A Voltage-Gated Cation Conductance Channel from Fragmented Sarcoplasmic Reticulum. Effects of Transition Metal Ions†

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ABSTRACT: The effects of transition metal ions on the voltage-gated K^+ conductance of a channel-type ionophore from sarcoplasmic reticulum membranes have been studied in an artificial planar bilayer system. Channels are inserted into the planar bilayer by a process resembling fusion with sarcoplasmic reticulum vesicles. K^+ conductance is modulated by transition metal ions in two ways. First, the conductance is irreversibly inhibited by certain ions added on either side of the membrane. The inhibition rate constants are ordered: $Ag^+ > Hg^{2+} > Cu^{2+} > Cd^{2+} \approx C_6H_5Hg^+ \approx CH_3Hg^+ > Pb^{2+} \approx Zn^{2+} > PHMBS^- >$ mersalyl$^-$. Mn^{2+}, Co^{2+}, and Ni^{2+} are inactive. The rate constant of inhibition by mersalyl is voltage dependent. Second, certain ions cause a reversible three- to fourfold stimulation of the K^+ conductance; the effect is seen only when the transition metal ion is added to the side of the membrane opposite to the side containing sarcoplasmic reticulum vesicles. The affinity constants for this stimulation fall in the order: $Cu^{2+} > Zn^{2+} \approx Cd^{2+} > Pb^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$. Organomercurials, Hg^{2+}, and Ag^+ fail to give this trans stimulation effect. These effects are seen only on the monovalent cation conductance channel, not on the anion conductance pathway, which can be assayed under different conditions. Neither effect is given by alkaline-earth cations. Single-channel fluctuation experiments demonstrate that the trans stimulation is an effect upon the probability of the channel's opening rather than on the conductance of the open channel. The possibility of a sulfhydryl group's involvement in the active channel is raised, and a simple model for the channel is presented.

In recent years, two independent lines of evidence have indicated that the sarcoplasmic reticulum (SR)[1] of vertebrate skeletal muscle contains a monovalent cation selective permeability pathway. By means of radioactive flux experiments on SR membrane vesicles, McKinley & Meissner (1977) found that the majority of the vesicles (50–70%) are highly permeable to Na^+, K^+, and Rb^+, but not to choline$^+$ or Tris$^+$. An alternative approach has been to cause SR vesicles to interact with an artificial planar phospholipid bilayer in a process resembling membrane fusion and then to examine the electrical conductance properties of the resulting "hybrid" membrane (Miller & Racker, 1976; Miller, 1978). While the former approach has the distinct advantage of studying native SR membranes, the latter method has the advantages of extreme sensitivity, millisecond time resolution, and control of the voltage across the membrane. Using the electrical technique, Miller (1978) has described the following properties of an ion conductance pathway from the SR membrane. (1) The pathway is monovalent cation specific, displaying high conductance for Na^+ and K^+ but not for Li^+, choline$^+$, or Tris$^+$. (2) The pathway is voltage-gated, being in a low-conductance state at certain voltages and a high-conductance state at other voltages. The intramembrane electrical field controls the state of the ionophore. (3) The ionophore is asymmetrically oriented within the artificial bilayer and, by implication, within the native SR membrane. (4) The ionophore operates by a two-state channel mechanism in which the voltage across the membrane controls the probabilities of existing in the "open" and "closed" states. (5) The channel contains a blocking site which, when occupied by certain divalent cations (Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}), reversibly inhibits the conductance of the channel to monovalent cations. This blocking site is accessible to divalent cations added only to one side of the artificial membrane.

In extending the above work on the blocking site, we came upon several unexpected effects of transition metal ions, and it is these effects with which this report is concerned. We have found that in addition to the reversible blocking site mentioned above, the channel contains two other sites which modulate the channel's K^+ conductance: an irreversible inhibitory site having properties suggestive of a sulfhydryl group and a reversible stimulating site, accessible from only one side of the membrane, the side opposite to the reversible blocking site.

Materials and Methods

Biochemical. SR vesicles were prepared from rabbit white dorsal and leg muscle by the method of either MacLennan (1970) or Meissner (1975), modified as follows. After an initial homogenization in either 0.15 M NaCl–5 mM Hepes or 0.18 M sucrose–5 mM Hepes, pH 7.5, homogenates were centrifuged at 9000*g* (av) for 12 min. The supernatant was adjusted to pH 7.0 with NaOH and was then centrifuged at 68000*g* for 45 min. The pellets were suspended in 0.4 M sucrose–2 mM Hepes–KOH, pH 7.5, and this suspension was centrifuged at 5000*g* for 10 min. The supernatant was then centrifuged at 68000*g* for 45 min, and the pellet was suspended in 0.4 M sucrose–2 mM Hepes–KOH, pH 7.5, to a protein concentration of 25–50 mg/mL. This preparation was then frozen in small aliquots at −70 °C. In a few cases, the preparation was further fractionated on discontinuous sucrose gradients according to Meissner (1975), and the "intermediate" fraction was collected and resuspended in 0.4 M sucrose–2 mM Hepes–KOH as above. SR made as above without sucrose gradient fractionation displayed the following activities measured at 25 °C: Mg^{2+}–ATPase, <0.1 μmol/(mg min); (Mg^{2+} + Ca^{2+})-ATPase, 0.2–0.3 μmol/(mg min) (without A23187 present); (Mg^{2+} + Ca^{2+})–ATPase + 3 μM A23187, 2.0–2.5 μmol/(mg min); Ca^{2+} uptake (oxalate independent), 80–180 nmol/mg; cytochrome oxidase, 1–3 nmol of O_2/(mg min).

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[1] Abbreviations used: EDTA, (ethylenedinitrilo)tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mops, morpholinopropanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PHMBS, *p*-hydroxymercuriphenylsulfonic acid; SR, sarcoplasmic reticulum; Tris, tris(hydroxymethyl)aminomethane. Mersalyl is the generic name for *N*-(γ-hydroxymercuri-β-methoxypropyl)salicylamide-*O*-acetic acid.

