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Comparative electrophysiological study of reconstituted giant vesicle preparations of the rabbit skeletal muscle sarcoplasmic reticulum K^+ channel

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Sarcoplasmic reticulum (SR) membranes isolated from rabbit skeletal muscle were reconstituted into two types of giant vesicles: (1) Giant proteoliposomes prepared by freeze-thawing of a mixture of SR vesicles and sonicated phospholipid vesicles without the use of detergent. (2) Giant SR vesicles prepared by fusion of SR vesicles using poly(ethylene glycol) (PEG) as a fusogen and without the addition of exogenous lipid. These giant vesicles were patch-clamped and properties of the single voltage-dependent potassium channel in the excised patch were studied. Single-channel conductance in a symmetrical solution of 0.1 M KCl and 1 mM $CaCl_2$ was 140.0 ± 10 pS ($n = 5$) for freeze-thawed vesicles and 136.4 ± 15 pS ($n = 7$) for PEG vesicles. Both types of vesicles exhibited a sub-conductance state having 55% of the fully open state conductance. The voltage-dependence of open-channel probability could be expressed in terms of thermodynamic parameters of $\Delta G_i = 0.95$ kcal/mol and $z = -0.77$ for freeze-thawed vesicles and $\Delta G_i = 0.92$ kcal/mol and $z = -0.87$ for PEG vesicles. These values correlated well with previous data obtained by fusion of native SR vesicles with a planar lipid membrane. Channel orientation was found to be conserved in both types of vesicles used in the present study.

Introduction

Ion channels of biochemically isolated biomembrane vesicles can be studied electrophysiologically when incorporated or reconstituted into a planar lipid membrane or a giant vesicle suitable for the application of a patch-clamp pipet. The planar membrane method has been widely used and it has been shown that properties of ion channels reconstituted into planar membranes are not significantly altered during the reconstitution procedure [1]. On the other hand, the development of the giant vesicle technique is still at an early stage and properties of ion channels in such reconstituted membranes have not been investigated in detail. For example, it is not clear whether the orientation of ion channel protein is conserved or randomized, or whether gating characteristics are modified.

Four methods of giant vesicle preparation have been reported. In the conventional method, small proteoliposomes are first prepared by the detergent/dialysis method and then fused into giant proteoliposomes by freeze-thawing [2–4]. We have reported the formation of giant proteoliposomes by freeze-thawing a mixed suspension of biomembrane vesicles and sonicated phospholipid vesicles, which permits omission of the detergent/dialysis step [5,6]. An essentially similar method was described by Correa and Agnew [7]. The third method is a dehydration/rehydration method reported successful in the preparation of giant vesicles from a mixture of biomembrane vesicles and liposomes [8], from proteoliposomes [9] and from rough endoplasmic reticulum membranes [10]. Rahamimoff et al. [11] reported the fusion of synaptic vesicles into giant vesicles by the use of poly(ethylene glycol) 1500 (PEG) as a fusogen. In order to demonstrate feasibility of these newly devised methods, it is important to examine characteristic properties of a well-characterized ion channel reconstituted by these methods.

The objective of the present study is to examine the properties of the voltage-dependent K^+ channel of the sarcoplasmic reticulum (SR) membrane reconstituted into giant proteoliposomes by freeze-thawing or into

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giant vesicles by PEG-induced fusion. We have chosen this ion channel because it is one of the most extensively studied and best characterized, thereby permitting evaluation of the feasibility of the two giant vesicle techniques. The results described below show that gating characteristics and orientation of the channel are not altered, but well-conserved in the giant vesicles prepared by these methods.

Materials and Methods

Chemicals

Asolectin (soybean phospholipid, type II-S) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and partially purified according to Kagawa and Racker [12]. Egg L-α-phosphatidylethanolamine (PE) and L-α-phosphatidylglycerol (PG) were purchased from Avanti Polar Lipids (Pelham, AL, U.S.A.). Poly(ethylene glycol)s were obtained from Boehringer Mannheim GmbH (West Germany).

Preparation of membrane vesicles

Light fraction of sarcoplasmic reticulum (SR) vesicles was prepared from rabbit white skeletal muscle as reported previously [13]. The protein concentration was determined by the method of Lowry et al. [14]. The phospholipid content of the SR vesicles was determined, by measuring phosphorus by the method of Gerlach and Deuticke [15], to be 980 nmol/mg protein. The weight ratio (w/w) of the total lipids to protein was estimated to be about one by assuming the average molecular weight of phospholipids is 800 and the phospholipid content in total lipids of SR is 80% [16].

