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# Simultaneous Optical and Electrical Recording of a Single Ion-Channel

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**Abstract:** In recent years, the single-molecule imaging technique has proven to be a valuable tool in solving many basic problems in biophysics. The technique used to measure single-molecule functions was initially developed to study electrophysiological properties of channel proteins. However, the technology to visualize single channels at work has not received as much attention. In this study, we have for the first time, simultaneously measured the optical and electrical properties of single-channel proteins. The large conductance calcium-activated potassium channel (BK-channel) labeled with fluorescent dye molecules was incorporated into a planar bilayer membrane and the fluorescent image captured with a total internal reflection fluorescence microscope simultaneously with single-channel current recording. This innovative technology will greatly advance the study of channel proteins as well as signal transduction processes that involve ion permeation processes. [Japanese Journal of Physiology, 52, 429–434, 2002]

*Key words*: bilayer, single-channel, BK-channel, single-molecule, imaging.

Recent remarkable developments in optical microscopy have enabled us to directly observe single fluorescent molecules. The single-molecule imaging technique has broad applications and has been used to study a variety of biological molecules as reviewed by Weiss [1]. This technique has been applied to study various types of proteins, especially motor proteins, and has revealed many new properties of both the actomyosin and kinesin-microtubule systems [2]. For example, we have performed experiments where the binding of single ATP molecules to a single myosin and the resulting force generations were simultaneously observed. Combining this technique with fluorescence resonance energy transfer (FRET) or the single-molecule spectroscopy technique, which act as molecular rulers, it is possible to optically detect the conformational changes in a single protein molecule [3, 4].

While a number of studies have been made on water-soluble proteins and nucleotides using the single-molecule imaging technique, very little has been done on imaging single-channel proteins despite the technique for single-channel current recording being well established. A channel protein is a membrane protein that is thought to have a psuedosymmetric structure and which spans the membrane. Selected ions move across the membrane, through the minute channel in the center of the protein, down the electrochemical potential gradient. This occurs when the gate of the channel is activated to open in response to specialized stimuli such as binding of the ligand molecule or a change in the membrane potential. The ionic current flowing through single-ion channel proteins can be routinely measured using patch-clamp or planar bilayer techniques. Many ideas on how to achieve the simultaneous recording of optical and electrical observations of single-ion channels have been proposed [5]. However, to date none have been successful. Recently, we have been successful in simultaneously observing a channel-forming antibiotic peptide, alamethicin, using a horizontal bilayer membrane formed on a thin agarose layer [6, 7] and

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recording the current from this single channel. In this paper, we report for the first time, that a single-channel protein incorporated into the horizontal bilayers could be imaged while simultaneously recording single-channel currents.

## METHODS

**Materials.** Diphytanoylphosphatidylcholine (DΦPC) was purchased from Avanti Polar Lipids, Inc. (AL, USA), and asolectin, agarose type VII, from Sigma (MO, USA). The polyclonal anti–BK-channel antibody raised in rabbit against a C-terminal part of a mouse Slo α subunit was purchased from Alomone Labs (Jerusalem, Israel). Cy5-monofunctional reactive dye was purchased from Amersham Pharmacia Biotech (Sweden). All of the other chemicals were commercial products of analytical grade. The smooth muscle sarcolemmal vesicles were prepared according to the method of Slaughter *et al.* [8].

**Fluorescent labeling of the BK-channel with Cy5-conjugated antibody.** The anti–BK-channel antibody was conjugated with Cy5 molecules using a Cy5-monofunctional reactive dye kit (Pharmacia Biotech). The vesicles from bovine tracheal smooth muscle were incubated with the Cy5-conjugated antibodies for 60 min at 22°C. In order to remove unreacted antibodies, the labeled vesicles were washed twice with 200 volumes of the appropriate buffer following centrifugation.

