na^+, K^+, Rb^+, and NH_4^+ (data not shown). Experience with the system shows that the distinction is wholly reproducible; K^+ conductance always proceeds with spikelike steps and Cl^- conductance always with staircaselike steps. This difference in the form of the quantal conductance increase suggests that the pathways involved in the movement of K^+ across the SR-modified artificial bilayer may be distinct from those involved in the movement of Cl^-.

The suggestion of two types of conductance pathway is further supported by the dependence of steady-state conductance upon applied voltage (g-V curve). Figure 2 shows this dependence for conductances of various ions. It is seen that Cl^- conductance is largely voltage-independent; other ions which show this simple ohmic behavior are: all anions tested, choline$^+$, and tris$^+$. On the other hand, the conductances of Na^+, K^+, and NH_4^+ are voltage-dependent. (Li^+ conductance shows a very weak voltage-dependence.) The conductance is low at large negative voltages, and it increases in a graded fashion as the applied voltage becomes more positive. No evidence of a maximum conductance level can be discerned, and above $+70$ mV, these membranes become unstable. It is important to realize that this voltage-dependent cation ionophore is oriented asymmetrically within the planar membrane; this is evident from the g-V curves of Fig. 2, which were measured with identical solutions on the two sides of the membrane. The asymmetry arises because the SR vesicles are added to only one side of the membrane; before the g-V curve is measured, the interaction of SR with the planar bilayer is stopped (*Materials and Methods*) to eliminate possible artifacts due to fusion of vesicles *during* the measurement of the g-V curve. Therefore, since asymmetry of the g-V curves is not due to asymmetry in the aqueous solutions, or in the lipid composition of the planar bilayer itself (as can be checked with valinomycin-mediated K^+ conductance), it must come about from preferred orientation of the ionophore within the membrane. The asymmetry in the g-V curve is stable up to at least several hours after fusion; thus, it is apparent that once incorporated into the artificial membrane, the K^+ ionophore is not free to reorient across the bilayer.

Figure 2 also points up a small but consistently observed effect of

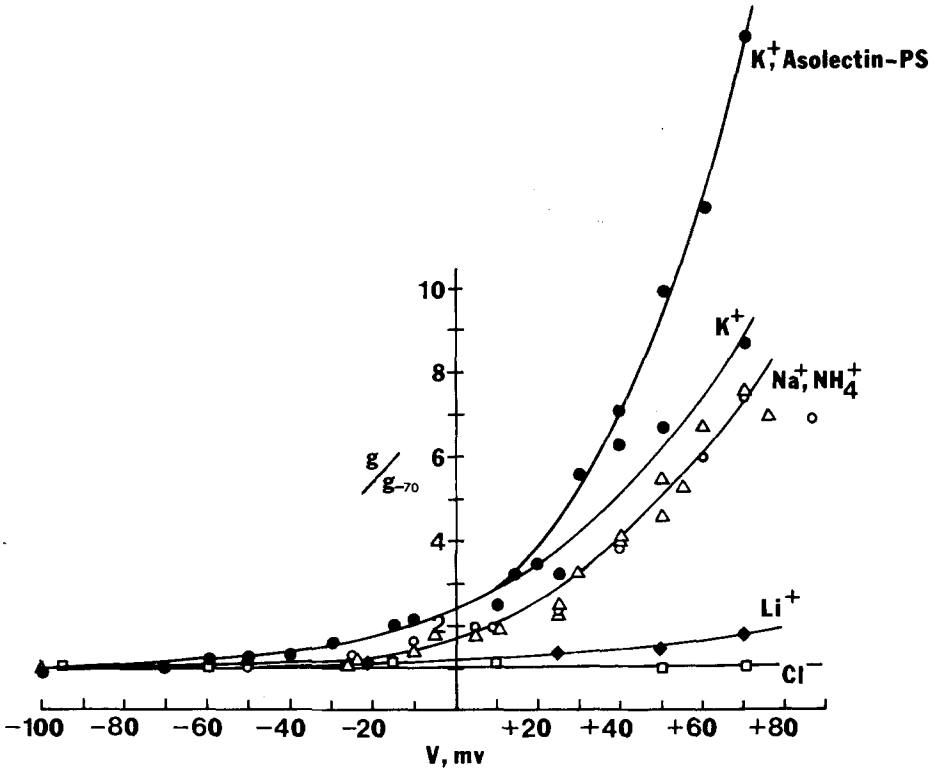

Fig. 2. Conductance-voltage relation for various ions. SR vesicles (50 μg/ml) were fused with planar bilayers in the presence of 0.7 mM Ca^{++} in buffers containing different ions (50 mM) as the major current-carriers (indicated on curves). Conductance, g, was measured after addition of 0.9 mM EDTA to stop fusion. Data are reported as conductance normalized to its value at −70 mV. In this experiment, values of g at −70 mV ranged from 2×10^{-8} mho/cm^2 (for Li^+) to 4×10^{-7} mho/cm^2 (for Na^+). As long as the −70 mV conductance is greater than 10^{-8} mho/cm^2, the normalized g-V curve is independent of absolute conductance for a given ion. Planar bilayers were 70% PE–30% PS, except for curve labelled "Asolectin-PS", which used asolectin in the place of PE

bilayer lipid composition on the position of the g-V curve along the voltage axis. In PE-PS membranes, higher positive voltages are required than in asolectin-PS membranes to achieve a given relative conductance level. This result indicates that the ionophore involved in the K^+ conductance is able to sense the lipid composition of the membrane.

