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Purification of a K^+-channel protein of sarcoplasmic reticulum by assaying the channel activity in the planar lipid bilayer system

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A K^+ channel protein of the sarcoplasmic reticulum (SR) was purified by assaying the channel activity in a planar lipid bilayer system. The light fraction of SR vesicles was solubilized in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and fractionated by an anion-exchange chromatography and followed by gel filtration chromatography and affinity chromatography with concanavalin A. All fractions in each steps were mixed with asolectin solubilized in CHAPS and reconstituted into vesicles by dialysis. The channel activity of each fraction was assayed after the reconstituted vesicles had been fused into a planar lipid bilayer. The final fraction which showed the K^+ channel activity contained only 100 kDa protein in a silver-stained gel after SDS-PAGE and an anti-Ca^{2+}-ATPase antibody did not recognize the protein. The characteristics of the K^+ channel were identical to those observed in native SR vesicles when using the same method. The channel showed a single-channel conductance of 120 pS in 0.1 M KCl and marked voltage dependence. The channel did not permeate Ca^{2+} and Cl^- and was blocked by neomycin B.

Introduction

In the last 10 years, a number of ion channel proteins were purified and their primary structures were determined from the DNA sequences of their clones, for example, Na^+ channels from several sources [1–3], a dihydropyridine receptor from skeletal muscle [4,5], an acetylcholine receptor channel from electric organ [6], and a GABA receptor channel from brain [7,8]. Further, the combination of the molecular genetic and biophysical studies allows us to identify specific residues in the channel protein that directly affect channel activity [9–11]. However, in order to deduce the general law of channel activity such as the molecular mechanisms of gating and ionic selectivity, it is necessary to know about the configuration of various kinds of channels. Thus, it is important to isolate many different kinds of channel proteins and to determine their amino acid sequences.

Miller and Racker made it possible to measure the electric current through channels in membrane vesicles by incorporating them into a planar bilayer [12]. By applying this method to sarcoplasmic reticulum (SR) vesicles, they found K^+ and Cl^- channels, and their properties were studied in detail on the single-channel levels [13–16], although molecular entity has not been made clear.

In the present study, we purified a K^+ channel from the light fraction of sarcoplasmic reticulum (LSR) of rabbit skeletal muscle using a combination of chromatographies by assaying the channel activity in the planar bilayer system. The purified protein had a molecular mass of 100 kDa and showed the same channel activity as observed in native SR vesicles in the planar bilayer method. Part of this work has already been reported [17].

Abbreviations: SR, sarcoplasmic reticulum; LSR, light fraction of sarcoplasmic reticulum; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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Materials and Methods

Materials

Asolectin, neomycin B, and proteinase inhibitors were purchased from Sigma, Con A-Sepharose and SDS gel molecular standards were from Pharmacia.

Asolectin was used without purification throughout the experiment. All other chemicals were commercial products of analytical grade.

Preparation of SR vesicles

LSR vesicles were prepared from rabbit skeletal muscle by the method of Kasai et al. [18] with slight modifications. Briefly, dorsal and leg muscles of the rabbit were homogenized with 4 volumes of 0.1 M NaCl and 5 mM Tris-maleate (pH 7.0), using a mixer MX-A30G (Toshiba, Japan) for 2 min. The homogenate was centrifuged at $4000 \times g$ for 30 min. The supernatant was centrifuged at $10000 \times g$ for 30 min to remove mitochondria, then at $53000 \times g$ for 60 min. This precipitate was suspended in 20–30 volumes of 0.6 M KCl and 5 mM Tris-maleate (pH 7.0), and the suspension was centrifuged again. The sediment was suspended in 0.1 M KCl, 0.3 M sucrose, and 5 mM Tris-maleate (pH 7.0), and stored at $-80\,^{\circ}C$. Throughout, care was taken to keep the temperature close to $4\,^{\circ}C$. The proteinase inhibitors (0.5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain) were included in all solutions used.

Solubilization

LSR vesicles were solubilized in 10 volumes of 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM EGTA, and 20 mM Tris-HCl (pH 7.0) in the presence of a mixture of proteinase inhibitors by stirring them at $4\,^{\circ}C$ for 2 h. The suspension was centrifuged at $100000 \times g$ for 30 min, and the supernatant was separated from the pellet.

Anion-exchange chromatography (HPLC)

The supernatant obtained above was applied to an anion exchanger column, COSMOGEL DEAE (Nacalai tesque, Japan), which was equilibrated in 1% CHAPS, 2 mM EGTA, the mixture of protease inhibitors described above, and 20 mM Tris-HCl (pH 7.0). Elution was performed with a discontinuous gradient of KCl in the same buffer.

Gel filtration chromatography (HPLC)

The anion-exchange fraction was injected into a size exclusion column, TSK-G4000SW (Tosoh, Japan), and the sample was eluted with an elution buffer containing 0.8% CHAPS, 0.1 M KCl, 2 mM EGTA, and 20 mM Tris-HCl (pH 7.0). Before use, the column was equilibrated in the same elution buffer.