**Formation of a bilayer on agarose-coated glass.** The bilayer apparatus consisted of two chambers. The upper chamber was made from a polypropylene tube with an inner diameter of 10 mm. This chamber could be moved using a piezo micromanipulator (PCS-5000, Burleigh Instruments, NY, USA). On the bottom of the upper chamber, a thin polypropylene film (0.2–0.3 mm thick) with a small pore in the center was attached. The pore on the film was made as follows: A heated stylus was pushed onto the film forming a projection. The top of the projection was shaved with a razor in order to make a circular pore of the appropriate diameter. This is the same method used to make an aperture for a conventional vertical bilayer. The projection used in this study, however, was taller than those conventionally used. The lower chamber consisted of a 35 mm glass dish with a hole, 12 mm in diameter, in the bottom. A coverslip was fixed over the hole with an adhesive just prior to the experiment. The upper and lower chambers were connected to a patch-clamp amplifier through Ag–AgCl electrodes. The coverslips (22×22 mm, 0.17 mm in thickness) were washed thoroughly, coated with agarose by painting with a warmed solution of 0.5–1% agarose type VII (Sigma) in water and then air-dried at room temperature. No special linkage was made between the agarose and the glass surface. The dried coverslip was fixed with an adhesive over the hole on the bottom of the chamber. The membrane was formed as follows: A thick membrane was formed across the hole by painting a lipid solution (20 mg DΦPC/ml *n*-decane) on the hole. The upper chamber was then moved in the downward direction until the membrane came into contact with the agarose-coated coverslip. By slightly increasing the pressure in the upper chamber, the membrane began to thin out and finally the center of the membrane became a bilayer. This process of thinning could be facilitated by applying membrane voltages (±75 mV) and could be observed using a normal bright-field microscope.

**Determination of a lateral diffusion constant from a single-molecule trajectory.** The mean square displacement ($\langle r^2 \rangle$) averaged over a trajectory at each time interval ($\Delta t$) was calculated from the trajectory of a particle. The diffusion constant, $D$, was calculated from the slope of the $\Delta t$–$\langle r^2 \rangle$ plot by least-squares fitting.

**Electrophysiology.** Recording of the current across the membrane was made with a patch-clamp amplifier (CEZ-2400, Nihon-Kohden, Tokyo, Japan) and stored on DAT tape with a DAT recorder (RD-125T, TEAC, Tokyo, Japan). The membrane potential was defined as the voltage of the upper side of the membrane with respect to the lower side that was held at virtual ground. Single-channel analysis was carried out with a personal computer using commercially available software (pClamp6, Axon Instruments, CA, USA).

**Microscopy.** The main specifications of the microscope used in this study have been previously described [6]. Modifications to the optical lenses and filters were made for this study. The laser beam was split by a polarizing beam splitter, and one of the beams was used for epi-fluorescence illumination and the other for evanescent field illumination. The incident angle at the specimen, which determined the penetration depth of the evanescent field, could be changed by sliding the mirror. Images were recorded using an 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. Video sequences were imported and analyzed by a personal computer using image analysis software (Image Command 4198, Nippon Avionics, Tokyo, Japan). A coor-

dinate $(x, y)$ of each particle was calculated as the center of intensity.

**Reconstitution of a channel protein into horizontal bilayers.** The experimental equipment is schematically shown in Fig. 1. Figure 2A illustrates the strategy used to incorporate the fluorescently labeled channel into the artificial membrane. The vesicles prepared from bovine trachea were incubated with monoclonal anti–BK-channel antibodies, which bind specifically to the BK-channel proteins. After removal of unreacted antibodies by ultracentrifugation, the channel in the vesicular membrane was transferred into the artificial planar bilayer by vesicle-fusion. In order to induce rapid fusion of the vesicle in the limited area of the bilayer, vesicles that had been osmotically loaded were puffed through a fine glass pipette as shown in Fig. 1. The solution contained 100 mM KCl, 1 mM $CaCl_2$, and 10 mM HEPES/Tris (pH 7.4).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western**

