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Ca^{++}-Induced Fusion of Fragmented Sarcoplasmic Reticulum with Artificial Planar Bilayers

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Summary. Addition of fragmented sarcoplasmic reticulum (SR) vesicles to the aqueous phase of a black lipid membrane (BLM) causes a large increase in BLM conductance within 10 min. The conductance increase is absolutely dependent on three conditions: The presence of at least 0.5 mM Ca^{++}, an acidic phospholipid such as phosphatidylserine or diphosphatidylglycerol in the BLM phospholipid mixture, and an osmotic gradient across the SR vesicle membrane, with the internal osmolarity greater than the external. These requirements are identical to conditions under which the fusion of phospholipid vesicles occurs.

When the early part of the time course of conductance rise is examined at high sensitivity, the conductance is seen to increase in discrete steps. The probability of a step increases with the concentration of Ca^{++} in the medium, with the fraction of acidic phospholipid in the BLM, and with the size of the osmotic gradient across the SR vesicle membrane. On the other hand, the average conductance change per step is independent of the above parameters, but varies with the type and concentration of ions present in the aqueous phase. For a given ion, the mean specific conductance per step is independent of the ion's concentration between 10 and 100 mM.

The probability distribution of the step-conductances agrees well with the distribution of SR vesicle surface areas, both before and after sonication of the vesicles.

The evidence indicates that SR vesicles fuse with the BLM, thereby inserting SR membrane conductance pathways into it. Each discrete conductance jump appears to be the result of the fusion of a single SR vesicle with the BLM. This technique may serve as a general method for inserting membrane vesicles into an electrically accessible system.

The basic events in excitation-contraction coupling in vertebrate skeletal muscle are now understood (Ebashi & Endo, 1968; Sandow, 1970; Weber & Murray, 1972; Ebashi, 1976). The action potential of the surface membrane, propagating into the transverse tubular system, causes a rapid release of Ca^{++} ions stored inside the sarcoplasmic reticulum (SR).

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The consequent rise of the myoplasmic Ca^{++} concentration is the primary biochemical signal triggering the contraction of the muscle. The process by which the surface membrane's electrical signal causes an increase in the SR membrane's permeability to Ca^{++} is, however, entirely unknown. Indeed, it is not known whether there are changes in the electrical potential difference between the myoplasm and the SR internal space during excitation, or even whether such a potential difference exists at rest.

In the past, attempts have been made to impose a potential across the SR membrane and study its effect on Ca^{++} efflux (Constantin & Podolsky, 1966, 1967; Kasai & Miyamoto, 1973; Nakajima & Endo, 1973; Thorens & Endo, 1975). These methods suffered from an inability to control or measure the imposed membrane potential. A more satisfactory direct approach by voltage-clamp methods is not feasible, because of the small dimensions of SR vesicles and SR *in situ*. Therefore, in order to attack the problem of Ca^{++} release from SR, we have attempted to cause fragmented SR vesicles to fuse with an electrically accessible system, the black lipid membrane of Mueller and Rudin (1969*a*).

Since it is known that SR vesicles are quite permeable to small monovalent ions (Duggan & Martonosi, 1970; Jilka, Martonosi & Tillack, 1975), we would expect the fusion of SR vesicles with a BLM to raise the electrical conductance of the latter, in the presence of simple monovalent electrolytes. We demonstrate in the present paper that such an effect occurs. By studying the parameters influencing the conductance rise and comparing them with the requirements for a known process of fusion of phospholipid vesicles, we conclude that the fusion of SR vesicles with a BLM has been achieved.

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 at $-70°$ in 0.25 M sucrose-10 mM tricine-KOH, pH 7.6. An aliquot was thawed for each day of experimental work.

Phosphatidylethanolamine (PE) was prepared from bovine heart mitochondria and purified by silicic acid chromatography, as decribed (Kagawa, Kandrach & Racker, 1973). Phosphatidylserine (PS) was extracted from bovine brain and purified on DEAE cellulose (Papahadjopoulos & Miller, 1967). Diphosphatidylglycerol (DPG) from bovine heart mitochondria was purchased from Gibco, Inc., and was used without further purification. All mixtures of lipids are reported in percent on a mole phosphorus basis.

Electrical

Black lipid membranes were formed on a 1-mm hole in a teflon cup (3-ml volume) inside a plexiglas dish (Mueller & Rudin, 1969a). The entire system was mounted on a concrete slab resting on a partially inflated motorcycle innertube. The phospholipid solution (10–20 mM lipid P in *n*-decane) was applied to the hole with a teflon rod. After thinning, membranes had resistances greater than 2×10^7 ohm-cm^2 and capacitances in the range 0.3–0.5 μF/cm^2.

The aqueous phase of the BLM system normally contained 50 mM of the appropriate salt, 5 mM HEPES[1], 0.1 mM EDTA, adjusted to pH 7.0 with tris base. When Ca^{++} was added to the buffer its concentration in excess of the EDTA is reported. Small calomel electrodes were connected to the aqueous chambers with glass salt bridges (0.1 M KCl or Li_2SO_4+2% agar).

In the early experiments, BLM conductance was measured by a voltage divider circuit; a known voltage was applied across the BLM and a standard resistor in series. The voltage appearing across the BLM was fed to a voltage-follower (Analog Devices 311J, input resistance $>10^{14}$ ohm) and recorded on a chart recorder. It was necessary to calculate the conductance from the chart record at discrete time points, since neither the conductance nor resistance is a linear function of the recorded voltage. The membrane conductance, g, is given by

$$g = \left(\frac{E}{V} - 1\right) \Big/ R_0,$$

where E is the voltage applied across the BLM and standard resistor in series, V is the voltage appearing across the BLM, and R_0 is the value of the standard resistor (10^7 ohm–10^9 ohm in these experiments). The conductance measurement was improved in later experiments by applying a known voltage across the BLM and measuring the resulting current with a circuit described by Montal and Mueller (1972), except that the operational amplifier used was Analog Devices 310J. In this way, conductance could be recorded continuously.