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Phospholipids were prepared from bovine heart muscle following a modified procedure of Eichberg & Burnham (1970). Four hundred grams of ground heart muscle was blended in a liter of chloroform–methanol (2:1, v/v) for three 15-s bursts at 4 °C. The homogenate was filtered on Whatman No. 1 paper, and the residue was blended as above in 2 L of chloroform–methanol. The filtrates were combined and enough chloroform–methanol was added to give a single-phase system. One-fifth volume of 0.1 M NaCl was then added and the mixture shaken vigorously. The phases were allowed to separate under argon in the cold. The lower phase was siphoned off and shaken against one half-volume of chloroform–methanol–0.1 M NaCl (3:48:47). The suspension was allowed to separate overnight in the cold, and the lower phase was reduced in volume to 300 mL on a rotary evaporator. The solution was chilled for 20 min at −70 °C and was added to a liter of diethyl ether–absolute ethanol (1:1) prechilled to −70 °C. After 2 h at −70 °C (or after 20 h at −20 °C) the precipitate was collected by centrifugation in glass bottles at 1000*g*, 5 min at −15 °C. The supernatant was decanted and the pellet was washed with ether–ethanol (1:1) at −70 °C. Again this was centrifuged as above, and the supernatants were combined and set aside for purification of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The pellet, which contains most of the acidic lipids, was dissolved in 400 mL of chloroform–methanol (2:1). After washing this solution with one-fifth volume of 0.1 M trisodium citrate and then with two-thirds volume of chloroform–methanol–0.1 M NaCl (3:48:47), the lower phase was evaporated to dryness and resuspended in 30 mL of chloroform–methanol (2:1). This suspension was centrifuged at 15000*g* for 15 min, and the supernatant was applied to a Sephadex LH-20 column (2.5 × 100 cm) equilibrated with chloroform–methanol (2:1). The acidic phospholipids were then eluted with this solvent running at 1–1.5 mL/min, emerging between 350 and 750 mL. Those fractions containing only acidic phospholipids were pooled and concentrated and were used as a mixed acidic phospholipid preparation. This preparation was about 60% diphosphatidylglycerol and 40% phosphatidylinositol; a small amount of phosphatidylserine was also present.

For the isolation of PE, the above "PC + PE" supernatant was evaporated, dissolved in chloroform, and fractionated on silicic acid (Kagawa et al., 1973). All phospholipid fractions were stored in chloroform solutions under argon at −70 °C.

Electrical. Artificial planar phospholipid bilayer membranes were formed by the method of Mueller & Rudin (1969). Briefly, the cell consisted of two aqueous chambers defined by a polystyrene cup seated in a figure-eight-shaped poly(vinyl chloride) dish. The planar bilayers were formed by applying a drop of phospholipid solution (25 mM in *n*-decane) to a hole (1.2-mm diameter) drilled in the cup. Thinning to a bilayer was monitored visually and by capacitance measurement. Further details of the system have been reported previously (Miller & Racker, 1976; Miller, 1978). All experiments reported here used a membrane phospholipid composition of 70% PE–30% acidic phospholipids (mole P basis).

For measurement of K^+ conductance, the aqueous phase of the artificial membrane system was 0.1 M potassium glucuronate, 10 mM Mops–KOH, pH 7.0, also containing 10–100 μM EDTA–Tris. In experiments in which divalent cations were added to the buffer, the free concentration calculated on the basis of complexation with EDTA is reported. The above buffer solution was chosen because it allows the measurement of K^+ conductance of the artificial membrane without interference from other ions (Miller & Racker, 1976; Miller, 1978). In some experiments Cl^- conductance was measured by using buffer containing 0.1 M choline or Tris-Cl^- (Miller, 1978). Each aqueous chamber was connected to a Ag/AgCl pellet-type electrode (Phipps & Lucchina, 1964; Martin et al., 1970) via a glass salt bridge containing 0.2 M KCl–2% agar. Experiments were carried out at room temperature, 20–22 °C.

The electrical conductance of the artificial membrane was measured by a current-to-voltage transducer circuit operating under voltage-clamp conditions, as described by Miller (1978). The voltage applied across the membrane was supplied by a 15-V dry-cell battery stepped down through a variable voltage divider.

Fusion of SR with the Artificial Membrane. The technique used to cause SR vesicles to insert conductance channels into the planar bilayer by a process resembling membrane fusion has been described in detail (Miller & Racker, 1976). Briefly, Ca^{2+} (as the glucuronate salt) was added to one side of the membrane (0.1–1 mM final concentration) with stirring. (Mixing time of the chamber under these conditions was 5–10 s.) SR vesicles (10–100 μg/mL) were then added to the same chamber, and membrane conductance was followed at +25-mV applied voltage. The interaction of SR vesicles with the membrane was stopped in one of two ways depending on the experiment to be performed. In experiments using divalent cation concentrations greater than 100 μM, fusion was stopped by addition of EDTA in excess of the Ca^{2+} present. In experiments using divalent cation concentrations less than 100 μM, fusion was stopped by perfusing away the SR vesicles using buffer free of EDTA (see below). After the fusion is stopped by either method, the membrane conductance does not change for at least 30 min (unpublished experiments). In all experiments to be reported, the cis side of the membrane is defined as the side to which the SR vesicles are added; the opposite side will be referred to as the trans side. Voltage across the membrane is defined with respect to the trans side (i.e., the trans chamber is virtual ground).

In some experiments, it was necessary to change solutions in the aqueous chambers during an experiment, with a planar bilayer in place. This was accomplished with the use of a perfusion apparatus consisting of two 10-mL syringes with plungers mounted back-to-back, the "delivery" syringe full of new solution to be added, and the "removal" syringe empty, with its plunger fully inserted. The syringes were connected to the chamber to be perfused with thin Tygon tubing. As the syringe plungers were moved from one extreme position to the other, equal volumes of new and old solutions were added and removed simultaneously, leaving the mechanically fragile membrane undisturbed. In this way, the 3-mL cis chamber could have 95% of its aqueous solution replaced (measured colorimetrically using dyes) in one pass of the syringe plungers, with a volume error of less than 30 μL. For a comparable extent of replacement, the 6.7-mL trans chamber was perfused with two passes of the plungers.