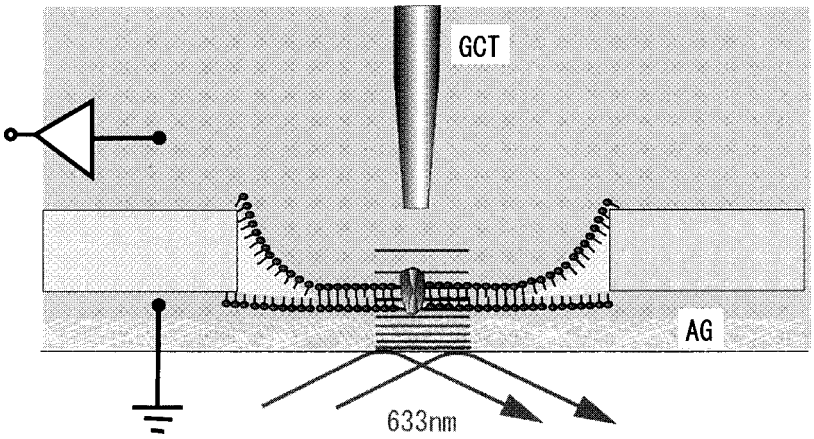


**Fig. 1. Schematic diagram of the horizontal bilayer.** Horizontal planar lipid bilayers were formed across an aperture pierced in the center of the bottom of the upper chamber, which was approximately 200 μm in diameter. The bottom of the lower chamber was made of thick glass (170 μm) that was coated with a thin agarose layer (AG) in order to prevent lipids adhering to the bottom. The fluorescent particles in the bilayers were visualized with evanescent field light ($\lambda$=633 nm) generated on the glass-agarose interface. Vesicles and drugs were added directly to the membrane through a fine glass capillary tube (GCT) with its tip positioned approximately 10 μm from the bilayers.