The comparisons made above between K^+ and Cl^- conductances suggest that two different types of conductance pathways can be inserted into a planar bilayer from the SR membrane. While both of these pathways may turn out to be of physiological interest, it is possible that

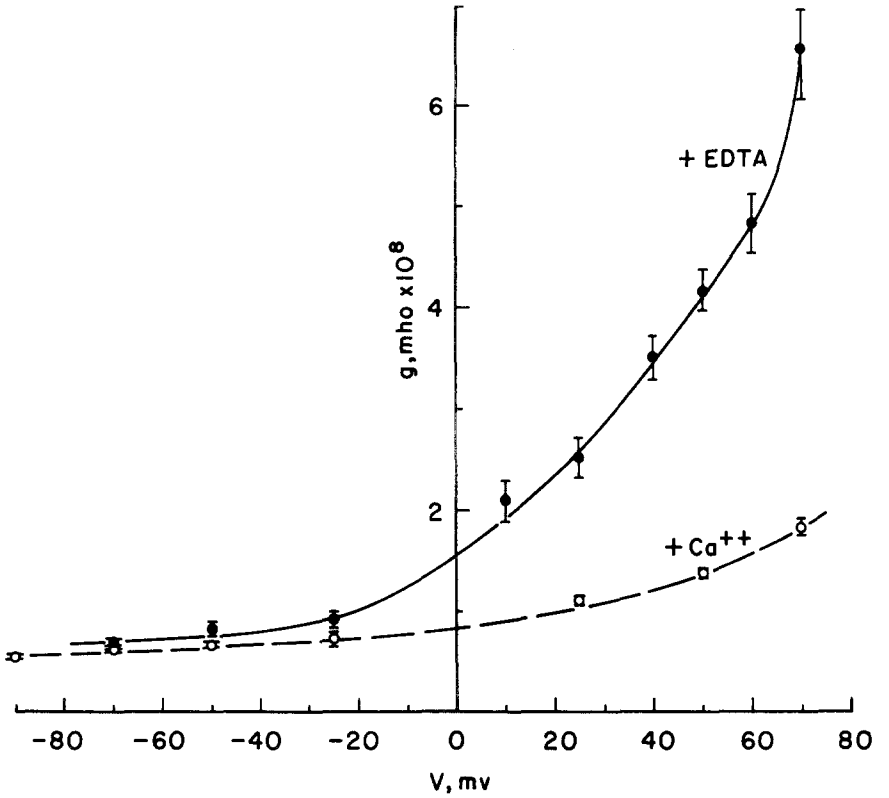

Fig. 3. Effect of Ca^{++} on K^+ conductance. Conductance-voltage curves were measured as described in the text using 100 mM K^+ buffer, and a planar bilayer of 70% PE–30% PS composition. SR vesicles (50 μg/ml) were fused in the presence of 0.3 mM Ca^{++}, and fusion was stopped with 0.4 mM EDTA. The *g-V* curve labelled *+EDTA* was determined. Ca^{++} (3.5 mM in excess of EDTA) was then added to the same side of the bilayer as the SR, and the *g-V* curve labelled $+Ca^{++}$ was determined. Points show means ±SE for 3–5 determinations of conductance at each voltage. All data were taken from the same planar bilayer

the voltage-independent pathway is a simple "leak," an artifact due to perturbation of the artificial membrane by fusion. Therefore, the remainder of this report will focus primarily on the voltage-dependent ionophore.

One property of this ionophore is its sensitivity to Ca^{++} and other divalent metal ions. Figure 3 presents the steady-state *g-V* curve for K^+ conductance in the presence or absence of Ca^{++}. In this experiment, Ca^{++} is added only to the same side of the planar membrane as are the SR vesicles ("*cis*" side). The presence of Ca^{++} on this side reduces the K^+ conductance measured at positive voltages, but has little effect at