Procedure for Fusion of SR Vesicles with BLM

After complete thinning of the BLM, stirring of the "inner" chamber with a teflon-coated magnetic flea was begun and a concentrated solution of $CaCl_2$ or HEPES-$Ca(OH)_2$ was added to this chamber to the desired final Ca^{++} concentration. Conductance was monitored between +25 and −25 mV ("outer" chamber defined as zero voltage). Under the conditions used here, there was no appreciable voltage-dependence of the conductance in this voltage range. The fusion reaction was initiated by addition of a small volume (1–5 μl) of SR vesicle suspension to the inner chamber; typical final SR protein concentrations were in the range 10–50 μg/ml. All experiments were done at room temperature, 20°–24°.

Electronmicroscopy of SR Vesicles

A suspension of SR vesicles at a protein concentration of 1 mg/ml in a solution containing (in mM) 50 KCl, 5 HEPES, 0.1 EDTA and 0.7 $CaCl_2$ was applied to a carbon-coated copper grid and negatively stained with 2% phosphotungstic acid. Electronmicroscopy was performed by Dr. J. Telford with an Allied Electronics Industries, Inc., electronmicroscope.

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

An estimation of the statistical distribution of the SR vesicle surface area was obtained by computing the product of the longest and shortest dimensions of each vesicle as measured on the electronmicrograph. All vesicles in the field were counted.

Results

The basic observation is shown in Fig. 1. Upon addition of SR vesicles to one side of a BLM made of 30% PS-70% PE, an increase in membrane conductance is observed after a 15–30 sec lag, provided that Ca^{++} (0.5 mM) is present on the same side of the BLM as the SR vesicles.

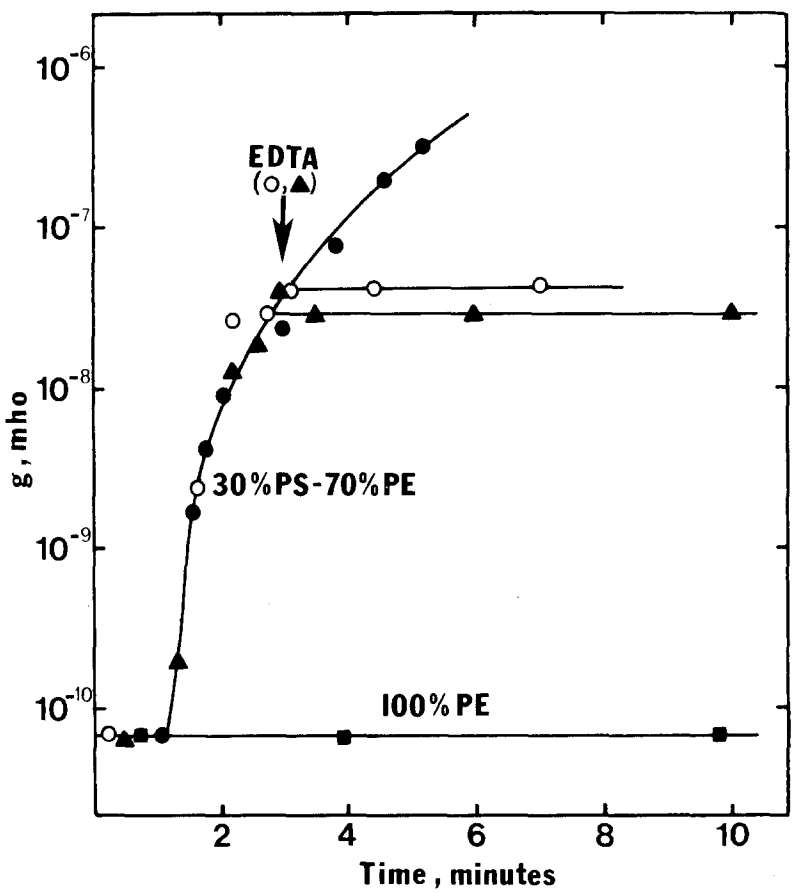


Fig. 1. Black lipid membrane conductance increase induced by sarcoplasmic reticulum. Black lipid membranes were made from either 30% PS-70% PE (●, ○, ▲) or 100% PE (■). The aqueous phase was 100 mM KCl-5 mM tris SO_4-0.1 mM EDTA, pH 7.0. $CaCl_2$ (0.5 mM) was added to the "inner" chamber, and SR vesicles (40 μg/ml) were added to this chamber at zero time. To two of the BLMs (○, ▲), EDTA (1.0 mM) was added at the arrow, and conductance, g, was monitored further. These membranes were stable for 15 min or longer, and experiments were terminated arbitrarily

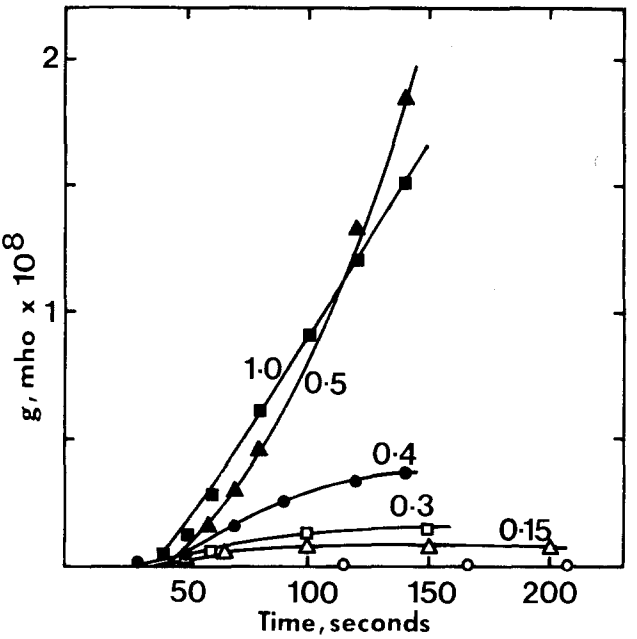


Fig. 2. Ca^{++}-dependence of conductance time course. BLM (40% DPG-60% PE) conductance time course was measured in 25 mM tris-Cl, 0.1 mM EDTA, containing the following concentrations of $CaCl_2$ in excess of the EDTA: (○) none; (△) 0.15 mM; (□) 0.3 mM; (●) 0.4 mM; (▲) 0.5 mM; (■) 1.0 mM. SR protein concentration was 35 μg/ml

If during the time course of the conductance increase excess EDTA is added to chelate the Ca^{++}, the conductance ceases to increase but does not diminish. This result demonstrates that under these conditions Ca^{++} is not a major carrier of current through the SR-modified BLM; the ion serves to control the process by which the conductance pathways are inserted into the artificial membrane. The figure also shows that with a BLM made of PE alone, i.e., lacking PS, no conductance change is seen. As subsequent data will show, the conductance increase is also observed with BLMs made of PE-DPG mixtures; in fact, DPG is somewhat more effective than PS in promoting the conductance increase, and during the course of the experiments, we adopted 40% DPG-60% PE as our usual BLM mixture.