Affinity chromatography

The gel filtration fractions were applied to a Con A-Sepharose column (10 mm × 7 mm), which had been equilibrated with 0.5 M KCl, 0.8% CHAPS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.0), and eluted with 1 M α-methyl-D-mannoside. Before transfer to an affinity column, EGTA in the gel filtration fractions was removed by a short gel filtration column, PD-10 (Pharmacia).

Reconstitution of the vesicles containing the channel

Before reconstitution, proteins solubilized in CHAPS were supplemented with asolectin solubilized in 1.0% CHAPS, 0.1 M KCl and 20 mM Tris-HCl to give a final concentration of 2–5 mg/ml and then dialyzed for over 72 h at $4\,^{\circ}C$ against 1000 volumes of the buffer solution containing 0.1 M KCl, 2 mM EGTA, and 20 mM Tris-HCl (pH 7.0). The buffer solution was changed at least three times during the dialysis. In some experiments, 0.3 M sucrose was added to the buffer solution to increase the fusion probability of the vesicles.

Bilayers and vesicle incorporation

Planar bilayers were formed by the painting method of Mueller and Rudin [19]. Asolectin solution (15 mg/ml in *n*-decane) was applied to a small hole made in a polypropylene film fixed in a polystyrene cup. In order to make the small hole (100–500 μm in diameter), a glass rod heated with a burner was pushed on a polypropylene film to make a projection, and the top of the projection was planed with a razor. The film was fixed in a polystyrene cup with adhesive. The planar bilayer separates two aqueous solutions. The vesicles containing channel-forming proteins were added to the aqueous solution on one side of the planar lipid bilayer (*cis* side) in the presence of 1 mM $CaCl_2$. Vesicles could fuse with a planar bilayer without osmotic gradient. The osmotic gradient between *cis* and *trans* solutions enhanced the fusion rate. Within a few minutes after the addition of the vesicles, the membrane conductance began to increase in discrete steps which reflected fusion of the vesicles with the planar bilayer. The recorded data were digitized by a 12 bit A/D converter at an appropriate sampling rate. Analysis was carried out using a microcomputer.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was carried out on 10% polyacrylamide gels using the buffer system of Laemmli [20]. Proteins solubilized in detergents or reconstituted into vesicles were supplemented with 3/16 volume of SDS-PAGE sample buffer (50% sucrose, 6% SDS, 0.01% Bromophenolblue, and 0.4 M Tris-HCl, pH 6.8) and 1/16 volume of 2-mercaptoethanol, and boiled for 2 min. Samples were electrophoresed on 10% polyacrylamide slab gels in buffer containing 0.1% SDS, 25 mM Tris, and 192 mM glycine (pH 8.3). The gels were stained with silver. Standard proteins were (in daltons): phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase

(30000), soybean trypsin inhibitor (20000), and α-lactalbumin (14400). Molecular weights of the peptides in the experimental samples were determined from a plot of log M_r versus relative migration for the standard peptides.

Dot blot analysis

A protein solution was spotted onto a nitrocellulose sheet. The sheet was incubated with 5% (w/v) skim milk and 5% (v/v) calf serum in 150 mM NaCl and 50 mM Tris (pH 7.6) for 1 h to block non-specific binding sites, and then it was washed with the NaCl-Tris buffer. The sheet was incubated with the antibodies in the same buffer for 3 h at room temperature. The nitrocellulose bound antibodies were reacted with horse radish peroxidase-conjugated anti-rabbit IgG and after washing the sheets, the proteins were visualized by incubation with dimethylaminoazobenzene (DAB) and H_2O_2.

Results

Purification of K^+ channel protein

LSR vesicles were solubilized in 1% CHAPS and the supernatant obtained after centrifugation was applied to an anion-exchange column. Fig. 1A shows a typical elution profile. The elution was accomplished with a discontinuous gradient of KCl: 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M KCl. The amount of eluted proteins was about 23, 3, 42, 20, 5, and 7% of total LSR proteins, respectively. Each fraction was analyzed by SDS gel electrophoresis followed by silver staining as shown in Fig. 1B. Most fractions contained a dark band protein which was about 100 kDa as a major component.

Asolectin solubilized in 1% CHAPS was added to all the DEAE fractions to a final concentration of 5 mg/ml, and proteins were reconstituted into vesicles by dialysis as described in Materials and Methods. Vesicles reconstituted from each fraction were added to the *cis* side of a planar bilayer to determine the fraction containing cation channels. Current fluctuations were observed when protein eluted with 0.5 M KCl was reconstituted into the planar bilayers by fusion of the vesicles (data not shown). In the presence of 0.1 M KCl on the *cis*/*trans* sides of the membrane, they were measured as a function of transmembrane potential. The frequency distribution of current fluctuations indicated the presence of a channel with a large single-channel conductance. The frequency in which channel events were observed was very high. Whenever 1/1000 volumes of the vesicle suspension (approx. 0.1 μg protein) was added to the *cis* solution, channel events could be observed (data not shown, but similar to Fig. 4). