Immobilizing BK-channels in artificial lipid bilayers using annexin V

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In this report, we show an improved method for the simultaneous measurement of optical and electrical properties of single-channel proteins for analysis of the gating mechanism. Large-conductance Ca\(^{2+}\)-activated potassium (BK) channels were isolated from porcine uterine smooth muscle and labeled with Cy5 via antibody against the N-terminal. These Cy5-labeled BK channels were incorporated into lipid bilayer membranes followed by single channel current measurements. Cy5-labeled BK channels possessed Ca\(^{2+}\) and voltage sensitivity. The orientation of the vesicles was determined to be outside-out. Charybdotoxin applied from the cis side blocked the channel current. For stable observations of ligand and channel binding, BK channel immobilization was also examined. The lateral diffusion coefficient of BK channels decreased over 200 fold in 1 µM annexin V while the open probability did not change. This study is a significant advancement in simultaneous measurements of ligand binding and current change at the single channel level. [DOI: 10.1380/ejssnt.2007.1]

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I. INTRODUCTION

Single ion-channel current recording is a pioneer technique in single molecule biophysics [1, 2]. Even so, it has not yet been able to directly detect the conformational changes of channel proteins accompanied by a change in activity. Channel gating is regulated by several factors such as voltage or the binding of specific ligands. Until now, the average binding constants for a large number of ligands or blockers have been used to analyze gating mechanisms. However, in such cases, the dynamic, kinetic and fluctuating properties of biomolecules remain hidden. Therefore, a new technology is required to image single channel interactions with ligands.

In BK channels, an increase in cytoplasmic calcium ions and depolarization of the membrane causes a synergistic effect on the opening of the BK channels [3]. Because of this, BK channels play a very important role in the excitability of many systems. BK channels are tetramers with a pore forming domain similar to other potassium channels whose structures have been crystallized [4]. In addition, the conductance of BK channels is very large. Thus, the BK channel is one of the more preferable channels to investigate gating mechanisms.

In order to investigate ligand related gating changes, it is important to examine the correlation between the channel-gating and channel-drug interaction. If a channel has several ligand binding sites, the real reaction kinetics will be obtained only at a single channel level, which has been done using the patch clamp technique. However, imaging of single ion channels is less developed. Recently we developed a novel experimental apparatus for the simultaneous measurements of optical and electrical properties of single ion channels [5–7]. The system combined total internal reflection fluorescence (TIRF) microscopy for single-molecule detection with horizontally made artificial black lipid (BL) bilayers for the functional recording of single-channels. The BL technique also offers advantages for investigating lipid-protein interactions.

Using these techniques, we have reported the moment that Cy5 labeled single BK channel was incorporated into the BL membrane and the opening of the channel [7]. These labeled BK channels from bovine trachea smooth muscle showed normal Ca\(^{2+}\) sensitivity and rapid movement on the lipid bilayer by thermal fluctuation. However, we still had two major obstacles to overcome for the stable observation of the ligand and channel binding. First, we needed to control the orientation of the channel protein in an artificial lipid bilayer. The BK channels from bovine trachea are oriented in an inside-out configuration in vesicles. Thus, the binding site of a blocker, such as charybdotoxin, is located along the extracellular facing surface. That is, in an inside-out vesicle orientation, outside of the channel located along the trans side after fusion to the bilayer. However, if fluorescently labeled charybdotoxin was added from the trans side, it would cause the binding of many fluorescently labeled blockers to the glass surface making it possible to detect the single dye imaging. Therefore we must use an outside-out oriented vesicle. Toro et al. [8] reported that most BK channels (80%) from porcine uterine smooth muscle were oriented outside-out. In this report, we isolated BK-channels from porcine uterine, fluorescently labeled them, estimated the orientation, and examined the effect of charybdotoxin applied from the cis side.

A second obstacle was the rapid lateral movement of the channel proteins. For the analysis of kinetics of the ligand binding and channel gating in a single molecule level, it is important to immobilize the ion channel and observe it at least several tens of seconds. Recently we reported

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that annexin V decreased the diffusion constant of cardiac ryanodine receptors as well as lipid molecules. 1 μM Annexin V significantly immobilized the ryanodine receptor without any direct effect on channel activity in the lipid bilayer [9]. Annexin V is a member of the annexin superfamily and binds specifically to phosphatidylserine in a Ca²⁺ dependent manner [10]. Some of the BK channels are mechanosensitive channels [11, 12], known to interact with cholesterol and lipid rafts [13, 14]. Therefore, it is important to examine the effects of immobilization of lipids on BK channel activity. In this paper, we examined the diffusion of Cy5-labeled single BK channel in a lipid bilayer under TIRF microscope, and investigated the immobilizing effects by annexin V.