Results

In this section we will describe three effects of transition metal ions upon the K^+ conductance of the SR cation channel. The first effect is an irreversible inhibition of the K^+ conductance when the ion is added to the cis side of the membrane; the second effect is a similar inhibition from the trans side of the membrane; the third effect is a reversible stimulation of K^+ conductance seen only when the metal ion is added to the trans side. In all the experiments, SR vesicles were fused

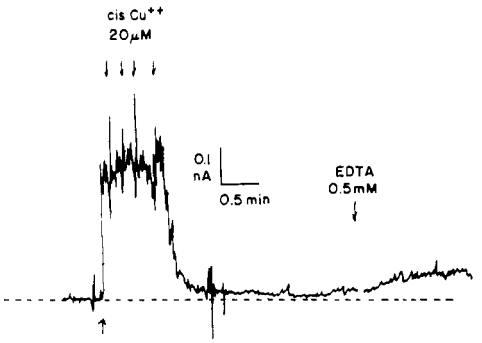

FIGURE 1: Irreversible inhibition of K^+ conductance by cis Cu^{2+}. SR vesicles were fused with the planar bilayer in the presence of 0.9 mM Ca^{2+}, and fusion was stopped by perfusion of the cis chamber with buffer containing 60 μM EDTA. At the arrow below the dashed line, 30 mV was applied across the membrane, and resulting current was followed. Additions of 20 μM (total concentrations) $CuSO_4$ were made where indicated by arrows. After inhibition was complete, 500 μM EDTA was added where indicated. Initial conductance was 1.8 μmho/cm^2.

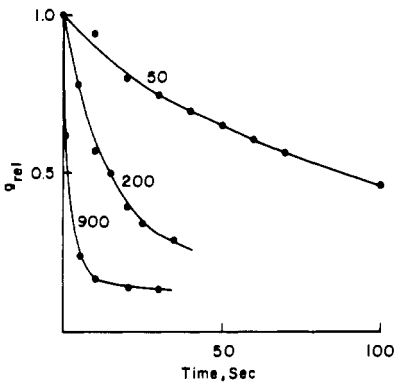

FIGURE 2: Inhibition by cis Zn^{2+} at different concentrations. Details are as in Figure 1, except that perfusion was done with buffer lacking EDTA. At zero time, $ZnSO_4$ was added to final concentrations indicated on graph in units of μM, and the conductance time course was followed. Time courses are from three different planar membranes, with initial conductance in the range 2.9–9.5 μmho/cm^2. Time courses are plotted with conductances, g_{rel}, normalized to the initial value before addition of Zn^{2+}.

with planar artificial membranes as described under Materials and Methods such that the conductance increased several orders of magnitude. The transition metal ions were then added to the appropriate side of the planar membrane, and the conductance response was followed.

Cis Inhibition. Figure 1 shows the effect of cis Cu^{2+} upon the channel's K^+ conductance. In this experiment, the 60 μM EDTA present initially prevented the first three additions of 20 μM Cu^{2+} from exerting an effect. The fourth addition, which brought the Cu^{2+} concentration to 20 μM in excess of the EDTA, caused a rapid inhibition of the K^+ conductance. Subsequent addition of a vast excess of EDTA did not reverse the inhibition. The small increase in conductance seen after this addition of excess EDTA is slow and variable; the voltage-dependent K^+ conductance properties of the channel (Miller, 1978) are completely lost after the inhibition (data not shown). It is possible to quantify this cis inhibition effect by the rate constant of inhibition, which we have found to be linearly dependent upon the inhibiting ion concentration (Figure 2). We can therefore define an apparent second-order rate constant of inhibition as the initial rate of conductance decrease normalized to the initial conductance level, divided by the inhibiting ion concentration. We emphasize that this is an apparent rate constant serving only as a relative index of effectiveness since we have not determined that all ions

Table I: Parameters of Transition Metal Ion Modulation of K^+ Conductance[a]

ions	cis-inhibition rate constant log k (M^{-1} s^{-1})	trans-inhibition rate constant log k (M^{-1} s^{-1})	trans-stimulation dissociation constant K_d (μM)
Mn^{2+}	N.A.	N.A.	>3000 (>800)
Co^{2+}	N.A.	N.A.	1500 (300)
Ni^{2+}	N.A.	N.A.	500 (100)
Cu^{2+}	3.6 (4.6)	2.4 (3.4)	1 (0.1)
Zn^{2+}	2.2 (2.9)	1.3 (2.0)	20 (4)
Cd^{2+}	3.0 (3.5)	1.7 (2.3)	20 (6)
Hg^{2+}[b]	4.4 (4.5)	4.3 (4.4)	N.A.
Pb^{2+}	1.9 (3.0)	0.5 (1.6)	400 (35)
Ag^+	>5.2 (>5.3)	>5.0 (>5.1)	N.A.
$C_6H_5Hg^+$	3.5	3.3	N.A.
CH_3Hg^+	3.3	3.0	N.A.
mersalyl$^-$	1.0	0.8	N.A.
$PHMBS^-$	1.5	1.4	N.A.

[a] Second-order rate constants for cis and trans inhibition were measured at an applied voltage of 35 mV by normalizing the initial rate of conductance decline to the zero-time conductance level and dividing by the inhibitor concentration. Rate constants measured in this way were independent of inhibitor concentration over the range of measurement; this range was adjusted such that reaction times of 30 s to 5 min were needed to give 50% inhibition. Dissociation constants for trans stimulation were measured by titration of the conductance as in Figure 4, using concentrations in the range $0.3K_d$–$3K_d$. Data represent the means of three–five determinations; the rate constants are reliable within ±0.2 log unit and the dissociation constants within ±30% at most (range of two–five determinations). "N.A." indicates that the ion is not active in the effect under consideration. Data in parentheses are corrected for estimated complexation of the transition metal ion by the 0.1 M glucuronate present in the assay medium (see Discussion). [b] Correction for Hg^{2+} complexation by glucuronate was estimated to be similar to that for Ag^+ since both ions are linearly coordinated in aqueous solution, where the mercuric ion exists primarily as $HgOH^+$.

tested follow exponential inhibition kinetics throughout the entire time course of inhibition; in the case of Hg^{2+}, however, we did make careful measurements (not shown) and found pseudo-first-order kinetics to at least 90% inhibition.

Second-order rate constants for cis inhibition were measured for a series of transition metal ions and organomercurials and were found to vary over several orders of magnitude (Table I). The effectiveness of inhibition is ordered: $Ag^+ > Hg^{2+} > Cu^{2+} > Cd^{2+} \approx C_6H_5Hg^+ \approx CH_3Hg^+ > Pb^{2+} \approx Zn^{2+} > PHMBS^- >$ mersalyl$^-$. It is noteworthy that Mn^{2+}, Co^{2+}, and Ni^{2+} were completely ineffective as irreversible inhibitors, even at very high concentrations (>3 mM).