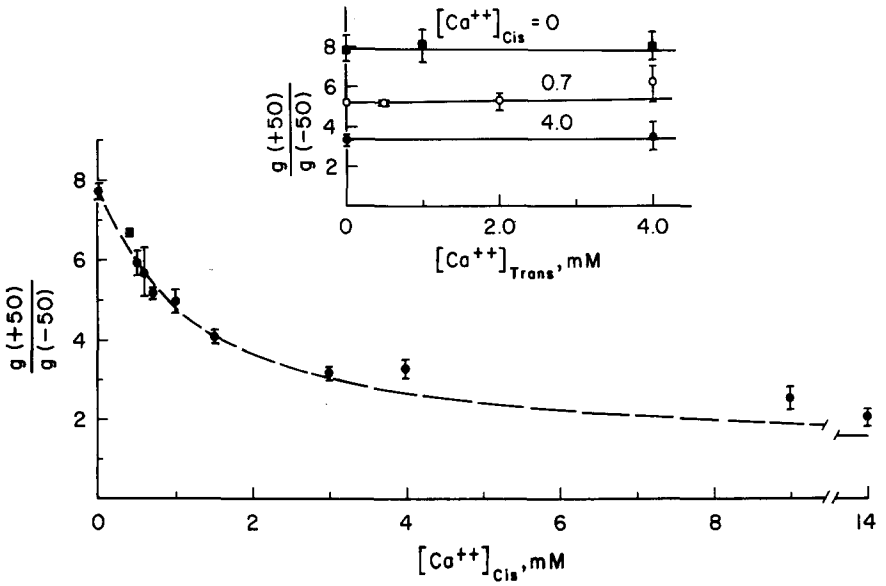

Fig. 4. Effect of Ca^{++} on K^+ rectification. The rectification ratio defined in the text, $g(+50)/g(-50)$, was measured for K^+ conductance as a function of Ca^{++} added either *cis* or *trans* to the SR. Planar bilayers were 70% asolectin-30% PS, and aqueous phase was 100 mM K^+ glucuronate buffer. With *cis* Ca^{++} addition (main curve), concentration of *trans* Ca^{++} was kept at zero. Solid curve is of the form:

$$\frac{r-1}{r_0-1}=\frac{1}{1+[Ca]_{cis}/K_i}$$

where r is the rectification ratio, r_0 is its value at zero Ca^{++}, and K_i is the "inhibition constant" (1.2 mM for this example). With *trans* Ca^{++} addition (inset), rectification ratios were measured with different concentrations of *cis* Ca^{++} (indicated on graph). Points represent means ± SE of 3–15 determinations on different artificial planar bilayers

voltages more negative than −50 mV. In order to explore this effect further, a convenient index of voltage dependence can be defined, the rectification ratio, the ratio of conductance at +50 mV to that at −50 mV. Figure 4 shows that this ratio is reduced by *cis* Ca^{++} but is unaffected by *trans* Ca^{++}. Furthermore, the rectification ratio is affected by Ca^{++} on *both* sides of the membrane in quantitatively the same way as it is when Ca^{++} is added to the *cis* side only. The inhibition by *cis* (or *cis* and *trans*) Ca^{++} follows a single-site Langmuir binding inhibition curve up to at least 60% inhibition, with some departure from the theoretical curve at higher Ca^{++} concentrations. The apparent inhibition constant for Ca^{++} is 1.2 mM, although this value varies (from 0.7 to

Table 1. Inhibition constants of various divalent cations for voltage-dependent K^+ conductance

Ion	K_i, mM
Ca^{++}	0.8
Mg^{++}	4.0
Ba^{++}	2.8
Mn^{++}	>9
Zn^{++}	<0.05[a]

[a] Inhibition not reversed by EDTA.

Inhibition constants (K_i) were measured by plots such as in Fig. 4, linearized by reciprocal plots. The best straight lines in reciprocal plots determined the inhibition constants within 20%. SR vesicles (60 μg/ml) were fused with planar bilayers (25% PS-75% asolectin) in 50 mM K^+-glucuronate buffer, containing 0.1 mM Ca^{++}. Before addition of test ion to the *cis* side, it was insured that the rectification ratio was in the range 7.0–8.5. Divalent ions were added to the *cis* side as the glucuronate or sulfate salts. All inhibition curves followed curves of the form:

$$(r-1)=\frac{r_0-1}{1+c/K_i}$$

where c is the concentration of divalent ion, and other symbols are defined as in legend to Fig. 4.

1.5 mM) with the SR preparation. Other divalent metal ions added *cis* to the SR vesicles also inhibit the voltage-dependent K^+ conductance, but with widely different inhibition constants, as Table 1 shows. The inhibition is completely reversed by excess EDTA, except for the case of Zn^{++}, which causes time-dependent, irreversible inhibition measured at *both* positive and negative voltages. The above effects of Ca^{++} appear only with the voltage-dependent cation conductance; the effects of Ca^{++} on Cl^- conductance are small and can be entirely accounted for by surface potential effects (data not shown).

A second property of the ionophoric systems here is sensitivity to low pH. Both the voltage-independent pathway (assayed by Cl^- conductance) and the voltage-dependent ionophore (assayed by K^+ conductance) can be inhibited by lowering the pH of the aqueous phase of the planar bilayer system. Figure 5 presents these data, which again demonstrate the different behavior of the two conductance pathways. The block of Cl^- conductance follows a single-site titration curve with apparent pK_a near 4.3, while the K^+ conductance is substantially inhibited at pH 6.0, and over 95% inhibited at pH 5.0. The data for K^+ conductance