II. EXPERIMENTAL

The polyclonal anti-BK channel antibody, MaxiKα(E16), raised in goat against the amino terminus of MaxiKα human origin, was purchased from Santa Cruz Biotechnology (California). Cy5-monofunctional reactive dye was purchased from GE Healthcare UK Ltd. (Little Chalfont, England). Phosphatidylethanolamine, Phosphatidylserine and Phosphatidylycholine were purchased from Sigma-Aldrich (Missouri). All of the other chemicals were commercial products of an analytical grade.

Fluorescent labeling of the BK-channel with Cy5-conjugated antibody

The vesicles containing BK channels were isolated from porcine uterine smooth muscle using a modification of the procedure of Toro, et al. [8]. The heavy microsomal fraction was centrifuged on a discontinuous sucrose gradient. Membrane fractions were obtained from 20:25% and 25:30 sucrose interfaces. The final pellets were resuspended in 300 mM sucrose, 100 mM KCl, and 5 mM Na-PIPES at pH 6.8.

The antiBK channel antibody raised at the amino terminus was conjugated with Cy5 molecules using a Cy5-monofunctional reactive dye kit. Free Cy5 was removed via NAP-10 gel filtration column (GE Healthcare). The Cy5-conjugated antibodies were incubated for 60 min at 22°C with BK-channel containing vesicles. In order to remove unreacted antibodies, the mixture was washed twice with 200 volumes of the appropriate buffer following centrifugation.

Formation of a bilayer on agarose-coated glass and incorporation of a channel protein

The apparatus to form artificial lipid bilayers was described in detail by Ide, et al. [6]. The apparatus consisted of upper and lower chambers. Over the hole of the bottom of the lower chamber, the coverslip was fixed. Before fixation, the coverslips (22 × 22 mm, 0.17 mm in thickness) were coated with agarose by painting with a warmed solution of 0.5-1% agarose and then air-dried at room temperature. This agarose layer prevents rupturing of the lipid bilayer. A thick membrane was formed horizontally by painting a lipid solution (20 mg DΦPC/ml n-decane) across the hole of upper chamber following the method by Ide, et al. [6]. The channel in the vesicular membrane was incorporated into the artificial lipid bilayer by vesicle-fusion as described [7]. The solution contained 300 mM KCl, 1 mM CaCl₂, and 10 mM HEPES/Tris (pH 7.4).

Electrophysiology

The current across the membrane was acquired with a patch-clamp amplifier (CEZ-2400, Nihon-Kohden, Tokyo, Japan) and recorded with a personal computer. Single-channel analysis was carried out using software pClamp8.1 from Axon Instruments (CA, USA). The membrane potential was defined as the voltage of the upper side (cis side) of the membrane with respect to the lower side (trans side) that was held at virtual ground.

Imaging

FIG. 1: The electrical activity of single BK-channel from porcine smooth muscle was measured after dye labeling. (A) Current at pCa5, (B) at pCa3. The artificial bilayer was clamped at +30 mV. Right side of each trace shows the frequency histogram of the current amplitude.
Single channel imaging was carried out following the method described previously using objective-type total internal reflection fluorescence microscope [5, 6]. Cy5-BK channels were excited with red (633 nm) laser. Images were acquired every 33 msec using ICCD camera, an image intensifier (VS4-1845, Video Scope, VA, USA) and an electron bombarded CCD camera (C7190-20, Hamamatsu Photonics, Hamamatsu, Japan), and stored on digital videotape.

III. RESULTS AND DISCUSSION

BK channels from porcine uterine smooth muscle were fluorescently labeled with Cy5 via antibody, which was raised against the N-terminal of the BK channel. The channel activity after labeled with fluorescent dye was investigated using the artificial lipid bilayer technique. The lipid bilayer was made horizontally and vesicles were incorporated by flushing just above the lipid bilayer through a glass capillary.

Fig. 1 shows the single channel recording of the BK channel. The current amplitude measured at +30 mV was over 10 pA, large enough to distinguish it from other types of channels. As shown in Fig. 1A, when the calcium concentration was as low as 10^{-5} M on the trans (lower) side, and the membrane clamped at +30 mV on the cis (upper) side of the bilayer, most BK channels were closed (Po = 0.18). On the contrary, at high calcium conditions such as 10^{-3} M on the trans side, almost all channels were open (Po = 0.98) as shown in Fig. 1B. This result suggests that the fluorescently labeled BK channels had normal calcium sensitivity.