Trans Effects. When transition metal ions are added to the trans side of the planar bilayer, two different types of effects are observed (Figure 3). Certain ions (Ag^+, Hg^{2+}, organomercurials) inhibit the K^+ conductance, as is shown for Ag^+ in Figure 3. Other ions (Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}) cause a rapid stimulation of the K^+ conductance, followed by slower inhibition, as shown in Figure 3 for Zn^{2+}. Finally, Ni^{2+}, Co^{2+}, and Mn^{2+} cause only a stimulation of K^+ conductance, never an inhibition, as is shown for Ni^{2+} in Figure 3. We propose that the stimulation and the inhibition on the trans side are two separate effects and that certain "dual effector" ions (Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}) cause both, while other ions cause only one of the two effects. Below, we demonstrate that these effects can be quantified separately.

Trans Inhibition. Like the cis inhibition effect, trans inhibition is irreversible (Figure 3). Second-order rate constants were measured for trans inhibition and are presented in Table I. There is good correlation between the rate constants for inhibition on the two sides of the membrane; the rate constants

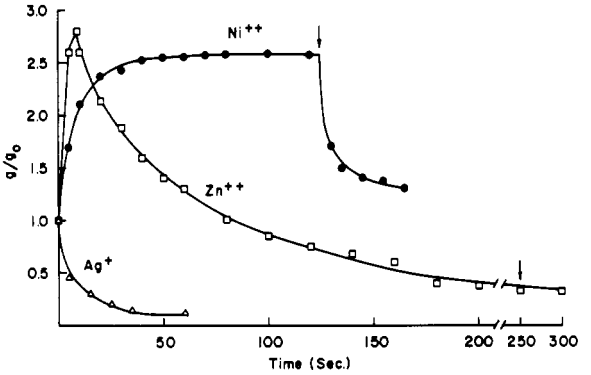

FIGURE 3: Trans effects of various transition metal ions. Details are as in Figure 1, except that fusion was stopped by cis addition of 1 mM EDTA. After the conductance had stabilized, metal ions indicated on the graph were added to the trans chamber, and time courses of the conductance changes were followed. The effectors were added to the following final concentrations: Ni^{2+}, 1.5 mM; Zn^{2+}, 0.9 mM; Ag^{+}, 0.4 μM. At the arrows indicated on the time courses, twofold excess EDTA was added to show the reversibility of the Ni^{2+} stimulation and the irreversibility of the Zn^{2+} inhibition. Conductance is plotted normalized to its value, g_0, before addition of effector.

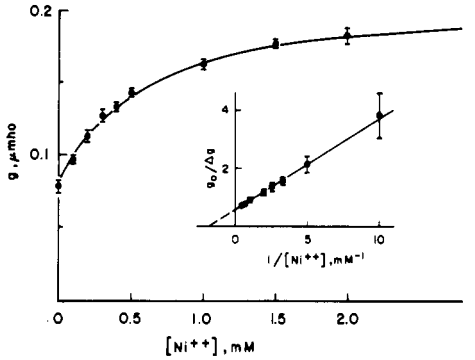

FIGURE 4: Titration of stimulation by trans Ni^{2+}. Details are as in Figure 3. After conductance had stabilized, $Ni(NO_3)_2$ was added to the trans chamber in increasing concentrations, and the conductance at each concentration was recorded. The new level of conductance was established within 15 s of each Ni^{2+} addition and was stable until the next addition. After conductance had been stimulated maximally by addition of 3 mM Ni^{2+} total, it was checked that subsequent addition of 1 mM Zn^{2+} caused no further stimulation, only a rapid inhibition. The inset shows a double-reciprocal plot of the fractional stimulation, $\Delta g/g_0$, against Ni^{2+} concentration. Parameters derived from this plot were K_d = 0.52 mM and maximal fractional stimulation $(\Delta g/g_0)_{max}$ = 1.8. These parameters were used to draw the solid curve in the main figure according to the following: $g = g_0 + \Delta g_{max} \cdot (Ni^{2+})/[K_d + (Ni^{2+})]$. Error bars on data represent the maximum range of electrical noise about the mean conductance.

vary over four orders of magnitude with the same relative effectiveness, although the absolute values of the rate constants are always larger for cis inhibition than for trans inhibition. This correlation suggests that the same type of site, if not the same site, is involved in the irreversible inhibition effects.

Trans Stimulation. This effect is qualitatively reversible, as is shown in Figure 3 for Ni^{2+}. Figure 4 presents the dependence of the relative stimulation upon Ni^{2+} concentration; a single-site binding curve is followed well, characterized by an apparent dissociation constant, K_d, and a maximal stimulation, which is 2.8-fold in this example. Although Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} show inhibition in addition to stimulation, it is nevertheless possible to estimate K_d's for stimulation by these ions as well as for "pure" stimulators such as Ni^{2+}. This is because for a given ion, the concentrations at which stimulation is observed are much lower than for those causing rapid inhibition. Thus, 2 μM Cu^{2+} gives nearly maximal stimulation, but the inhibition at this concentration takes over

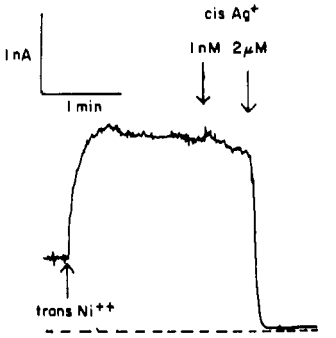

FIGURE 5: Inhibition and stimulation effects are in series. Fusion of SR with the planar bilayer was induced by 1 mM Ca^{2+}, and the reaction was stopped by addition of 1.5 mM EDTA. Initial conductance stabilized at 3.7 μmho/cm^2. A trans addition of 2 mM Ni^{2+} was made at the first arrow, and after the membrane conductance had stabilized, cis Ag^{+} was added to a final concentration as indicated on the graph. Dashed line marks the level of zero current.

10 min to proceed to 50% completion (data not shown). Thus, by rapidly titrating these dual effector ions, it is possible to obtain a rough estimate of the stimulation constants. These constants, which vary over several orders of magnitude, are compiled in Table I.

A question arises about the stimulation shown with the dual-effector ions: is this stimulation the same effect as that given by the "pure" stimulators, Ni^{2+}, Co^{2+}, and Mn^{2+}? Two lines of evidence support an affirmative answer. First, the stimulation effect by the dual effectors Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} is reversible, as for the pure stimulators. This can be demonstrated by adding EDTA after the immediate stimulation by the dual effector is seen, but before the time-dependent inhibition has proceeded to a significant degree. In such an experiment (not shown), the stimulated conductance returns to within 20% of its value before dual effector was added. The second line of evidence is that all the ions stimulate to the same maximal level, three- to fourfold. The stimulation effects by two different ions are additive only well below the K_d's for both ions; if a high concentration of a pure stimulator such as Ni^{2+} is first added to stimulate maximally, then the subsequent addition of a dual effector such as Zn^{2+} gives only inhibition (see legend to Figure 4).