The Ca^{++}-dependence of the conductance rise is shown in Fig. 2. Below 0.1 mM Ca^{++}, no conductance increase is observed, while concentrations in the range 0.5–1.0 mM give rapid and reproducible increases. It should be noted that in these experiments Ca^{++} is effective only if added to the same side of the BLM as are the SR vesicles. In fact, if Ca^{++} is added to both sides of the BLM, the rate of conductance rise is strongly inhibited (data not shown).

Effect of Osmotic Gradients

It is known that SR vesicles are slowly permeable to sucrose (Duggan & Martonosi, 1970; Jilka *et al.*, 1975). During standard preparation and storage conditions, therefore, the vesicles become loaded with 0.25 M sucrose. Since the aqueous phase of the BLM system has an osmotic strength on the order of 100 mosM, it was necessary to ascertain whether the osmotic gradient across the SR membrane had any effect on the BLM conductance increase. We found that such an osmotic gradient is in fact an absolute requirement for the effect of SR vesicles on the BLM. Fig. 3 shows that addition of sucrose in concentrations greater than 100 mM to the aqueous phase of the BLM system completely inhibits the BLM conductance rise. That this is an effect of an osmotic gradient and not of sucrose itself is shown in Fig. 4. Here we compare the effect of 100 mM external sucrose in two different SR vesicle preparations, loaded (a) with 0.25 M sucrose and (b) with 0.4 M sucrose. With the 0.25 M sucrose vesicles (Fig. 4 *A*), 100 mM external sucrose abolishes the BLM conductance increase. With the 0.4 M sucrose vesicles, the conductance time course in 100 mM external sucrose (Fig. 4 *B*) is comparable to that of low-sucrose vesicles with no external sucrose present. The highest rate of conductance rise is seen with 0.4 M sucrose vesicles and no external sucrose.

We can contrast the effect of sucrose with that of glycerol, which by virtue of its small size is expected to be more permeant to the SR membrane. Vesicles loaded with 0.25 M glycerol instead of sucrose are not able to raise the BLM conductance (Fig. 5). This is as expected, since the osmotic gradient initially present should be rapidly dissipated upon addition of the glycerol-loaded vesicles to the 50 mM KCl medium of the BLM. Furthermore, addition of 0.25 M glycerol externally causes no inhibition of the effect of 0.25 M sucrose-loaded vesicles on BLM conductance (data not shown).

These observations show that an osmotic gradient across the SR vesicle membrane is necessary for the BLM conductance increase induced by SR vesicles in the presence of Ca^{++} and negatively charged lipids.

Quantization of BLM Conductance Increase

In the experiments presented above, conductance was measured at periodic intervals, using a slowly responding voltage divider circuit. In