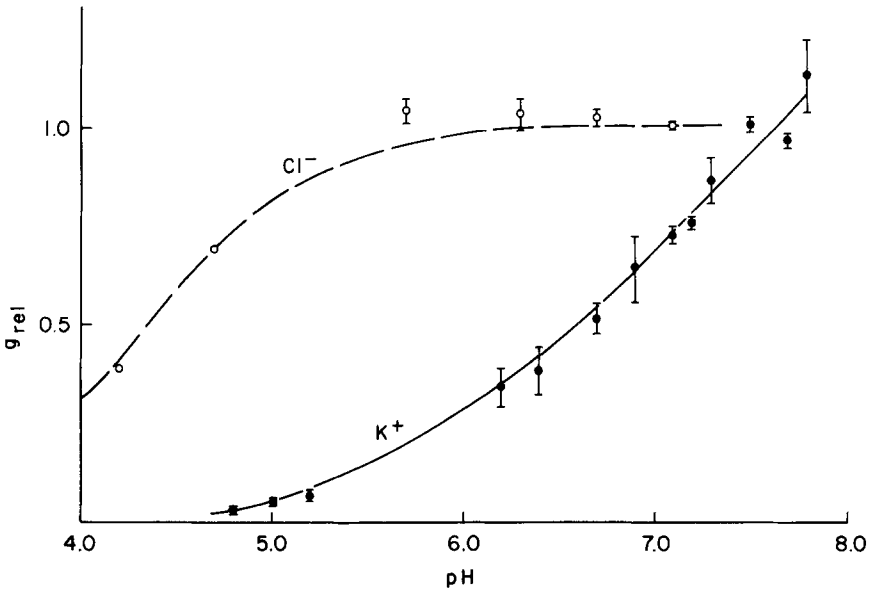

Fig. 5. Effect of pH on K^+ and Cl^- conductances. K^+ and Cl^- conductances were measured at +50 and −50 mV in 100 mM K^+ or Cl^- buffers, with excess EDTA added after fusion of SR (50 μg/ml) in the presence of 0.7 mM Ca^{++}. Conductance, g_{rel}, is reported relative to its value measured at pH 7.3 for K^+ conductance and pH 7.1 for Cl^- conductance. The pH was changed by symmetrical addition of glucuronic acid or tris base. No differences in g_{rel} were found when measured at +50 or −50 mV, and data collected at *both* values of applied voltage are included in averages. Planar bilayers were made of 70% PE and 30% of either PS, PA, or DPG. For K^+ conductance, all of these lipids gave similar results and were included in averages. For Cl^- conductance, however, PS gave spurious results below pH 5.0 due to titration of the lipid, and so only PA was used. Dashed line drawn through Cl^- data is a single-site titration curve of pK_a 4.3. Points represent mean ± SE for 3–6 determinations on different membranes; points without error bars are single measurements

do not fit a simple titration curve, nor is a maximum conductance level reached even as high as pH 7.6. The inhibition is immediate and fully reversible. The inhibition of K^+ conductance is identical with membranes using either PS, PA, or DPG as the anionic lipid; this shows that the reduction of conductance is not due to surface potential changes with pH. For measurement of Cl^- conductance inhibition, however, PS could not be used, since its carboxyl group titrates with a pK_a of 4.7 in these bilayers (measured by valinomycin-mediated K^+ conductance—data not shown); therefore, PA, which does not titrate in this pH range, was used for Cl^- conductance measurements. The degree of inhibition of both K^+ and Cl^- conductance is the same when measured at +50 or −50 mV.

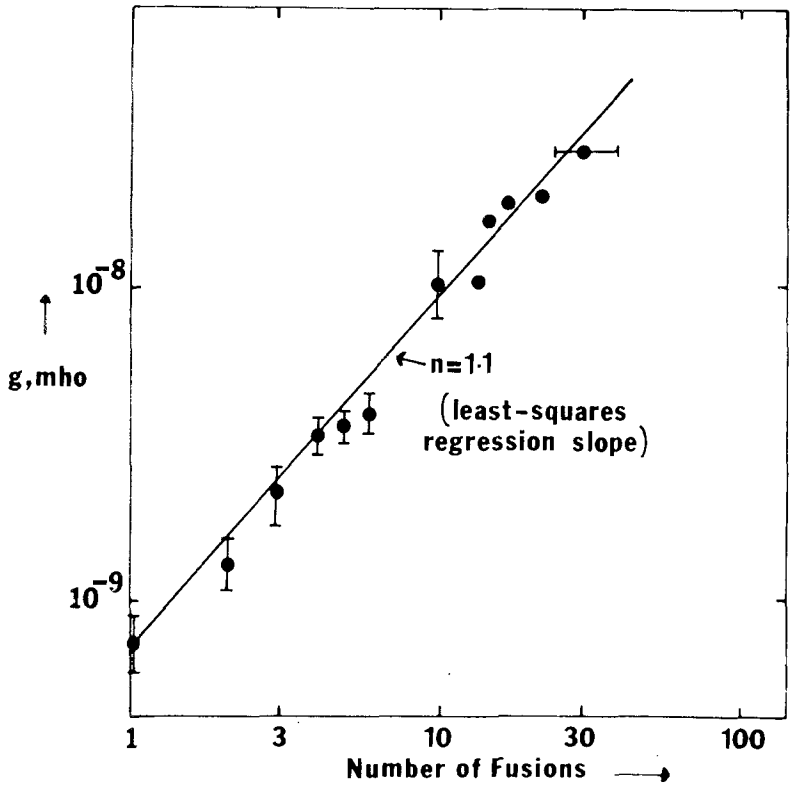

Fig. 6. Dependence of K^+ conductance on ionophore concentration. Conductance, g, in 100 mM K^+ buffer containing 0.3 mM Ca^{++}, was determined at +50 mV as a function of the number of conductance jumps (fusion events). SR vesicles (2–50 μg/ml) were added, and the quantal conductance increase was carefully followed with time. The number of conductance jumps leading to a given level of steady-state conductance was counted and plotted on the horizontal axis. The membrane conductance reached after the initial spike had relaxed (Fig. 1) was plotted on the vertical axis. Planar bilayer was composed of 70% asolectin–30% DPG. Points represent mean ±SE of 3–10 determinations. Points without error bars represent single determinations

While this result is not surprising for the Cl^- conductance, it was not expected for the K^+ conductance. It raises the question of whether the K^+ conductance at −50 mV is due to passage of K^+ ion through the same pathway assayed by Cl^- conductance or through a "low conductance state" of the K^+ ionophore itself. The fact that the K^+ conductance at −50 mV is almost completely inhibited at a pH where the Cl^- conductance is almost fully active suggests that the latter possibility is correct, and that the voltage-independent pathway is somewhat selective for Cl^- over K^+.