Another important question to arise from the data is the following: are the two effects, stimulation and inhibition, in parallel operating on different systems or in series operating on the same channel? Figure 5 demonstrates that the two effects are in series. The K^{+} conductance i first stimulated maximally by trans Ni^{2+}; then a "pu-e" i hibitor, Ag^{+}, is added on the cis side of the membrane. Ev[illegible] nM Ag^{+}, a slow inhibition is observed, and when the A $^{+}$ concentration is increased to 2 μM, a rapid, irreversible, a id complete inhibition ensues. The fact that this inhibition is complete shows that Ag^{+} inhibits both the basal K^{+} conductance and the Ni^{2+}-stimulated K^{+} conductance. The simplest explanation for this result is that the two effectors are operating upon the same conductance pathway. In this experiment, the Ag^{+} was added cis rather than trans so that it cannot be argued that the effect of Ag^{+} ion was to displace Ni^{2+} from the trans-facing stimulatory site; identical results are obtained with Ag^{+} added to the trans side.

It might be argued that possibly the trans stimulation is due to conductance by the transition metal ion itself, not due to modulation of K^{+} conductance. That this is not the case is confirmed in control experiments (not shown) in which trans additions of ions are made in solution free of small monovalent ions; for instance, in 0.1 M Tris-Cl, a medium which allows

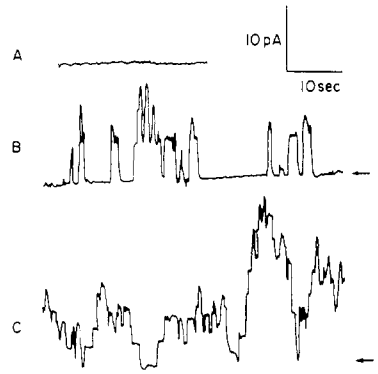

FIGURE 6: Effect of trans Ni^{2+} on single-channel fluctuations. Conditions for observing single-channel fluctuations of K^+ conductance were achieved as has been described (Miller, 1978). In this experiment, a low concentration of SR (2 μg/mL) was allowed to interact with the planar bilayer in the presence of a low concentration of Ca^{2+} (0.1 mM). Immediately after a single fusion was observed (3 min after adding vesicles), further fusion was prevented by perfusing away the SR vesicles and adding 0.1 mM EDTA. Conductance fluctuations were examined at −50 mV, an applied voltage at which the channel is normally in its closed state most of the time. Trace A was recorded before addition of SR vesicles or Ca^{2+}. Trace B was recorded after fusion of a single vesicle and subsequent perfusion of the chamber, as described above. Immediately after trace B was recorded, 2.3 mM Ni^{2+} was added, with stirring. Ten seconds after this addition, the stirrer was turned off, and trace C was recorded. The arrows in traces B and C mark the "zero-level" of conductance with no channels in the open state. This zero-level conductance was about 30% higher than the conductance of the unmodified membrane in trace A. Time-averaged conductance was estimated by integrating the traces, using the zero-level as base line, by cutting and weighing the traces. Mean single-channel conductances were roughly calculated from these traces by using only transitions which could be clearly distinguished by eye, given the time resolution of the chart recorder. The values were as follows: before Ni^{2+} addition, 120 pmho ± 3 pmho (SE of 16 measurements); after Ni^{2+} addition, 62 pmho ± 2 pmho (SE of 10 measurements).

the Cl^- conductance of the SR-doped planar bilayer to be measured, trans addition of Zn^{2+} or Cu^{2+} gave no stimulation effect whatever, measured at 50 or −50 mV.

Since it is known that the K^+ channel under study operates by a two-state mechanism (Miller, 1978), there are two conceivable ways in which trans stimulation could occur. The stimulating ion could either increase the conductance of the open state of the channel or it could increase the probability of the channel's existing in the open state. The experiments presented above cannot distinguish the two possibilities since they are concerned with the average behavior of a large number of channels. However, the question can be attacked by examining single-channel conductance fluctuations in planar membranes into which only a very small number (30–100) of channels has been inserted. The technique for observing single channels in this system has been described (Miller, 1978), and a typical experiment is shown in Figure 6. Trace A shows that before interaction with SR vesicles, there is very little conductance "noise" in the system. After interaction of the planar bilayer with a single SR vesicle, conductance fluctuations due to the random opening and closing of individual channels are observed (trace B). At the voltage applied in these experiments (−50 mV), the channels are all in the "closed" state most of the time. Occasionally one or two channels randomly open, raising the membrane conductance by unitary jumps. The average single-channel open state conductance in trace B is 120 pmho, in good agreement with that reported previously (130 pmho) for this channel (Miller, 1978). Trace C was taken after addition of a maximal concentration of trans Ni^{2+}, added immediately after trace B

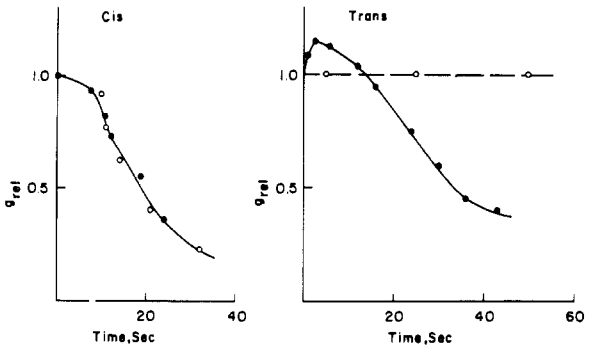

FIGURE 7: Effect of cis and trans mersalyl at 50 and −50 mV. Details of SR fusion and stopping are as in Figure 3. After conductance had stabilized, Tris–mersalyl was added either on the cis or trans side of the planar membrane to a final concentration of 1 mM, and the conductance time course was followed at either 50 or −50 mV. Data here are taken from four separate planar bilayers, one for each combination of the two applied voltages, with additions on the two sides. Data are normalized in each case to the conductance value before addition of mersalyl, and this relative conductance, g_{rel}, is plotted. Conductances at 50 mV were five- to sevenfold higher than those at −50 mV. Closed circles, 50-mV applied voltage; open circles, −50-mV applied voltage.

was recorded. The time-averaged conductance (measured by integrating the traces and dividing by the total time in each trace) was increased 3.3-fold by Ni^{2+}, in agreement with the stimulations observed in the macroscopic experiments above. However, the single-channel open-state conductance was actually decreased to 62 pmho, an inhibition of 48%. The stimulation of the time-averaged conductance was due to a greatly increased probability of the channel's existing in the open state. Whereas before addition of Ni^{2+} the system fluctuated between zero and two channels being open, after Ni^{2+} addition at least two channels were open most of the time and occasionally as many as five were open. Thus, the trans stimulation is attributable to two effects; the effector actually blocks the open channel conductance by about 50%, but it more than compensates for this blocking by making the open state of the channel thermodynamically more favorable.