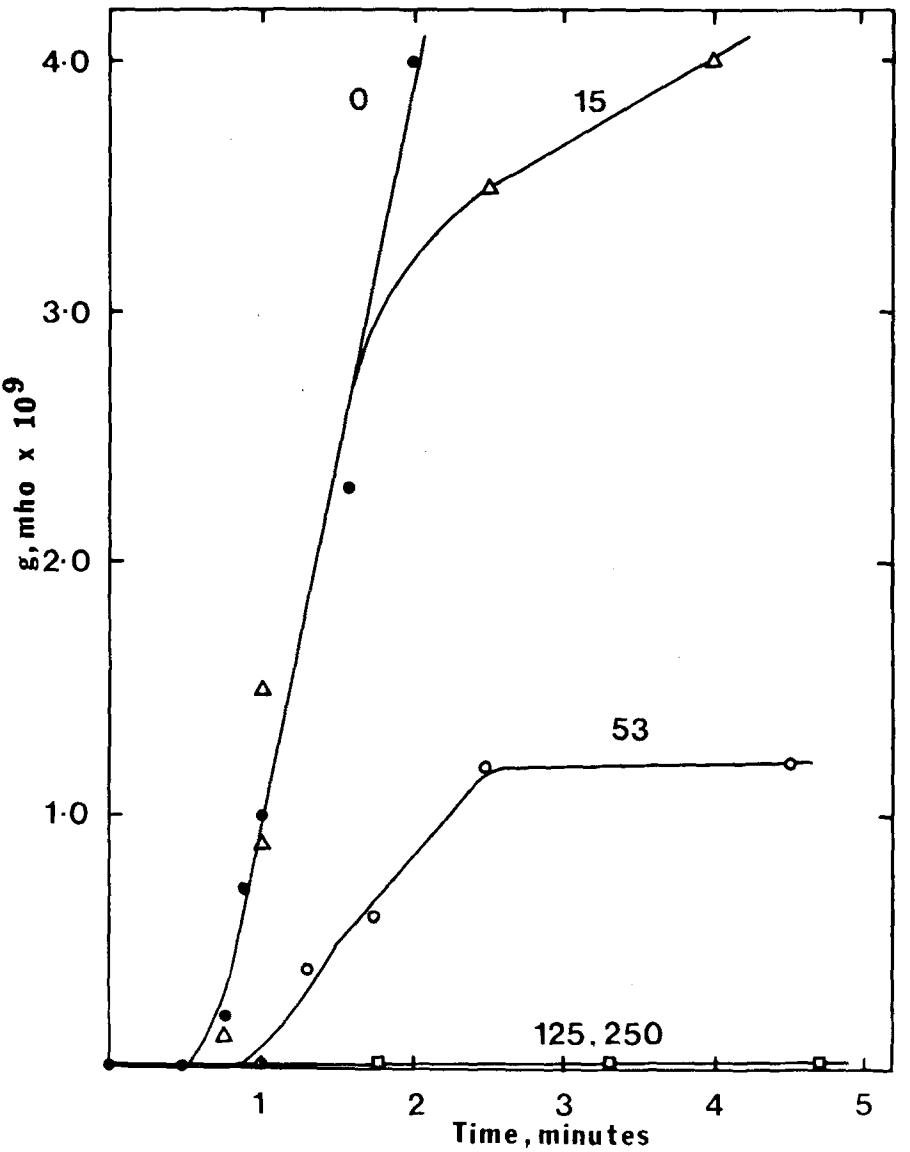


Fig. 3. Effect of sucrose on conductance time course. Sucrose (concentration indicated in mM on graph) was added to 50 mM KCl, 5 mM HEPES, 0.1 mM EDTA, containing 0.5 mM $CaCl_2$. BLM mixture was 40% PS-60% PE. Zero time is defined by addition of SR protein (40 μg/ml)

these experiments, concentrations of vesicles greater than 30 μg protein per ml were used and the conductance appeared to increase smoothly with time. However, when conductance is monitored continuously using a current amplifier at high sensitivity with SR protein concentrations

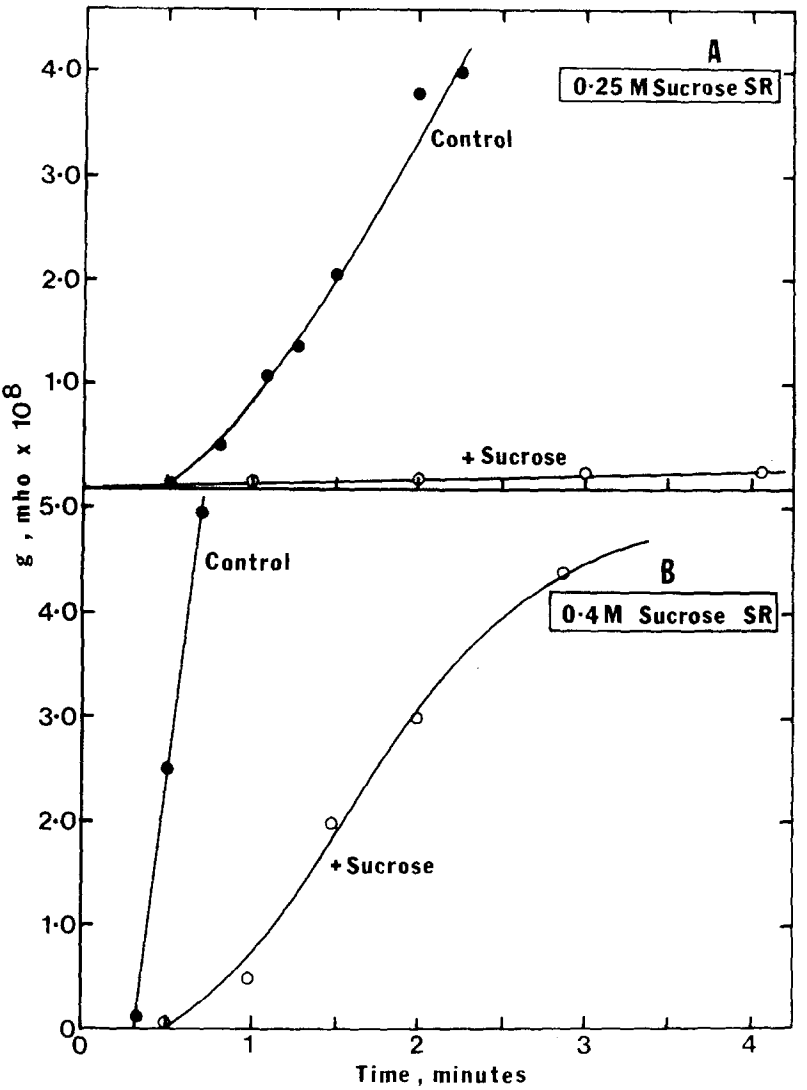


Fig. 4. Effect of osmotic gradients on conductance time course. Effects of vesicles loaded with either 0.25 or 0.4 M sucrose on BLM conductance are compared. The 0.4 M sucrose vesicles were prepared by slowly adding 2 M sucrose to a suspension of 0.25 M sucrose SR vesicles to a final concentration of 0.4 M, followed by 1 hr incubation at room temperature. SR vesicles prepared with 0.25 M sucrose according to MacLennan (1970) were also incubated at room temperature for an hour. Conductance of the BLM (45% PS-55% PE) was measured in 50 mM KCl, 5 mM HEPES, 0.1 mM EDTA, 0.7 mM $CaCl_2$ (labelled "control") or the same medium with 100 mM sucrose added (labelled "+sucrose"). (*A*) 0.25 M sucrose SR vesicles (40 μg/ml) added. (*B*) 0.4 M sucrose SR vesicles (40 μg/ml) added

between 3 and 10 μg per ml, an interesting feature of the early part (first 2 min) of the time course becomes apparent (Fig. 6). Whereas at high concentrations of vesicles there appears to be a continuous rise of conduc-