Perhaps the most basic property to be determined for any newly-discovered ionophore is the concentration dependence of the conductance. In this system it is possible to obtain an independent measure of ionophore concentration in the artificial membrane, since the number of fusion events leading to the high-conductance membrane can simply be counted (as in Fig. 1). Thus, the determination of conductance *vs.* concentration becomes trivial, and such a result is shown in Fig. 6 for the K^+ conductance at +50 mV. The data show that the voltage-dependent K^+ conductance follows a first-order dependence on concentration, at least over a 30-fold concentration range. Thus, there is no evidence that this ionophore relies upon an oligomeric aggregation process for its operation, as is the case for the model voltage-dependent ionophores alamethicin (Eisenberg, Hall & Mead, 1973; Baumann & Mueller, 1974), E.I.M. (Bean, Chan & Eichner, 1969; Ehrenstein *et al.*, 1974), and monazomycin (Bamberg & Janko, 1976). However, this linearity does not directly argue in favor of a monomeric structure for the conducting state of the ionophore (*see Discussion*).

While this report is mainly concerned with the steady-state properties of these conductance pathways, it is notable that the voltage-dependent K^+ ionophore displays single-channel conductance fluctuations of the type seen for gramicidin (Hladky & Haydon, 1970), alamethicin (Gordon & Haydon, 1972; Eisenberg *et al.*, 1973), and other "channel forming" model ionophores. In order to observe clear single-channel behavior here, it is imperative that only a very small number of channels be inserted into the artificial membrane. This condition is achieved by stopping the fusion of SR vesicles with the membrane immediately after the first observed fusion event by adding excess EDTA. The fluctuations in conductance are then observed at high gain, using an applied voltage at which the ionophore is expected to be in the low conductance state most of the time (e.g., in the range −50 to −100 mV). Figure 7 shows a recording of such fluctuations measured at −50 mV, with 0.1 M K^+ in the aqueous phase. The conductance fluctuates between discrete values, and occasionally integral multiples of the unit conductance change are seen. The unit single-channel conductance is $1.4 \pm 0.2(\text{SD}) \times 10^{-10}$ mho in 0.1 M K^+. Under the conditions of Fig. 7 a channel's mean "on time" is 150 msec. While this on time is extremely sensitive to conditions such as lipid composition, voltage and temperature, the open state conductance changes very little with these parameters, as will be detailed in a subsequent report (*in preparation*). Single-channel behavior has been observed only with ions which display voltage-dependent conductance in the steady-state.

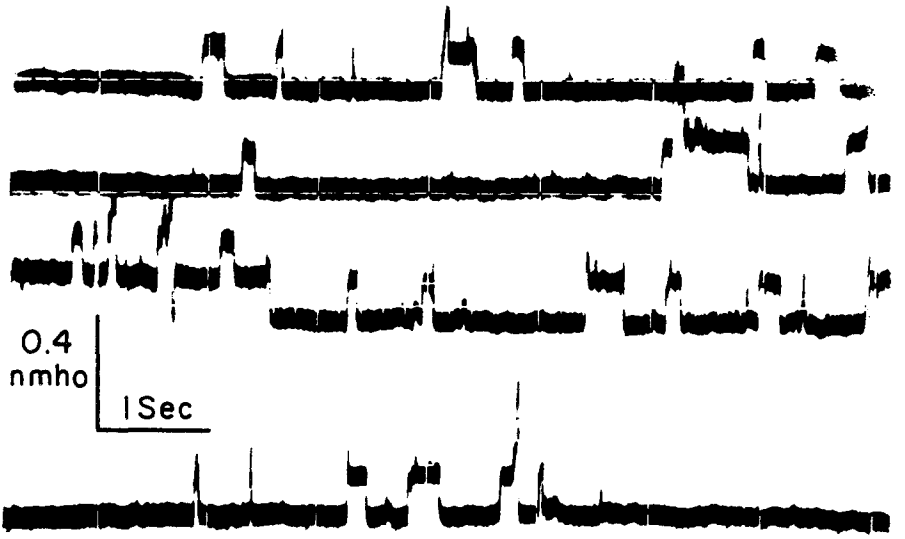

Fig. 7. Single-channel fluctuations of K^+ conductance. Incorporation of a single SR vesicle into the planar bilayer was achieved as described in the text. Conductance was recorded at -50 mV applied voltage. Artificial bilayer was 70% PE–30% PS, and aqueous phase was 50 mM K_2SO_4 buffer. Temperature was 28° in this experiment