Giant proteoliposomes were prepared by the freeze-thawing method described elsewhere [5,6]. Briefly, a suspension of SR vesicles and sonicated phospholipid vesicles both in a solution of 150 mM KCl and 10 mM Hepes-KOH (pH 7.0) were admixed with a weight ratio of protein/exogenous lipid of 1:40–1:10. The mixture was rapidly frozen in liquid nitrogen and then thawed on ice. Giant proteoliposomes (freeze-thawed vesicles) ranging 10–30 μm in diameter were formed. Giant SR vesicles (PEG vesicles) were prepared by PEG-induced fusion of SR vesicles. A suspension of SR vesicles (1–2 mg protein) was centrifuged and the pellet covered with 1 ml of a poly(ethylene glycol) 1500 (PEG) solution (25% PEG, 20% dimethylsulfoxide, 150 mM KCl, 20 mM Hepes-KOH, pH 7.2). After incubation for 10 min at 30°C, the PEG solution was removed by decantation and the pellet gently suspended with a Pasteur pipet in 1 ml of 150 mM KCl solution buffered with 10 mM Hepes-KOH at pH 7.2. The suspension was incubated at room temperature for several hours to obtain fused giant SR vesicles up to 50 μm in diameter.

Patch-clamp recording

A giant vesicle suspension (5–20 μl) was placed in a plastic dish and covered with a few ml of appropriate bath solution. A standard patch-clamp technique was used [17]. A patch-pipet microelectrode was made from a Pyrex glass capillary of o.d. 1.5 mm with a pipet puller (PP-83, Narishige, Tokyo, Japan). The pipet tip was fire-polished with a Narishige microforge (MF-83) Its resistance was 7–9 MΩ when filled with 150 mM KCl solution. The vesicles were observed with an inverted microscope (IMT-2 with phase-contrast or Nomarski differential interference-contrast optics, Olympus, Tokyo, Japan) and manipulated with a three-dimensional micromanipulator (MO-103, Narishige, Tokyo, Japan). Current measurements were carried out in the inside-out patch configuration using a patch-clamp amplifier (EPC-7, List-Electronic, Darmstadt-Eberstadt, West Germany or CEZ-2200, Nihon Kohden, Tokyo, Japan). Signals were stored on video tape for later analysis. Stored data were retrieved through a low-pass filter (FV-664, NF Electronic Instruments, Yokohama, Japan) and digitized with a 12-bit A/D converter (PCN-2198, Neolog Electronics, Tokyo, Japan) for computer analysis (PC-9801VM, NEC, Tokyo, Japan) [6].

Results

Reconstitution of SR vesicles into giant vesicles

It is easier to obtain a large number of giant vesicles of 10–20 μm diameter with the freeze-thaw method than the PEG method. In the latter, the longer the incubation time at room temperature, the larger number and size of PEG vesicles obtained. Giant PEG vesicles were often formed attached to aggregates of small SR vesicles. Poly(ethylene glycol)s of molecular weights higher than 1500 (i.e., 4000, 6000, 8000) were tested but ineffective in the formation of vesicles larger than 10 μm in diameter.

Patch-clamp recording from the freeze-thawed vesicle

Single-channel current fluctuations were recorded with a standard inside-out configuration. Typical examples of single-channel current traces of a K^+ channel of SR membrane incorporated into a giant proteoliposome are shown in Fig. 1. Current levels due to fully open and sub-conductance states of the channel are seen. From the amplitude histograms (not shown), single-channel currents were determined. Fig. 2 shows plots of the single-channel current against membrane potential (potential of the pipet internal solution with respect to the bath solution). From the straight *I-V* relationships, single-channel conductances of fully open and sub-conductance states were found to be 140.0 ± 10 (mean $\pm$ S.E., $n = 5$), and 79.3 ± 6 ($n = 5$) pS, independent of membrane potentials, in a symmetrical solution