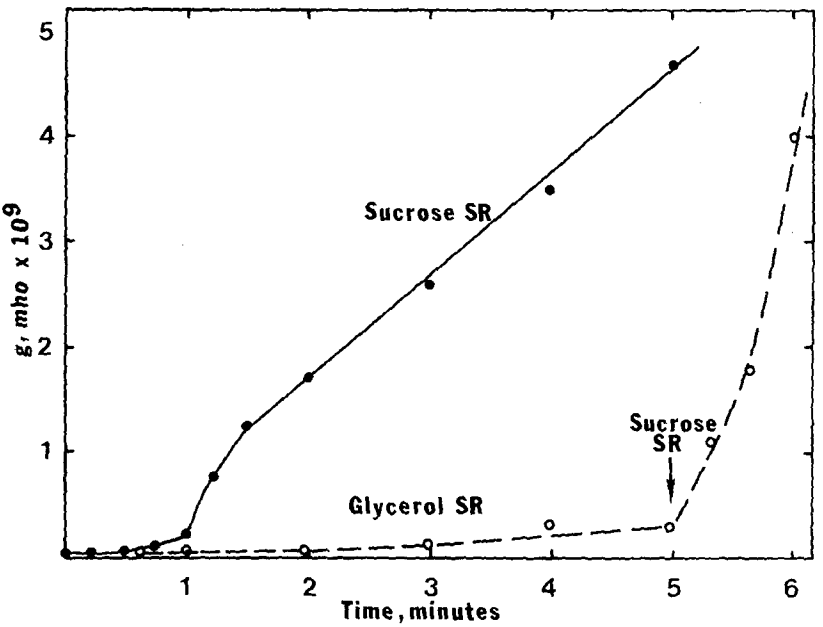


Fig. 5. Lack of effect of glycerol-loaded SR on BLM. Glycerol-loaded SR were prepared by suspending the SR vesicles in 0.25 M glycerol – 10 mM Tricine-KOH instead of in sucrose. Either 0.25 M *sucrose* SR (•) or *glycerol* SR (○) were added (15 μg/ml) to the BLM inner chamber at zero time. At the arrow on the glycerol SR time course, *sucrose* SR (15 μg/ml) were added in addition. BLMs were 40% PS-60% PE, with the aqueous phase 50 mM KCl, 5 mM HEPES, 0.1 mM EDTA, 0.4 mM $CaCl_2$

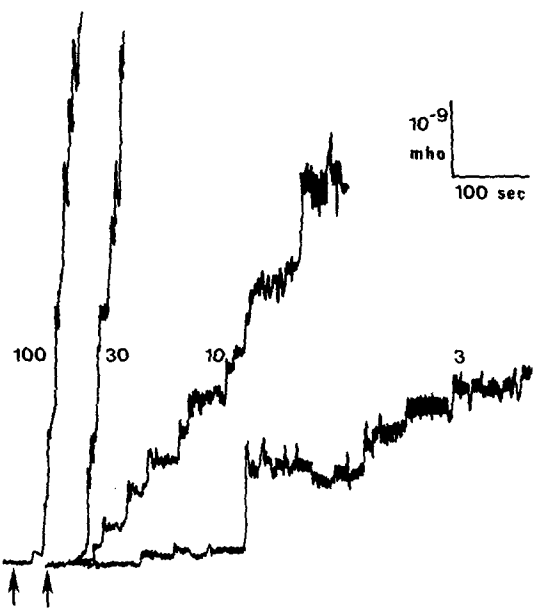


Fig. 6. Discrete conductance transitions induced by SR vesicles. The early part of the conductance time course was monitored continuously after addition (at arrows) of SR vesicles (protein concentrations in μg/ml) indicated on figure. BLMs were 40% DPG-60% PE; aqueous phase was 50 mM tris-Cl, 0.1 mM EDTA, 0.7 mM $CaCl_2$

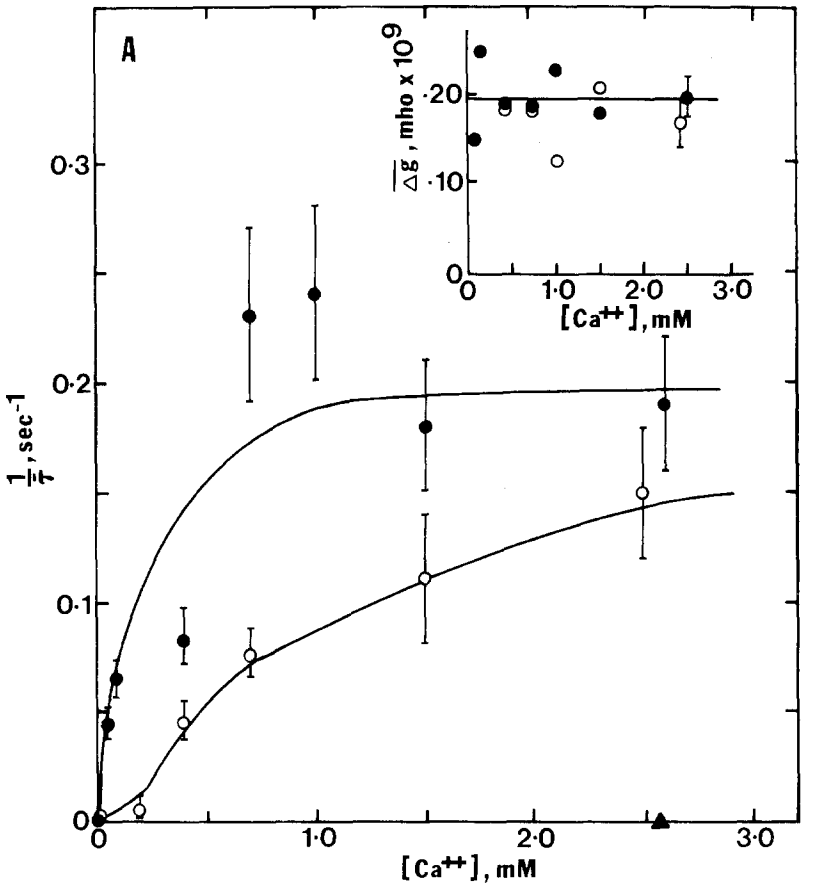
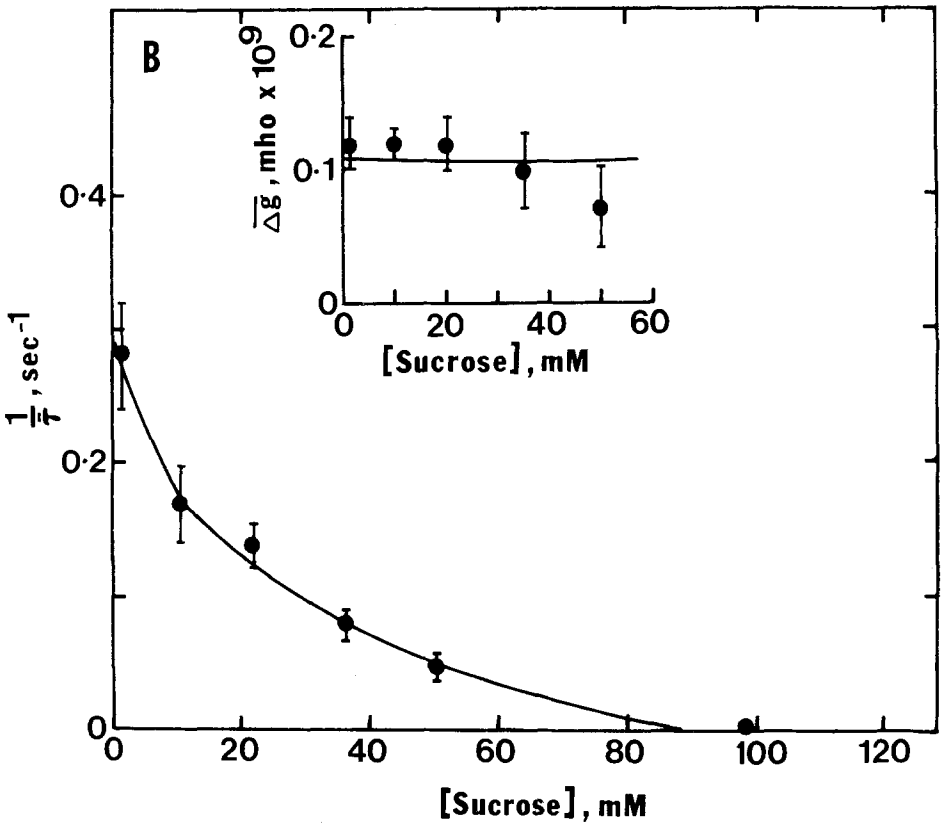