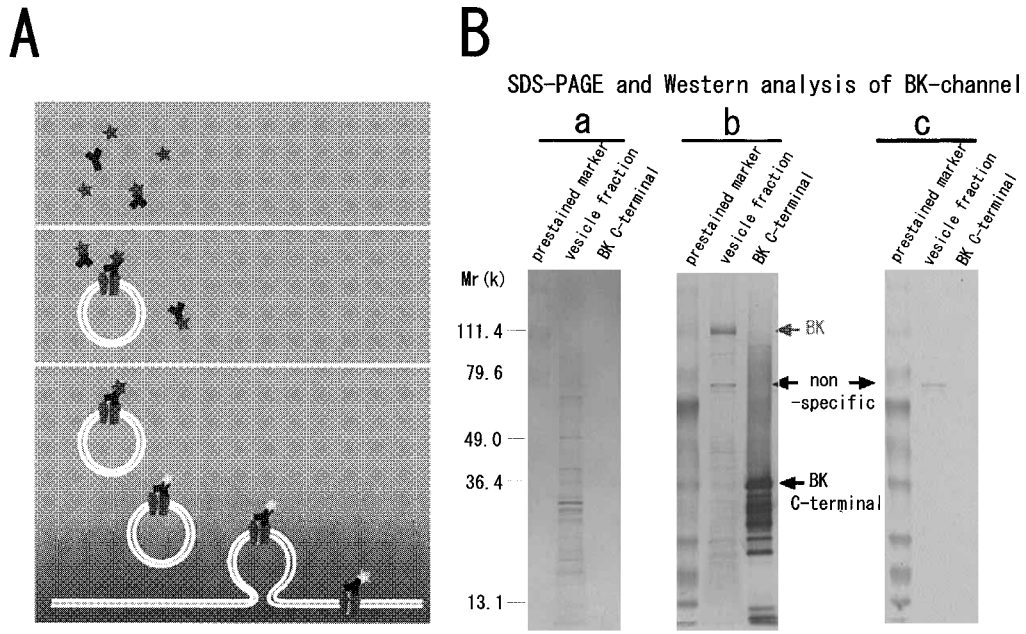


**Fig. 2. A: Schematic diagram of how channel proteins become incorporated into a membrane.** The anti–BK-channel antibodies were labeled with Cy5-dye molecules. The vesicles were incubated with the labeled antibodies. After the removal of unreacted antibodies by ultracentrifiguation, the vesicles were added directly to the bilayer through a fine glass capillary. The channel protein was transferred into the planar bilayer by fusion between the vesicular membrane and planar bilayer. **B: The specificity of anti–BK-channel antibody investigated using SDS-PAGE and Western blotting.** Column a shows CBB staining of SDS-PAGE (4–20%). Columns b and c represent the Western blotting against anti–BK-channel antibody in b and secondary antibody for negative control in c, respectively. Each column shows lane 1: prestained molecular marker from GIBCO Invitrogen Corp. Japan (Tokyo, Japan); lane 2: 4 μg of sarcolemmal vesicle proteins; and lane 3: 20 ng of the fusion protein for positive control.

**blotting.** SDS-PAGE gels with Laemmli's buffer system (4–20%) from Dai-ichi Pure Chemical Co. (Tokyo, Japan) were stained with Commassie Brilliant Blue (CBB). Proteins were transferred onto polyvinylidene difluoride (PVDF) transfer membranes from ATTO Bioscience & Biotechnology (Tokyo, Japan) by the method of Towbin *et al.* [9]. Anti-rabbit IgG, goat-poly, biotin-conjugated antibody from Vector Laboratories Inc. (CA, USA) was used as the secondary antibody, followed by incubation with horse radish peroxidase (HRP)–conjugated streptavidine. Development was accomplished by adding dimethylaminoazobenzene.

## RESULTS

Figure 2B shows the specificity of anti–BK-channel antibody investigated using SDS-PAGE and Western blotting. Column a shows CBB staining of SDS-PAGE (4–20%). Columns b and c represent the Western blotting against anti–BK-channel antibody and secondary antibody for negative control, respectively. For the positive control, the fusion protein of *Schistosoma japonicum* glutathione-S-transferase (GST) and a C-terminal part of a mouse α subunit from Alomone Labs were used in lane 3 in each column. The control fusion protein of 37 kDa was only stained intensely against anti–BK-channel antibody. The 70 kDa protein represented by the staining both in b and c refers that this 70 kDa is a non-specific protein. In the sarcolemmal vesicle fraction, the 120 kDa protein was stained intensely against the anti–BK-channel antibody (column b, lane 2). A protein of this size corresponds with the expected $M_r$ (125 kDa) of BK [10]. As shown in column a, the sarcolemmal vesicle fraction contained very little 125 kDa BK protein. These results confirm