The observation of single-channel fluctuations allows us to ask about the nature of the steady-state voltage dependence of the K^+ conductance. Figure 8*A* shows the results of an experiment in which the probability of the appearance of different conductance levels is plotted as a function of voltage applied. While the open-state conductance is independent of voltage in the range -100 to $+50$ mV (data not shown), the probability of a channel's existing in the open state is voltage-dependent. At highly negative voltages, the probability of opening is low, and as the voltage is made more positive this probability increases. Using these data coupled with the ohmic behavior of the open state conductance, it is possible to construct a time-averaged curve of the voltage-dependent part of the K^+ conductance, unobscured by the "background" conductance (i.e., that conductance level measured at -100 mV). This average curve is shown in Fig. 8*B*; it demonstrates that between -70 and -25 mV, the average conductance of the voltage-dependent K^+ ionophore increases by a factor of about 10, and between -70 and $+25$ mV by a factor of 60–100. Of course, such large stimulation is not seen in the steady-state curve of Fig. 2 because of the low, but nonzero, background conductance.

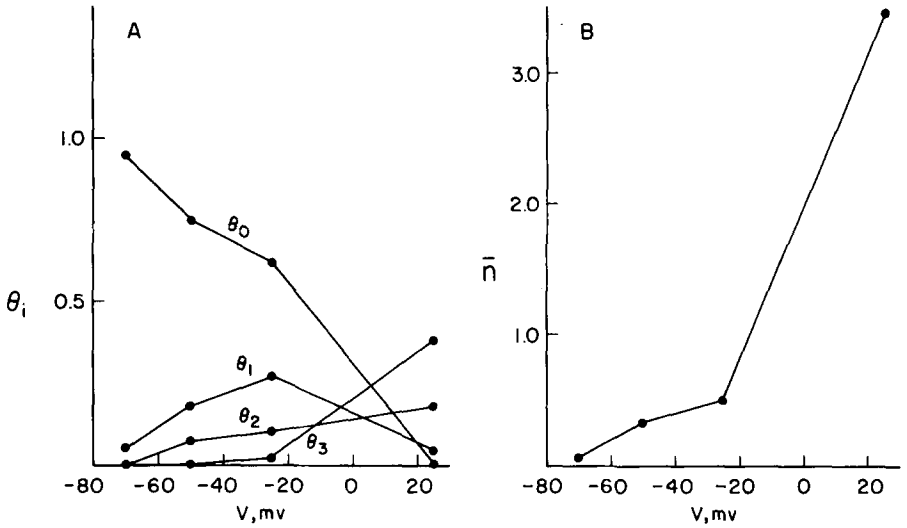

Fig. 8. Voltage-dependence of single-channel fluctuations. Experimental procedure was as in Fig. 7, except that fluctuations were examined at various applied voltages in the range -70 to $+25$ mV. (*A*): Voltage dependence of probability, θ_i, of i channels being in the open state. Probability was calculated as the fraction of time spent in the i^{th} conductance level. For clarity, data are shown only for θ_0, θ_1, θ_2, and θ_3; at voltages more positive than -25 mV, up to 7 channels could be detected and discriminated. (*B*): Time-averaged conductance, $\bar{n}$, calculated from data in *A*, as well as from additional conductance levels not shown in *A*, in units of average number of channels open, averaged over time. This curve was calculated according to:

$$\bar{n} = \sum_i i\theta_i$$

where θ_i are the probabilities displayed in *A*

Discussion

The purpose of this work has been to begin a characterization of the electrical properties conferred upon an artificial planar bilayer by the fusion of SR vesicles. The steady-state conductance measurements made here lead to the conclusion that at least two different types of conductance pathways are inserted into the bilayer. One of these is voltage-independent and possibly anion-selective, while the other is voltage-dependent and selective for certain cations such as Na^+ and K^+. This latter ionophore is a channel-former. Below, the discussion will concentrate on the evidence supporting this model and other conclusions arising from the data.

1. The K^+ and Cl^- Pathways are Separate and Distinct

This proposition is supported by three lines of evidence, each demonstrating differences in behavior for the Cl^- and K^+ conductance pathways. First, the time course of conductance increase for each proceeds in quantal conductance jumps, but the form of these jumps is determined by the type of ion carrying the current. All ions displaying voltage-independent conductance give rise to a staircaselike time course, while ions showing voltage-dependent conductance give a large spike at the beginning of each step. The explanation for the spike is not known. One possibility is that the state of the K^+ ionophore is sensitive to the membrane environment, and when this changes upon fusion of an SR vesicle, relaxation to a new average conductance level occurs. Whatever the explanation for the spikes, however, the point can be made that it is seen only with ions with voltage-dependent conductances, and never with ions showing ohmic behavior.

The second difference between K^+ and Cl^- conductances is found in their dependence upon pH. The K^+ conductance is strongly inhibited below pH 6.5, and is over 95% inhibited at pH 5.0. In contrast, the Cl^- conductance is nearly fully active at pH 6.0, substantial inhibition occurring only below pH 4.5. The pH effects in the range 4.0 to 7.1 are not artifacts due to possible titration of phospholipid head groups, although the further increase in K^+ conductance above pH 7.5 could be a result of an increase in negative surface charge as the PE amino group begins to lose its proton.