Fig. 7. Effect of Ca^{++} and osmotic strength on quantal conductance parameters. (*A*) Effect of Ca^{++}. Conductance was monitored in 50 mM choline-Cl, 5 mM HEPES-tris, 0.1 mM EDTA, containing the appropriate Ca^{++} concentration using BLMs made from 100% PE (▲), 20% DPG-80% PE (○), or 40% DPG-60% PE (●). (*B*) Effect of osmotic strength. Conductance was monitored in 25 mM choline-Cl, 5 mM HEPES-tris, 0.1 mM EDTA, containing 0.7 mM Ca^{++}, to which the appropriate concentration of sucrose was added. BLMs were 40% DPG-60% PE. In both figures, SR vesicle protein concentration was 20 μg/ml. For each BLM, conductance time courses were monitored for not more than 5 min after addition of SR vesicles. Each point represents the mean ± standard error of 20–40 conductance transitions observed on several BLMs. The mean time between transitions, $\bar{\tau}$, and the mean conductance of the transitions, $\Delta\bar{g}$, were determined from the chart record

tance, at the lower concentrations a sequence of discrete conductance jumps or steps is observed. It is evident from the figure that the probability of a step's occurrence increases with SR vesicle concentration, while the average step size remains approximately the same.



We can now study two important parameters of this quantized conductance increase: the average step *size*, and the average step *frequency*. The latter is given by the inverse of the average time between conductance jumps, $\bar{\tau}$. Fig. 7*A* shows these parameters as a function of Ca^{++} concentration in the medium (50 mM choline-Cl, 5 mM HEPES-tris, 0.1 mM EDTA). The jump frequency increases with Ca^{++} concentration in DPG-PE membranes; membranes containing 40% DPG require considerably less Ca^{++} than do 20% DPG membranes. No conductance steps occur at all in 100% PE membranes, even at high Ca^{++} concentrations. With the DPG-containing membranes, there is a suggestion of saturation behavior (i.e., the existance of a maximum frequency) at high Ca^{++} concentrations, but the error in the frequency determinations is too great to state this with certainty. The mean jump size is independent of Ca^{++} concentration under these conditions.

Fig. 7*B* shows how the step-conductance parameters vary with osmotic strength of the medium (adjusted by addition of sucrose to 25 mM

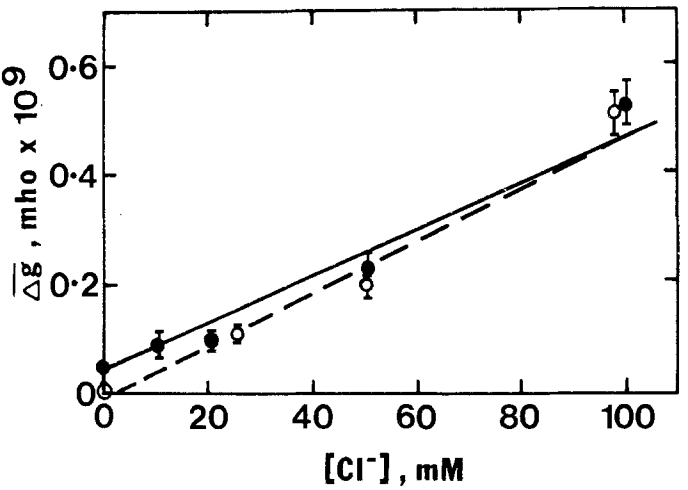


Fig. 8. Dependence of conductance step-heights upon Cl^- concentration. Mean step-conductance, $\Delta\bar{g}$, was determined as a function of Cl^- concentration, in buffers containing Cl^- as the major current-carrying ion. BLMs were 40% DPG-60% PE. SR protein concentration was 20 μg/ml; the conductance increase was initiated by addition of 0.7 mM $CaCl_2$ to the appropriate buffer. ●—Variation of Cl^- at constant ionic strength (solid line). Cl^- concentration was varied by making appropriate mixtures of 100 mM choline-Cl and 100 mM choline-glucuronate, each containing 5 mM HEPES-tris and 0.1 mM EDTA. The nonzero $\Delta\bar{g}$ at zero Cl^- concentration is due to the conductance of choline glucuronate. ○—Variation of Cl^- at varying ionic strength (dashed line). Cl^- was varied without compensation by other ions. Buffers were composed of varying choline-Cl + 5 mM HEPES-tris + 0.7 mM $CaCl_2$. Each point represents the mean ± standard error of 10–30 conductance transitions observed on several different BLMs

choline-Cl medium). The step frequency decreases with increasing external sucrose, until at 100 mM no conductance increase occurs. The mean conductance step size is largely unaffected by sucrose addition.