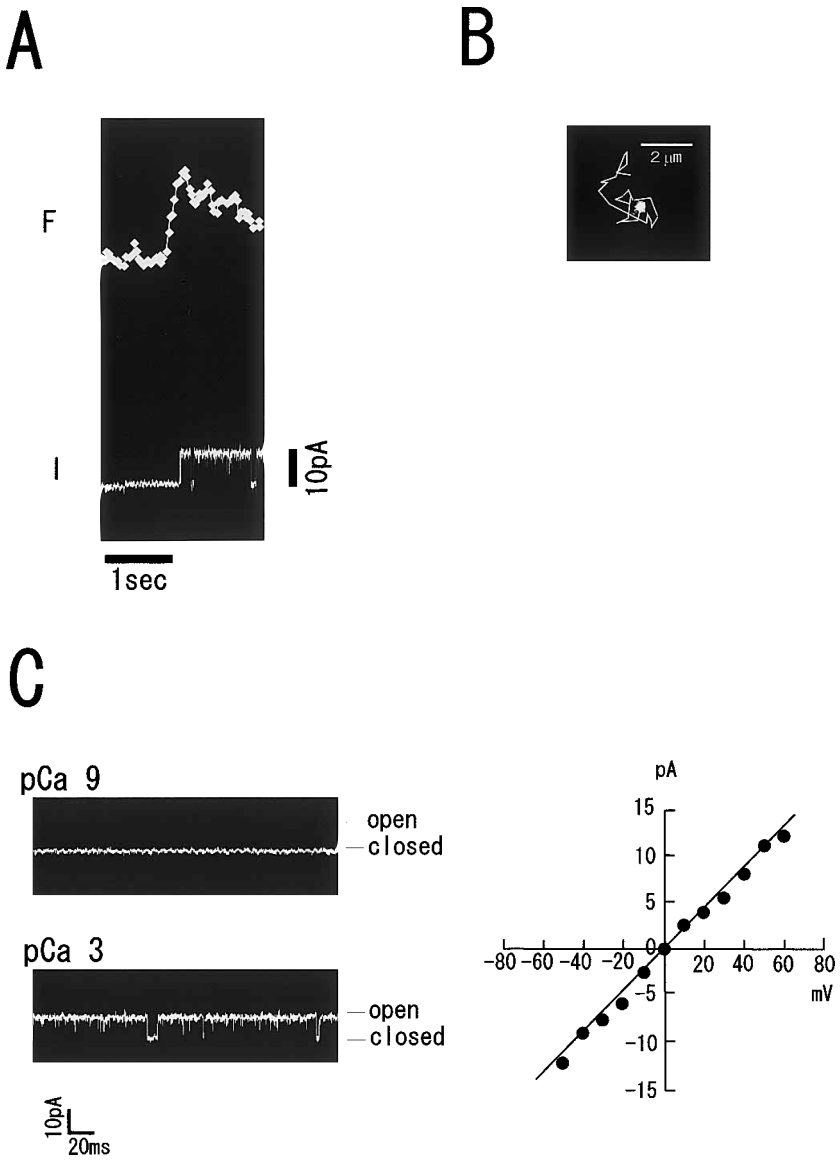


**Fig. 3. Simultaneous electrical and optical recording of a single BK-channel. A**: The BK-channel was labeled with Cy5-dye molecules and incorporated into the bilayer using vesicle fusion techniques. The top trace shows the fluorescence intensity and the bottom trace shows the single-channel current record recorded simultaneously. The decrease in fluorescence intensity represents the photobleaching effect of the Cy5-dyes. The channel was in the open state before it was transferred into the planar bilayer because the solution contained 1 mM $CaCl_2$. The number of Cy5-dyes attached to the channel was estimated to be approximately 25 from the fluorescence intensity. **B**: Thermal motion of the channel protein in the membrane. The channel protein moved thermally in the membrane decreasing its fluorescence intensity as a result of photobleaching. The diffusion constant was determined to be $4.0\times10^{-8}$ $cm^2/s$, which corresponds well to predicted values of small particles moving freely in the membrane. This indicates that there was no strong interaction between the channel and agarose layer. **C**: Single-channel recording of the BK-channel incorporated into the horizontal planar bilayer. The traces show single-channel fluctuations taken from the same bilayer shown in A and B (**left**). The free calcium concentration in the upper chamber was controlled by adding EGTA to the solution. Each trace shows the current fluctuation at pCa 9 and pCa 3, respectively. The single-channel conductance was determined to be 225 pS (**right**).

the specificity of this anti–BK-channel antibody against BK in the vesicle fraction.

Figure 3 shows the results of the experiments in which the smooth muscle BK-channel was reconstituted into the bilayer by vesicle fusion, and simultaneous optical and electrical recordings were performed. The channel was labeled with Cy5-dye molecules and applied to the upper side of the bilayer. A bright spot appeared just before the current across the membrane began to fluctuate as shown in Fig. 3A. After incorporation, the spot moved rapidly in the bilayer as shown in Fig. 3B. The diffusion constant, $D$, was calculated to be $3.0\pm1.5\times10^{-8}$ cm$^2$/s ($n=5$), which agrees well with the value for a channel moving freely in a membrane [6, 7]. We did the experiment 27 times, of which 20 experiments were successful in observing the synchronous increase in optical and electrical signal. In this study, we did not observe that more than two channel proteins were incorporated into the membrane at the same time since the density of the BK-channel in the tracheal membrane was very low [11] and each vesicle contained no or only one channel protein.