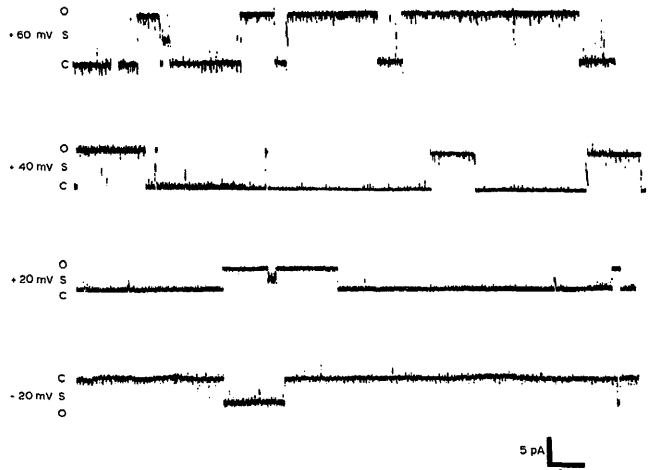

Fig. 1. Single K^+ channel current traces from a freeze-thawed vesicle. Currents were recorded from an inside-out patch in symmetrical solution of 100 mM KCl, 0.1 mM $CaCl_2$ and 10 mM Hepes-Tris (pH 7.2). Currents were filtered at 320 Hz. Letters O, S and C represent the fully open, sub-conductance and closed level, respectively. Holding voltages are indicated left of the corresponding traces.

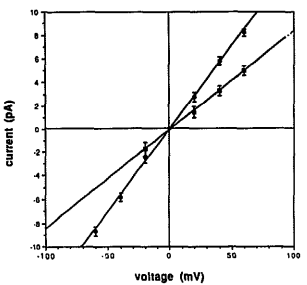

Fig. 2. Single-channel current-voltage relationship for the K^+ channel of sarcoplasmic reticulum reconstituted in a freeze-thawed proteoliposome. Experimental conditions were the same as in Fig. 1. Single-channel current was obtained from the peak intervals of the amplitude histograms of the current traces. Straight lines for fully open state (●) and sub-conductance state (■) were obtained by a linear regression analysis of the data. From the slopes of the lines, single-channel conductances were determined to be 141.2 and 84.3 pS for fully open and sub-conductance states, respectively.

of 100 mM KCl, 0.1 mM $CaCl_2$ and 10 mM Hepes-Tris (pH 7.2) Sometimes several channels were observed in a single patch.

It is apparent from Fig. 1 that open-channel probability depends on membrane potential. The channel tends to open at more positive potentials. Fig. 3A is a quantitative presentation of the voltage dependence of the open probability. Open-channel probability was calculated dividing the sum of open time (fully open and sub-conductance levels) by total sampling time (more than 30 s). The time the channel spends in the sub-conductance state was less than 5% of the total open time at any membrane potential tested. Thermodynamically open-channel probability (P_0) can be expressed as a function of membrane potential (V) as follows [18],

$$P_0(V) = [1 + \exp(\Delta G_i + zFV)/RT]^{-1} \quad (1)$$

where ΔG_i refers to 'internal free energy of opening', z refers to 'effective gating charge', and F, R and T are Faraday's constant, gas constant and absolute tem-

perature, respectively. Eqn. 1 can be rewritten to Eqn. 2

$$\ln[(1 - P_o)/P_o] = (\Delta G_i + zFV)/RT \quad (2)$$

When the left hand side of Eqn. 2 was plotted vs. V, values for ΔG_i and z could be determined. The plot is shown in Fig. 3B and linear regression analysis provides values for the two parameters, $\Delta G_i = 0.95$ kcal/mol and $z = -0.77$.

Patch-clamp recording from PEG vesicles

Single K^+ channel current fluctuation was also recorded from giant SR vesicles prepared by fusion of SR vesicles using PEG as a fusogen, as shown in Fig. 4. Frequency of giga-seal formation and frequency of encountering ion channels in PEG vesicles did not greatly differ from those in freeze-thawed vesicles. Single-channel current-voltage relationships are shown in Fig. 5. From the slopes of these linear plots, single-channel conductances of fully open and sub-conductance states were determined to be 136.4 ± 15 ($n = 7$) and 74.1 ± 9 pS ($n = 7$) in a symmetrical solution of 100 mM KCl, 5 mM $MgCl_2$ and 10 mM Hepes-KOH (pH 7.0). The voltage-dependence of open-channel probability was plotted in two ways in Figs. 6A and 6B. The thermodynamic parameters, $\Delta G_i = 0.92$ kcal/mol and $z = -0.87$, were derived from the plot in Fig. 6B.