Finally, the most obvious difference in behavior of the two systems is the voltage-dependence of the K^+ channel in contrast to the ohmic nature of the Cl^- conductance. In addition, the single-channel conductance fluctuations, which are patently associated with the voltage-dependent pathway, are not seen with Cl^- as the current-carrying species. If Cl^- were traversing the same pathway as K^+, we would be able to detect Cl^- conductance fluctuations easily, since it is known (Miller & Racker, 1976) that around 0 mV the Cl^- conductance per SR vesicle is in the same order of magnitude as the K^+ conductance per vesicle.

The identification of two such ionophoric systems raises the question of whether part of the conductance measured is due to a simple leakiness brought about by local damage done to the planar bilayer as a result of the fusion process. This is a particularly pertinent question in view of the recent work of Düzgüneş and Ohki (1977), who demonstrated a con-

ductance increase in a 100% PS planar bilayer induced by phosphatidylcholine liposomes in the presence of Ca^{++}. It is unlikely that the voltage-dependent K^+ conductance is such a leak, because of the specific properties displayed by this ionophoric pathway. It is possible that the Cl^- conductance is a reflection of such membrane damage, though the results here do not demonstrate this with any degree of certainty.

One question arising from the data is whether the "background" K^+ conductance measured at highly negative voltages represents K^+ movement through the same pathway that Cl^- moves, i.e., a nonspecific leakage pathway. Although this picutre is simple and appealing, it does not explain the fact that the K^+ conductance at -50 mV follows that same pH inhibition curve as the K^+ conductance at $+50$ mV. If the -50 mV conductance were an indication of a nonspecific leak, we would expect it to be inhibited similarly to the Cl^- conductance. This is not the case (Fig. 5), and the conclusion emerges that K^+ current flowing at voltages which give rise to the low conductance state does not go through the same permeability pathway as Cl^- current. This in turn suggests that the K^+ ionophore may exist in two states: one which displays high conductance (the "on" state), and one which displays low but nonzero conductance; furthermore, these considerations imply that the pathway accounting for the Cl^- current is not merely a nonspecific leak, but actually shows some anion selectivity. Whether or not this Cl^- conductance is due to fusion-induced membrane damage or is due to the operation of a true ionophoric molecule is open to question.

2. *Inhibition of K^+ Conductance by Divalent Metal Ions*

We have seen that addition of Ca^{++} and other divalent metal ions to the *cis* aqueous chamber of the planar bilayer inhibits the voltage-dependent K^+ conductance; its value measured at $+50$ mV is reduced, while at -50 mV Ca^{++} has little effect. In order to interpret this result, it is necessary to consider several effects of Ca^{++} which may be operating. First, since the artificial membrane carries a negative surface charge, addition of Ca^{++} will reduce the magnitude of the surface potential (by virtue of charge screening or specific binding), and this will reduce the local K^+ concentration near the membrane surface. If Ca^{++} is added asymmetrically, the differences in local K^+ concentrations on the two sides of the membrane might be able to account for the inhibition of voltage-dependence caused by *cis* Ca^{++} (Fig. 4). However, calculations of

surface potentials and control experiments (not shown) with valinomycin-mediated K^+ conductance show this effect to be small (on the order of 20%). Furthermore, experiments using *symmetrical* addition of Ca^{++} on both sides of the membrane (Fig. 4) rule out local K^+ concentration effects as an explanation for the inhibition of voltage-dependence by Ca^{++}.

A second effect of Ca^{++} will be upon the gating mechanism of the K^+ channel. Asymmetric addition of Ca^{++} (by changing surface potentials asymmetrically) will change the electric field within the membrane at a fixed total transmembrane potential (Frankenhaeuser & Hodgkin, 1957; Muller & Finkelstein, 1972). In the system described here, this explanation predicts that *cis* addition of 2 mM Ca^{++} should cause a shift of the g-V curve to the left by about 10 mV; thus at +50 mV, a substantial *increase* of K^+ conductance is predicted, not the observed decrease. Of course, this "gating effect" also demands that symmetric addition of Ca^{++} should have no effect on the voltage-dependence of K^+ conductance, in contrast to observation.

Therefore, a third possibility must be seriously considered, namely, that the K^+ channel contains a specific inhibitory site which binds divalent cations. The data demand that this site be accessible only from the *cis* side of the membrane. Such a suggestion is in harmony with all the data on divalent ion inhibition. The agreement of the inhibition data with a single-site inhibition curve is probably coincidental, however, and should not be given great weight. The two surface potential effects discussed above operate in opposite directions; that they should approximately compensate is fortuitous.

3. *Apparent Monomeric Nature of K^+ Channel*

The observed linearity of voltage-dependent K^+ conductance with ionophore concentration deserves some comment. Previous work (Miller & Racker, 1976) has shown that the size of each quantal conductance jump observed upon fusion of SR with the planar bilayer is correlated with SR vesicle surface area. If we assume that the ionophoric material is derived from the SR membrane, then it is valid to use the number of conductance jumps as a relative measure of the number of ionophores inserted into the membrane. This is an extremely convenient method of estimating ionophore concentration in the membrane, and it forms the basis of the measurement of the conductance-concentration relation.