The typical current traces recorded with the horizontal bilayer are shown in Fig. 3C. The open probability, $P_O$, of the BK-channel was $<0.01$ at pCa 9 and $>0.95$ at pCa 3, respectively. The single-channel conductance was determined to be $229\pm8$ pS ($n=5$).

## DISCUSSION

The artificial planar bilayer technique has been used to study many types of channels and is a potential tool to study ionic channels which have revealed both pharmacological and dynamic behaviors [12]. Conventional planar bilayers can be formed vertically in aqueous environments and channel proteins are incorporated into the bilayers most commonly by vesicle fusion. In this study, bilayers were formed horizontally so single fluorescent particles in the membrane could be imaged using a total internal reflection fluorescence microscope (TIRFM) [13]. To prevent vertical movement of the membrane and possible breakage of the membrane by touching the glass bottom of the chamber, the bottom of the chamber was coated with a thin layer of agarose. The bilayers were positioned so as to make contact with the agarose layer. The experimental equipment is schematically shown in Fig. 1. The TIRFM allowed us to visualize single fluorophores in the membrane by reducing the background noise and also to detect single fluorescent molecules in the solution when they came very close to the membrane.

In previous papers [6, 7], we reported the results of simultaneous optical and electrical recordings of the channel-forming peptide, alamethicin. Channel proteins cannot be reconstituted into bilayer membranes simply by adding them to the aqueous solution. This is in direct contrast to small amphipathic peptides that spontaneously become incorporated into bilayers. We utilized vesicle-fusion techniques to reconstitute channel proteins into the artificial bilayers. In a specified area of the membrane within a small time frame, only one channel protein is expected to be incorporated into the membrane. However, to observe the moment the channel becomes incorporated into the artificial membrane, more sophisticated techniques are required. The vesicles were added directly to the bilayers through a fine capillary tube. This was immediately followed by an instantaneous increase in ionic concentration in the vicinity of the membrane by puffing a small amount of solution with a high salt concentration. The optimum conditions for successfully incorporating the channel into the membrane were not the same for all channel types. In fact, even for proteins of the same type, the conditions changed from one protein preparation to the next. Thus, the experimental conditions such as the amount of protein to be used and the most suitable ionic concentrations had to be determined for every vesicle preparation.

The techniques described above were applied to achieve the image of a channel protein. Figure 3 shows the results of the simultaneous optical and electrical recording of the smooth muscle BK-channel. This result shows that the fluorescently labeled channel protein in the vesicular membrane had moved thermally in the solution, and when in close proximity to the horizontal planar bilayer, had become incorporated into the bilayer through vesicle fusion. In other words, the bright spot shown in Fig. 3 corresponds to the channel protein itself. The channel properties obtained in this study, shown in Fig. 3C, were consistent with the results obtained in patch-clamp or conventional planar bilayer experiments [11], showing that it is possible to record the natural properties of channel proteins with the method described here.

We have developed a novel method for simultaneously recording the optical and electrical properties of single-ion channels. This method is so sensitive that we can image single fluorophores in the membrane and should prove to be applicable to a wide variety of channel proteins. It is also possible, with this method, to observe single fluorescent molecules in the solution when they are very close to the membrane. This means that we can directly observe the interaction between a single-channel protein and its ligand mole-

cules labeled with a fluorescent dye as we did in ATP-myosin coupling [2]. Such observations will greatly increase our understanding of the ligand-receptor interaction dynamics and the activation mechanisms at the single-molecule level in ligand activated receptor channels such as nACh and glutamate.

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