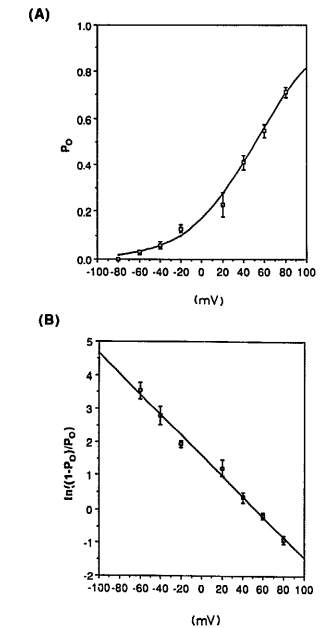

Fig. 3. (A) Voltage dependence of open-channel probability of the K^+ channel of a freeze-thawed vesicle. The plot was obtained from six patches. Each point and bar represent mean and standard error, respectively. The solid line is a theoretical curve using parameter values determined by linear regression analysis as shown in (B). (B) The same data replotted for linear regression analysis. The solid line gives values for the parameters $\Delta G_i = 0.95$ kcal/mol and $z = -0.77$.

Asymmetry of channel-gating voltage-dependence

Since the K^+ channel of SR membrane has an asymmetric voltage dependence, orientation of the channel in the reconstituted membrane can be investigated. Open-channel probability increased with increased membrane potential (potential of the pipet solution with respect to the bath solution) in all experiment runs with both freeze-thawed vesicles ($n = 20$) and PEG vesicles ($n = 13$) as shown in Figs. 3 and 6. In no case was reversed asymmetry of voltage dependence observed.

Discussion

Single-channel conductance of the fully open state of the SR K^+ channel in a symmetrical solution of 100 mM KCl was found to be 140.0 ± 10 ($n = 5$) and 136.4 ± 15 ($n = 7$) pS for the freeze-thawed and PEG vesicles, respectively. Although conductance is known to be variable depending on salt concentrations [19–21], membrane surface charge [20] and pH [18], the above values can be compared to the 130–148 pS in 100 mM K-glutamate (pH 7.0) [18] obtained with SR vesicles incorporated into a planar lipid membrane. SR K^+ channels were also previously reconstituted into giant proteoliposomes by the conventional method involving the detergent/dialysis step [22]. Conductance was reported to be 174.5 ± 19 pS in 100 mM KCl (pH 7.4), not much different from the data above.

Conductance of sub-conductance state was determined to be 79.3 ± 6 ($n = 5$) and 74.1 ± 9 ($n = 7$) pS with the freeze-thawed and PEG vesicles, respectively, under the same conditions for the determination of fully open-channel conductances. The ratio of sub- to full-conductance was 0.57 for the freeze-thawed vesicles and 0.54 for the PEG vesicles. These values correlated well that reported by Fox [21] who demonstrated that with SR vesicles incorporated into a planar lipid membrane, the ratio of the two conductances is 0.60 and that this value is independent of ion concentrations.

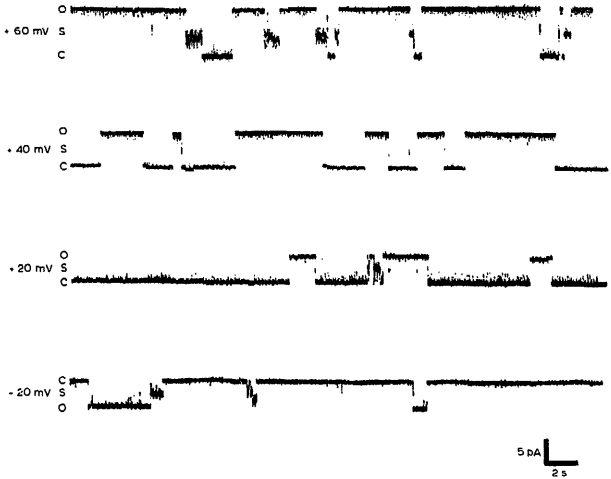

Fig. 4. Single K^+ channel current traces from a PEG vesicle. Currents were recorded from an inside-out patch in a symmetrical solution of 100 mM KCl, 5 mM $MgCl_2$ and 10 mM Hepes-KOH (pH 7.0). Other experimental conditions and symbols are the same as in Fig. 1.