Voltage-Dependent Inhibition by Mersalyl. Mersalyl, a carboxylic organomercurial frequently used as a sulfhydryl reagent, inhibits the K^+ conductance when added to either side of the membrane (Figure 7). When the addition is made to the cis side of the membrane, the inhibition behaves in a way qualitatively similar to that of the other agents described above; this cis inhibition is equally effective at −50 mV. However, with mersalyl added to the trans side, a major difference between the 50 and −50 mV time courses is apparent. At 50 mV mersalyl inhibits as expected, but at −50 mV, no change in K^+ conductance occurs.

There are two possible reasons for the failure of trans mersalyl to inhibit at −50 mV; either the reaction of mersalyl with the appropriate site does not occur at this voltage or the −50-mV conductance is not sensitive to mersalyl complexation at this site. Figure 8 demonstrates that both of these possibilities are correct. In this experiment, trans mersalyl is added at an applied voltage of −50 mV. After 45 s, during which time no change in the −50-mV conductance is seen, the voltage is changed to +50 mV. Immediately after the voltage jump, the +50 mV conductance is as high as it was before mersalyl was added; thus, during the 45 s at −50 mV, the reaction of mersalyl with the trans inhibitory site failed to occur. However, after the voltage jump was made, inhibition of the +50-mV conductance commenced at once, at a rate similar to that shown in Figure 7. The voltage was held at 50 mV for 2.9 min, and the inhibition proceeded nearly to

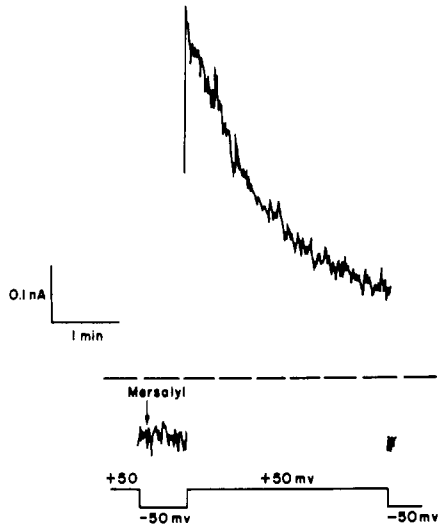

FIGURE 8: Effect of voltage jump on inhibition by trans mersalyl. SR vesicles were fused and stopped as in Figure 3. The K^+ conductance was monitored at 50 and −50 mV as shown on the lower "voltage-command" trace. Initial values of conductance at both voltages were obtained as indicated on the chart recorder tracing. Tris-mersalyl (1 mM) was added at −50 mV, as indicated by the arrow, and voltage was held at −50 mV for 45 s. The voltage was then jumped to 50 mV and held at that value for 2.9 min, during which time inhibition occurred. The voltage was then jumped back to −50 mV to check the −50-mV conductance after the inhibition reaction had occurred. The dashed line represents zero current level.

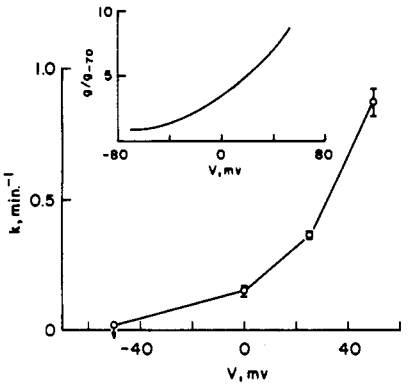

FIGURE 9: Voltage dependence of rate constant of trans mersalyl inhibition. SR fusion and stopping were carried out as described in Figure 3, and the pseudo-first-order rate constant of inhibition by trans mersalyl (1 mM concentration) was measured at different voltages as follows. After the conductance had stabilized, the appropriate holding voltage was applied for 10 s; a control value of the conductance was then obtained by pulsing to the "assay" voltage of 50 mV for 4 s and then returning to the holding voltage. Mersalyl was then added to the trans side, and every 30 s thereafter, the effect of the mersalyl was assayed by a 4-s pulse to 50 mV. The 4-s pulse was short enough so that no substantial inhibition occurred during the pulse. It was necessary to perform the experiment in this way so that the effect of the reaction of mersalyl with the channel was always assayed at the same voltage. In this way, the voltage dependence of the *progress* of the inhibition reaction could be separated from the voltage dependence of the *effect* of the reaction. Pseudo-first-order apparent rate constants were measured as described in Table I. Inset: Conductance–voltage relation measured under the same conditions as in the inhibition experiments, but without mersalyl present. Steady-state conductance is plotted relative to its value at −70 mV as a function of applied voltage, as detailed previously (Miller, 1978).

completion. The voltage was then returned to −50 mV in order to assay the effect of this inhibition reaction upon the −50-mV conductance. Surprisingly, no inhibition was seen.

In order to understand the differences in the effect of mersalyl at the two different voltages, it was necessary to realize that the 50-mV conductance assays the open state of the voltage-gated K^+ channel, whereas the −50-mV conductance assays the low, but nonzero, conductance of the closed state of the channel (Miller, 1978). The experiment in Figure 8, then, leads to two conclusions. First, it indicates that mersalyl can react only with the open conformation of the channel, not with the closed conformation; second, it demonstrates that even after the irreversible inhibition reaction has taken place, the closed state's conductance is unaffected. If the above proposal of selective reactivity with the open state is correct, then the inhibition rate constants of trans mersalyl should vary with applied voltage in the same way as does the probability of the channel's existing in the open state. That this is the case, at least qualitatively, is shown in Figure 9, which plots the dependence of inhibition rate constant on applied voltage as well as the steady-state conductance-voltage relation. This latter plot has been shown to measure the voltage dependence of the open-state probability (Miller, 1978; Coronado and Miller, unpublished experiments). Although the two plots are not superimposable at all voltages, the fit is close enough to strengthen the hypothesis that the mersalyl inhibition site is accessible to the reagent from the trans side of the membrane only in the channel's open conformation.