Fig. 8 shows that the mean step conductance size varies approximately linearly with Cl^- concentration, in buffers containing Cl^- as the only small ion in appreciable concentration. Linearity has also been observed (in the range 10–100 mM) for K^+ conductance (data not shown).

Table 1 reports the dependence of jump frequency and conductance on the *type* of ion making up the major ionic portion of the medium. Here, we use the HEPES salt of the cation and the tris salt of the anion under study; control experiments (not shown) confirm that neither $HEPES^-$ nor $tris^+$ contributes significantly to the conductance under these conditions. It is apparent that the size of the step-conductance is dependent upon the ion type, while the jump frequency is not. Again we see the stimulation of the jump frequency by lowering the osmotic

Table 1. Ionic selectivity of step conductances[a]

Buffer (mM)	$\Delta\bar{g}$, mho $\times 10^9$	$1/\bar{\tau}$, sec^{-1}
K^+ (50)	0.83 ±0.09 (8)	0.06±0.02
K^+ (25)	0.39 ±0.03 (36)	0.14±0.03
Na^+ (50)	0.63 ±0.13 (12)	0.03±0.01
Na^+ (25)	0.36 ±0.05 (12)	0.17±0.03
Li^+ (50)	0.11 ±0.02 (14)	0.04±0.01
Li^+ (25)	0.047±0.006 (16)	0.14±0.03
Cl^- (50)	0.24 ±0.02 (18)	–
Cl^- (25)	0.14 ±0.01 (20)	–
$SO_4^=$ (25)	0.09 ±0.01 (18)	–

[a] Mean conductance step sizes, Δg, and jump frequencies, $1/\bar{\tau}$, were determined in buffers of various compositions, using BLMs of lipid composition 40% PS-60% PE. The "cation" buffers were 50 or 25 mM KOH, NaOH, or LiOH neutralized to pH 7.0 with HEPES. The "anion" buffers were 50 or 25 mM HCl or H_2SO_4 neutralized with tris base. The conductance increase was initiated by addition to the inner chamber of HEPES-$Ca(OH)_2$ to a final Ca^{++} concentration of 0.5 mM. (Jump frequencies were determined only in comparing the "cation" buffers, since only here were the osmotic strengths comparable.) Data are reported as mean ± SEM, with the number of determinations in parentheses.

strength of the medium. The ionic selectivity of the jump conductance is in the order $K^+ \cong Na^+ > Cl^- > Li^+ \cong {}^1/_2 SO_4^=$.

From these data it appears that the conductance increase proceeds by the incorporation of discrete quanta of conductance pathways into the BLM. The probability of incorporation is affected by such variables as Ca^{++}, osmotic strength, negative charge in the BLM, and vesicle concentration, while the quantal conductance size is dependent on the type of ion in the medium.

Correlation of Quantal Conductance with Vesicle Size

It is apparent from Fig. 6 that the quantized conductances are by no means uniform in size. There is a wide distribution of values for a given ion. Fig. 9 compares the distribution of conductance step sizes (normalized to the mean) with the distribution of apparent vesicle surface area (normalized to the mean) as measured by electronmicroscopy. The similarity of the two distributions is remarkably close, considering the limits of the method of estimating vesicle surface area. Both distributions show a characteristic skewing out to large values. Sonication of the vesicles sharpens the surface area distribution, substantially reducing the fraction of large vesicles; likewise, the distribution of conductance

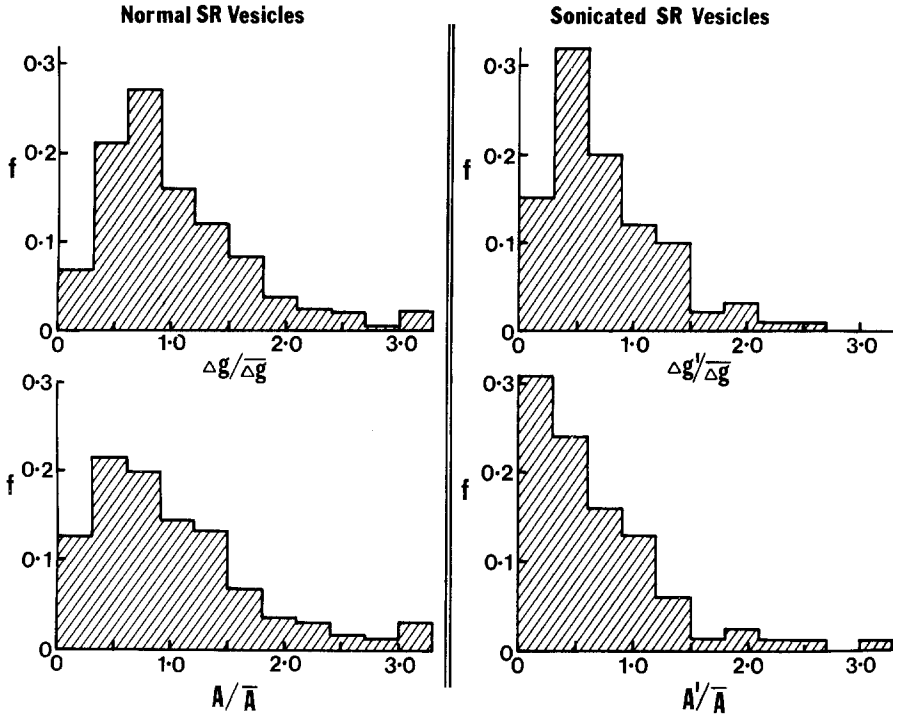


Fig. 9. Conductance step-height and vesicle surface area distributions. Histograms (f=frequency of occurrence) of relative conductance step-heights, $\Delta g/\bar{\Delta g}$, and vesicle surface areas, $A/\bar{A}$, are compared for normal and sonicated SR vesicles, as indicated on the graphs. Unprimed symbols represent unsonicated vesicles, while primed quantities refer to sonicated vesicles. The distributions for the sonicated vesicles are normalized to the means of the unsonicated vesicles, so that the distributions for the two types of vesicles can be compared directly. The conductance steps were taken as compiled data from a variety of buffers, BLM compositions, and SR vesicle concentrations, each set of data normalized to its own mean. The area distributions were calculated as described under *Materials and Methods*. Sonication of SR vesicles (0.1 ml) was carried out in 0.25 M sucrose for 30 sec at 20 °C in a bath-type sonicator (Miller & Racker, 1976). The conductance distributions were calculated on a sample of 416 observations for the unsonicated vesicles, and on 202 observations for the sonicated vesicles. Area distributions were calculated on samples of 150–180 observations for the two types of vesicles

quanta also sharpens upon sonication, so that again the two distributions agree well. Sonication reduced the mean value of both the conductance and the area distributions by 20%–30%.