Figure 2A shows the I-V relationship of the Cy5-BK channel at pCa3. Almost all channels were open with a single channel conductance of 398 pS calculated from the slope of the I-V curve. Orientation of the vesicles was determined more clearly by the voltage dependency at low calcium concentration. When the clamp voltage on the cis side (outside of the vesicle) was shifted toward the positive direction at pCa5, the open probability of the channels decreased (Fig. 2B). Therefore depolarization of the lower side of the lipid bilayer opened the channels, similar to how intact channels would behave. These results confirm that the BK channels from porcine uterus smooth muscle had a normal conductance and open probability and had an outside-out configuration in the vesicles.

For simultaneous optical and electrical measurements, a blocker such as charybdotoxin was applied from the cis side, since fluorescently labeled free blocker sedimentation on the trans side (lower chamber) interfered with single molecule observation. The Cy5-BK channels from porcine uterine smooth muscle showed a significant response to charybdotoxin (Fig. 3). 10 nM charybdotoxin applied from the cis side completely abolished the opening of BK channels. However, when we used the vesicles from bovine tracheal smooth muscle, there was no response to charybdotoxin applied from the cis-side (data not shown). The reason for different organs having different BK channel orientations is unknown but may be due to the membrane orientation in the cells. The majority of BK channels in tracheal smooth muscle might be located at the invasinated plasma membrane. In contrast, BK channels in uterine are located in lipid rafts and interact with the lipid raft microdomains and the sarcoplasmic reticulum, possibly in a regulative manner for uterine contractions [13]. Artificial bilayer technique has the potential to examine this mechanism between the interaction of the channels and lipid directly.

Ion channels in artificial lipid bilayers move rapidly since there is no plasma membrane associated proteins to anchor the channels. For stable optical recordings of BK channels and ligand binding, we immobilized the channel protein. Otherwise the channel protein would leave the visual field. If we decreased the size of the bilayer area, the lipid bulk layer caused scattering of the laser beam, making it impossible to conduct single molecule imaging. To immobilize, we applied annexin V, which is known...
FIG. 3: Single channel recording of the BK channel from porcine uterine smooth muscle in the artificial bilayer clamped at 0 mV before (A) and after (B) the application of 10 nM charybdotoxin. Charybdotoxin was applied from the cis side (outside of the vesicle), inhibiting single BK-channel activity.

FIG. 4: Influence of annexin V on the diffusion coefficient was examined. (A) Trajectory of a single BK channel labeled with Cy5 in an artificial lipid bilayer. Left side shows the trace before application of 1 µM annexin V, while right side shows the trace after application. (B) Mean square displacement $< r^2 >$ plotted against the time interval $\Delta t$. Closed circles represent the values before annexin V application. Closed squares represent the values after 1 µM annexin V application. From the slope of the line, the diffusion coefficient, $D$, of this single molecule was determined to be $1.96 \times 10^{-8}$ cm$^2$/s (without annexin V) and $1.48 \times 10^{-10}$ cm$^2$/s (with annexin V). Lipid composition of the bilayer was PE:PS:PC = 5:3:2 (50 mg/ml phospholipids in n-decan).

FIG. 5: Single channel recording of the BK channel from porcine uterine smooth muscle in the artificial bilayer clamped at 0 mV before (A) and after (B) the application of 10 nM charybdotoxin. Charybdotoxin was applied from the cis side (outside of the vesicle), inhibiting single BK-channel activity.

In conclusion, we examined the orientation and immobilization of BK channels prepared from porcine uterus smooth muscle cells using artificial lipid bilayer and TIRF microscopy. The BK channels fluorescently labeled with Cy5 possessed normal electrical activities as controls and showed an outside-out configuration in the vesicle. The electrical activities of the BK channels were inhibited by the application of charybdotoxin from the upper side of the bilayer. In addition, these BK channels were immobilized by annexin V without changing their electrical properties. This makes it possible to continuously observe ligand and channel binding with simultaneous electrophysiological measurements. These progresses should open new insight for single molecule physiology.

IV. CONCLUSIONS

In conclusion, we examined the orientation and immobilization of BK channels prepared from porcine uterus smooth muscle cells using artificial lipid bilayer and TIRF microscopy. The BK channels fluorescently labeled with Cy5 possessed normal electrical activities as controls and showed an outside-out configuration in the vesicle. The electrical activities of the BK channels were inhibited by the application of charybdotoxin from the upper side of the bilayer. In addition, these BK channels were immobilized by annexin V without changing their electrical properties. This makes it possible to continuously observe ligand and channel binding with simultaneous electrophysiological measurements. These progresses should open new insight for single molecule physiology.

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