It is puzzling that after mersalyl has reacted fully, the −50-mV conductance is inhibited by cis mersalyl but not by trans mersalyl. This may indicate that the sites of reaction from the two sides of the membrane are different; however, additional evidence would have to be offered on this point before the proposal could be made seriously.

Finally, it should be mentioned that mersalyl is unique among all the agents tested in this study in showing a voltage-dependent rate constant. Careful measurements on the rate constants of trans Hg^{2+} show that the inhibition rate constant at −50 mV is within 20% of that at 50 mV (Miller, unpublished experiments); this is also the case for cis mersalyl (Figure 7).

Discussion

We propose that the channel contains a separate site for each effect described here: the irreversible inhibition from the cis or trans side and the trans stimulation. In order to justify this proposal we discuss below the characteristics of each effect, the possible chemical nature of the sites, and the possible artifacts involved in the measurements.

Irreversible Inhibitory Effect. Certain transition metal ions added to either side of the membrane cause inhibition of K^+ conductance, with apparent rate constants varying over four orders of magnitude (Table I). For a given ion, the cis rate constant is higher than the trans rate constant, but the order of effectiveness is the same for inhibition from either side. Thus, there is no evidence that the cis and trans inhibitory effectors are acting on different sites. Of course, this is not to claim that the cis and trans effects are on the same site either, but for the sake of simplicity, we will assume that only one site, accessible from both sides of the membrane, is involved.

Whereas the inhibitory reaction can be prevented by excess EDTA and/or 2-mercaptoethanol, once the reaction has taken place, it cannot be reversed by these agents. This fact, taken along with the rapidity with which Ag^+ and Hg^{2+} inhibit the conductance, leads us to suggest that a critical sulfhydryl residue is involved in the inhibition reaction. The inhibition by monovalent organomercurials also supports this view. In addition, the ordering of the divalent transition metal cations as above departs clearly from the Irving-Williams series (Irving & Williams, 1953; Williams, 1959; Vallee & Wacker, 1970),

which describes the interaction of divalent cations with nitrogen- and oxygen-containing ligands; instead, the order follows the affinity of these ions for model ligands containing sulfur as an electron-donating group (Vallee & Wacker, 1970). Particularly striking in this latter respect is the lack of inhibitory effectiveness of Ni^{2+}, which acts similarly to Cd^{2+} and Zn^{2+} in the Irving-Williams ordering scheme, but which interacts considerably less strongly with sulfur ligands than do Cd^{2+} and Zn^{2+}. However, *N*-ethylmaleimide, *N*-cyclohexylmaleimide, and iodoacetamide (5–10 mM, 1–3-h reaction time, 20 °C) failed to have any effect upon the K^+ conductance of the channel (data not shown). It is possible that these reagents are not accessible to the inhibitory site.

In no case have we observed 100% inhibition of the conductance. Under our usual conditions, with the inhibition reaction taking place at an applied voltage in the range 30–50 mV, the extent of inhibition is about 90%. The voltage dependence of the conductance, however, is completely eliminated; thus, the inhibitors convert the artificial membrane from a rectifier to an ohmic resistor. We do not know whether the residual conductance after inhibition is due to a background "leakiness" of the membrane or to a residual conductance of the channel in its inhibited state. Finally, it is important to note that the irreversible inhibition is distinct from the reversible inhibition at a cis blocking site described previously (Miller, 1978) for Ca^{2+}, Mg^{2+}, Ba^{2+}, and Mn^{2+}.

The case of inhibition by mersalyl, a negatively charged organomercurial, is particularly interesting because this agent displays voltage dependence of the rate constant of the inhibition reaction. However, this voltage dependence is seen only with mersalyl added to the trans side of the membrane. While cis mersalyl inhibits equally well at 50 and −50 mV, trans mersalyl shows rapid inhibition at 50 mV and very slow, if any, inhibition at −50 mV. Since the conductances at 50 and −50 mV assay the open and closed states of the channel, respectively (Miller, 1978), it is reasonable to conclude that trans mersalyl is accessible to the inhibitory site only when the channel is in the open state. This conclusion is supported by the dependence of the inhibitory rate constant of trans mersalyl upon applied voltage, which follows qualitatively the steady-state conductance–voltage relation; this latter relation describes the voltage dependence of the probability of the channel being in the open state (Miller, 1978). According to this explanation, mersalyl added to the cis side would be accessible to the inhibitory site in both the open and closed states. It is not clear why mersalyl is the only inhibitor which shows voltage dependence; possibly, because of its relatively large size and zwitterionic character, the rate-limiting step in its effect may be its accessibility to the inhibitory site, while that of the smaller cationic inhibitors is the reaction with the site itself.

Reversible Stimulating Effect. This effect, documented in Figure 3 and Table I, is seen only with trans addition of certain divalent cations, with effectiveness in the order $Cu^{2+} >> Zn^{2+} \approx Cd^{2+} > Pb^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$. Neither the "soft" ions Ag^+ and Hg^{2+} nor the "hard" alkaline-earth cations are effective. The ions follow single-site titration curves with maximal stimulations of three- to fourfold. The apparent dissociation constants vary over four orders of magnitude.

The stimulation effect is in series with the inhibitory effect. This is shown by the fact that dual effector ions give stimulation followed by a complete inhibition (Figure 3). The claim is more clearly demonstrated, however, by Figure 5, in which the conductance is first stimulated maximally by trans Ni^{2+} and then fully inhibited by 2 μM Ag^+. We cannot make any serious proposal about the chemical nature of the stimulating site.

Table II: Effect of Transition Metal Ions on Valinomycin-Mediated K^+ Conductance[a]

ion	concn required for 20% inhibn (mM)
Ag^+	>0.1
Hg^{2+}	>0.1
Cu^{2+}	0.2
Cd^{2+}	1.0
Ni^{2+}	>3.0
Zn^{2+}	2.0
mersalyl	>2.0

[a] K^+ conductance was measured in the absence of SR vesicles but in the presence of 2 nM valinomycin, under conditions identical with those used for SR-mediated conductance. This concentration of valinomycin gave membranes with conductances in the same range as those used in the SR experiments, 10^{-6}–10^{-5} mho/cm^2. The conductance was allowed to stabilize for 5–10 min after valinomycin addition, and then transition metal ions were added from stock solutions to varying final concentrations. Inhibitory effects, when present, were immediate and reversible. Alternatively, effects of these ions (with both cis and trans additions) on membranes first modified by SR vesicles to a conductance level of about 10^{-6} mho/cm^2; 10–15 nM valinomycin was then added to swamp out the SR-induced conductance. Results obtained were identical with those observed on valinomycin-treated membranes unmodified by SR.