The most serious disagreement between the conductance and area distributions is seen at low values; it is possible that this is due to a failure to detect conductance steps smaller than 15% of the mean value. When the area distributions are recalculated neglecting all vesicles

with areas less than 15% of the mean of the total population, the area distributions fit the conductance distributions more closely at all values.

The data in Fig. 9 suggest that each conductance-jump event is the result of the interaction of a single SR vesicle with the BLM, and that the size of the conductance step is proportional to the size of the SR vesicle, most likely to its surface area.

Discussion

The insertion of conductance pathways from biological membranes into the black lipid membrane system is by no means a trivial problem. Since its development, the BLM has been used almost exclusively for the study of a multitude of ionophores produced by microorganisms. These toxins attack other microorganisms and incorporate easily into the membranes of the target cells. They therefore do not present problems of incorporation into the BLM. These ionophores have proven valuable for the study of ion transport mechanisms in general, and in analogy to excitation phenomena in particular (Mueller & Rudin, 1969*b*; Haydon & Hladky, 1972; Mueller, 1975).

Only recently, however, has the BLM been used to study proteins derived from mammalian membranes (Drachev *et al.*, 1974; Shamoo & MacLennan, 1974; Shamoo & Eldefrawi, 1975; Bradley *et al.*, 1976). These studies have not been concerned with the factors influencing the insertion of the proteins under study into the BLM, but rather with the possible physiological relevance of the ionophoric systems.

The data which we have presented show clearly the incorporation into a BLM of conductance pathways derived from the sarcoplasmic reticulum membrane. As a first step towards understanding this system as it applies to the physiology of the muscle, this paper addresses the question of how the ionophores are inserted into the BLM. We propose that the most likely explanation for the conductance increase caused by fragmented SR vesicles is the fusion of the vesicles with the BLM. There are two lines of evidence leading to this conclusion.

First, the requirements for the BLM conductance increase are the same as those for a known process of fusion of phospholipid vesicles. Liposomes (Papahadjopoulos, Poste, Schaeffer & Vail, 1974) and liposomes reconstituted with membrane proteins (Miller & Racker, 1976) fuse with each other if an acidic phospholipid such as PS is included as a major membrane constituent ($>30\%$ of total phospholipid). DPG, required

in only 15% concentration, is somewhat more effective than PS in promoting the fusion (G. Eytan and C. Miller, *unpublished observations*). Liposome fusion is completely dependent upon Ca^{++} (>0.5 mM) and varies with the phospholipid composition. Since 10%–15% of SR lipids are acidic (Owens, Ruth & Weglicki, 1972), it is understandable that these vesicles can undergo Ca^{++}-induced fusion with BLMs containing substantial concentrations of negatively charged lipid.

One apsect of the liposome fusion system which initially seemed to argue against SR-BLM fusion is that only small (~ 300 Å diameter) liposomes could be made to fuse; liposomes which had grown to diameters over 1000 Å lost the ability to fuse (Miller & Racker, 1976; Miller *et al.*, 1976). It appeared that this was an effect of membrane curvature, that the thermodynamic driving force for fusion is an excess surface energy difference which becomes negligible as liposome diameter increases above 1000 Å. Since SR vesicles have diameters in the range 1000–2000 Å, and since the BLM is actually planar, it was difficult to see how the liposome fusion process could serve as an adequate explanation of the SR-BLM interaction.

This problem became resolved with the discovery that large liposomes (>1000 Å) can be made to fuse if they are presented with a substantial osmotic gradient, with the internal osmolarity higher than the external (Miller *et al.*, 1976). The osmotic gradient-dependent fusion still requires Ca^{++}, but for liposomes of negligible curvature it is also absolutely dependent upon an osmotic gradient on the order of 0.25 osM. Taken together with the dependence on Ca^{++} and acidic lipid of both the liposome fusion process and the SR-induced BLM conductance, such an observation strengthens the hypothesis that the SR-BLM interaction is in fact achieved by membrane fusion.

The second line of evidence leading to the same conclusion is the quantal nature of the BLM conductance increase. We find it strongly suggestive that the variables known to increase the probability of liposome fusion (osmotic gradient, acidic phospholipid, Ca^{++}, vesicle concentration) also control the frequency of the conductance jumps, without affecting the individual jump size. Further, the close correlation between the SR vesicle surface area and the conductance jump size suggests that each jump represents the incorporation into the BLM of the membrane of a single SR vesicle. It is difficult to envision a process other than fusion which would account for such "all or none" behavior.

It should be noted that these experiments are possible not because SR-BLM fusion is a highly probable process, but because the BLM

is an extraordinarily sensitive assay system. In fact, SR-BLM fusion is improbable even under the most favorable conditions; in a typical experiment, with about 10^{11} SR vesicles added to the BLM chamber, only about 100 fusion events occur over a period of 10 min. Therefore, in drawing an analogy between the liposome fusion process (in which average properties of a large liposome population are measured) and the SR-BLM interaction, we should not be disturbed by quantitative differences in the variables involved, e.g., the 30% acidic lipid requirement for the liposome system *vs.* the 15% acidic phospholipid content of the SR.

This paper is meant only to describe the process of incorporation of conductance pathways from the SR membrane into the BLM. We consider it important that this process appears to be membrane fusion, because this is undoubtedly the gentlest possible way to "reconstitute" the SR membrane proteins into an electrically accessible membrane system. We do not yet wish to make any claims as to the physiological significance of the electrical properties of the SR-BLM hybrid membrane.

Finally, we have not lost sight of the possibility that the approach described here might find general applicability as a technique for gaining access to electrically elusive transport systems, such as those of oxidative or photoactive energy transducing membranes.

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