Labarca et al. [18] analyzed the voltage-dependence data using Eqn. 1 assuming the two-state (open and closed) model and determined the thermodynamic parameters ΔG_i and z. Although it is now known that the channel has sub-states, the channel spends most time at either closed or fully open states. The channel resides in its sub-state for only a small fraction of the total time. Therefore, the two-state model, as performed by Tomlins and Williams [22] can still be used. Accordingly, values for the two parameters determined in the present study were $\Delta G_i = 0.95$ kcal/mol and $z = -0.77$ for the freeze-thawed vesicles at pH 7.2 and $\Delta G_i = 0.92$ kcal/mol and $z = -0.87$ for the PEG vesicles at pH 7.0. These values correlate well with the $\Delta G_i = 1.16$ kcal/mol and $z = -1.1$ reported by Labarca et al. [18] in the planar lipid membrane experiment at pH 7.0. The positive value for ΔG_i indicates that the closed state is favored at zero potential and the negative value for z means that a net negative charge moves towards the pipet solution.

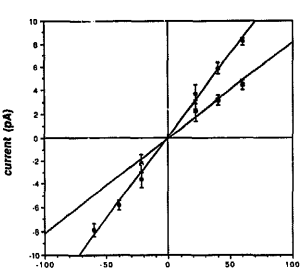

Fig. 5. Single K^+ channel current-voltage relationship for a PEG vesicle. From the slopes of the straight lines, single-channel conductances are estimated to be 139.5 and 81.6 pS for fully open (●) and sub-conductance (■) states, respectively.

From planar lipid membrane experiments the K^+ channel of the SR membrane is known to exhibit higher open-channel probability with higher potentials of cytoplasm side with respect to the SR lumen [18]. In the present experiments open-channel probability increased with increased membrane potential. Taking account of the inside-out patch configuration in the present experiments, the pipet solution corresponds to the cytosol for native SR membranes. Therefore, we can conclude that orientation of the channel in the reconstituted giant vesicles of the present study is neither randomized nor reversed but conserved.

Tomlins and Williams [22] reported with the giant proteoliposomes prepared by the method involving sol-

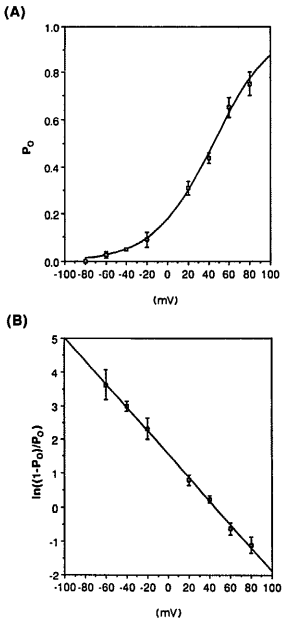

Fig. 6. (A) Voltage dependence of open-channel probability of the K^+ channel of a PEG vesicle. Each point represents a mean ± standard error ($n = 7$). (B) The same data replotted for linear regression analysis. $\Delta G_i = 0.92$ kcal/mol and $z = -0.87$.

ubilization/dialysis that virtually all channels in the membrane patch are in one orientation. However, they measured the macroscopic current through the membrane patch containing a large number of the ion channels and conducted analysis under the assumption that minimum conductance obtained at sufficiently negative potentials was 'background conductance' and that maximum conductance measured at potentials higher than +80 mV corresponds to conductance of all the channels in the fully open state. This analysis may inherently miss a small fraction of ion channels in the reversed orientation; it has not been clearly shown that orientation of the SR K^+ channel is conserved in giant proteoliposomes prepared by the conventional method of detergent/dialysis followed by freeze-thawing.

The frequency of encountering ion channels in PEG vesicles did not differ from that in freeze-thawed vesicles. Initially we expected a much higher frequency with PEG vesicle membranes because channel density should be 10–40-times that in the freeze-thawed vesicle membranes, where membrane proteins are diluted by exogenous lipids. The reason for this low frequency is not clear.

In conclusion, the two methods of giant vesicle preparation, freeze-thawing without use of detergent and PEG-induced fusion, are useful in the study of ion channels of vesicular biomembranes. They do not significantly alter gating characteristics of the channel, and orientation of the channel is conserved. The freeze-thawing method is particularly advantageous in that it is simple and rapid.

Acknowledgments

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