Although the order of the steady-state conductance-concentration curve has been often used as a reasonable indication of the degree of molecular association of the conducting state of model ionophores (Eisenman *et al.*, 1973; Ehrenstein & Lecar, 1977), in the present case it is dangerous to conclude from the linearity of this relation that the K^+ channel acts as a monomer. The reason for caution here is that it is entirely possible that the ionophores do not mix with the planar bilayer after insertion, but rather remain in patches. Thus the *local* ionophore concentration would not change with the number of fusion events, and linearity in the conductance-concentration relation would be expected for *any* model of the ionophore's conducting state.

One piece of evidence argues that the above possibility of SR-like patches does not apply. This is that the ionophore's steady-state properties are sensitive to the particular lipid composition of the artificial membrane, i.e., that the ionophore senses the chemical composition of the membrane. Figure 2 shows that the position of the g-V curve along the voltage axis is dependent upon phospholipid composition. The shift shown in this example is small but consistently observed. Furthermore, voltage-jump experiments in progress show a strong dependence of lipid composition on the kinetics of turning on and off of the K^+ channel. These experiments, then, show that the membrane lipids mix upon fusion, but the possibility remains that the membrane proteins remain in patches because of local cross-linking. Until the ionophore can be isolated and reconstituted, it will be difficult to rule out this explanation. Therefore, while the evidence appears to favor a monomeric model for the K^+ channel, this conclusion must remain tentative.

4. Observation of single channels

Using conditions allowing only a small number of ionophores to enter the artificial membrane, it is possible to detect fluctuations in K^+ conductance which follow now-classical single-channel behavior (Bean *et al.*, 1969; Hladky & Haydon, 1970; Ehrenstein & Lecar, 1977). These single-channel fluctuations are associated with the voltage-dependent K^+ conductance. The data show clearly that the voltage-dependence of the steady-state conductance is due to the voltage-dependence of the probability of the ionophore being in the conducting state. The conductance of the open channel itself is independent of voltage. The fluctuations occur between only two states, "open" and "closed". This behavior is in

contrast to that of the voltage-dependent model ionophores, E.I.M. (Ehrenstein, Lecar & Nossal, 1970; Ehrenstein *et al.*, 1974), alamethicin (Eisenberg *et al.*, 1973; Boheim, 1974; Gordon & Haydon, 1976), hemocyanin (Latorre *et al.*, 1975), and monazomycin (Bamberg & Janko, 1976), which give single channels fluctuating between multiple open states. The two-state behavior described here is more reminiscent of the discrete fluctuations detected in the acetylcholine-gated cation channel of frog muscle in its native membrane (Neher & Sackmann, 1976).

The open-state conductance of the K^+-channel from SR is about 10^{-10} mho in 0.1 M K^+, a value much larger than could be accounted for by a mechanism other than a channel (Armstrong, 1975). Given this value of single-channel conductance, and knowing the conductance associated with a single SR vesicle fusion (Miller & Racker, 1976), we can conclude that an average sized SR vesicle contains at least 30 channels; given the failure to detect any intimation of a maximum conductance in the steady-state g-V curve, it is likely that each SR vesicle contains well over 100 channels.

This report has described several basic steady-state electrical properties of artificial membranes modified by ionophores from SR vesicles. A voltage-independent system (possibly anion-selective), and a voltage-dependent cation channel have been detected. The cation channel maintains a high degree of asymmetry within the membrane, as shown by the steady-state g-V curve and the sidedness of Ca^{++} inhibition. This orientation is most likely due to the process of incorporation, which is probably fusion of the SR vesicles with the planar bilayer. If this picture is correct, the *cis* side of the membrane is topologically equivalent to the myoplasmic side of the SR membrane *in situ*, while the *trans* side is equivalent to the internal SR space. It is, therefore, difficult to avoid noting that voltage changes which cause opening of the cation channel in the artificial membrane are in the equivalent direction as those which lead to Ca^{++} release in the SR membrane *in vivo*.

However, at this stage of this study, it is impossible to make serious proposals for the role of this ionophore in SR function. The system certainly induces Na^+ and K^+ permeability in a voltage-dependent fashion, but its Ca^{++} conductance has still not been measured because of technical problems involved. Until this measurement has been made, the relation of this ionophore to excitation-contraction coupling must remain unknown. Neither is it known what relation this ionophore might bear to the operation of the Ca^{++}-ATPase, the major SR protein. This protein in its purified state (Shamoo & MacLennan, 1974) or recon-

stituted in liposomes (Miller & Racker, *unpublished*) has no effect on planar bilayer conductance. However, Shamoo and MacLennan (1974, 1975) have shown that a Ca^{++}-specific ionophore can be detected in a controlled tryptic digest of Ca^{++}-ATPase. The properties of this ionophore differ vastly from those of the systems described here, and so it is unlikely that the two systems are identical.

Finally, it is important to realize that these studies should not be considered reconstitution experiments. The ionophores under study have been introduced into an artificial membrane not via the usual procedures of isolation, solubilization, and membrane reconstruction, but rather by direct incorporation from the natural membrane. Such a process of insertion carries less inherent risk of damage to the molecular species involved than does the usual reconstitution approach, and this advantage may account for the gratifying quantitative reproducibility of the system. However, a distinct disadvantage here is that since the conductance properties of the natural SR membrane are entirely unknown, we cannot be sure to what extent they are modified by the artificial membrane system. Ultimately, then, this approach must give way to resolution and reconstitution of the ionophores on one hand, and more physiological understanding of the native SR membrane on the other.

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