The nature of the stimulating effect is made apparent by the single-channel fluctuation experiment shown in Figure 6. Here, we see that in a membrane containing only a very small number of channels, the major effect of a maximal concentration of trans Ni^{2+} is to increase the probability of the channel existing in the open state; the single-channel conductance of the open state is actually reduced by trans Ni^{2+}.

Possible Artifacts. In any study of the modulation of ion conductance by divalent cations in negatively charged phospholipid membranes, it is imperative to consider the effects these cations might have upon surface potentials. The planar bilayers used in these experiments are 30% negatively charged lipids, and calculations according to Muller & Finkelstein (1972) show that the surface potentials here may be as high as −80 mV. Since the K^+ conductance is determined by the local K^+ concentration at the membrane surface, and since this will vary sharply with surface potential, we must be certain that the effects described here cannot be attributed to surface potential changes caused by specific binding of and charge screening by the transition metal cations. There are several lines of evidence which rule out this possible artifact.

First, addition of divalent cations can only reduce the magnitude of the surface potential; hence, the trans stimulation effect cannot be attributed to this cause. The cis and trans inhibition cannot be due to surface potential changes either since the effects are irreversible. Second, the effects described are specific for monovalent cation (K^+) conductance; no such effects are seen on Cl^- conductance measured under the same conditions. Finally, we have studied the effects of all of the ions used here upon the valinomycin-mediated K^+ conductance under identical conditions to those used in the SR experiments. As Table II shows, these ions do have an inhibitory effect upon the valinomycin-mediated conductance (which is sensitive to surface potential), but the concentrations needed to observe only 20% inhibition are far higher than those which inhibit the SR channel fully.

Another artifact which must be considered in ordering the selectivity of the ions for both the inhibitory and stimulating effects is the complexation of the ions with glucuronate anion,

which is the major aqueous anion in the system. If there is a high degree of selectivity of the complexation reaction, then the apparent selectivity of the reactions with the SR channel will be spurious. The stability constants of transition metal ions with the glucuronate anion have not been measured. However, complexation of a few transition metal ions with the gluconate anion has been determined; these stability constants are similar to the stability constants of the same ions complexing with acetate (Sillén & Martell, 1964, 1970), and so we may use the widely studied acetate anion as an adequate model for complexation of transition metal cations by glucuronate. Using these constants to calculate the concentrations of free metal ions present in our system leads to two conclusions. First, the absolute values of the corrected rate constants are two- to tenfold higher than the apparent values; second, the order of selectivity of the ions is unchanged by correcting for complexation. In addition, experiments (not shown) in which the noncomplexing NO_3^- ion replaces glucuronate as the major anion confirm the validity of the use of the acetate complexation constants for correction of the rate constants. (Because of substantial background NO_3^- conductance, it is not practical to use this anion routinely.)

Model of the Channel. By using transition metal ions as probes for the structure of the monovalent cation channel from SR, we have added several new features to the picture of the channel described in the introduction. We propose that the channel is a protein complex which may exist in two conformational states: conducting (open) and nonconducting (closed). The equilibrium between the two states is sensitive to applied voltage because the conformational change involves a rearrangement of the protein's electrical charge such that when the channel opens, positive charge moves toward the trans side of the membrane. Thus, as the electrical potential of the cis side is made increasingly positive, the conformational equilibrium is driven toward the open state, and the conductance increases.

As described previously, the cis side of the channel carries a reversible divalent cation blocking site (Miller, 1978). The trans side of the channel contains the stimulating site specific for divalent transition metal cations. Finally, the channel contains at least one sulfhydryl group, accessible from both sides; bonding of this group with transition metals destroys the conducting structure irreversibly.

Besides leading to the above model of the channel, the data presented here strengthen several conclusions about this system which have been reached already (Miller, 1978). The first of these is that the channel is highly oriented within the artificial bilayer. The data here allow us to estimate a lower limit on the degree of asymmetry. Addition of 10 μM Cu^{2+} (or 100 μM Zn^{2+}) to the trans side of the membrane causes an immediate maximal stimulation of K^+ conductance of about 250% (Figures 3 and 4, Table I), whereas the same concentration of the ion added to the cis side causes a slow inhibition, with no discernible immediate stimulation of K^+ conductance. We would have been able to detect stimulation of 5%, and since this was not seen with cis addition, we conclude that the channel is at least 98% oriented within the planar bilayer. This high degree of asymmetry is a particularly advantageous aspect of this system. It is a consequence of the process of insertion of the channel into the artificial membrane, which is most likely the fusion of SR vesicles with the planar bilayer, such that the asymmetric native SR membrane becomes part of the artificial planar bilayer.

The second conclusion strengthened by these data is that the channel under study here is separate and distinct from the Ca^{2+} ionophore isolated by Shamoo & MacLennan (1974) from a tryptic digest of the $(Ca^{2+} + Mg^{2+})$-ATPase of the SR membrane. Miller (1978) had made this suggestion on the basis of differences between the two systems in their ion selectivities, voltage dependence, and single-channel behavior. We now see an additional important difference: while organomercurials such as CH_3Hg^+ are potent inhibitors of the SR channel, they do not affect the Ca^{2+} conductance of Shamoo's ionophore even at high concentrations (Shamoo & MacLennan, 1975; Shamoo & Goldstein, 1977). It seems likely that the channel under study here is related to the Na^+ and K^+ permeability of native SR vesicles described by McKinley & Meissner (1977). The effects of transition metal ions will provide a means of tracking this component in future purification studies.

As a final note, we should mention that our analysis of the phenomena reported here is based upon the assumption that channels are incorporated into the planar membrane irreversibly; hence, any changes in K^+ conductance are attributed to effects on the channel itself rather than on the process of incorporating the channel. This assumption remains unproven, but its validity is supported strongly by the following evidence. First, after interaction of vesicles with the planar membrane is stopped (either by chelation of Ca^{2+} or by removal of Ca^{2+} and vesicles by perfusion), the K^+ conductance is stable. Second, while SR vesicles induce conductances for K^+ and Cl^- which may be distinguished experimentally (Miller, 1978), the agents used here modulate only the K^+ conductance as described. Finally, the single-channel experiment (Figure 6) shows directly that the stimulating ions exert their effect upon properties of individual channels.

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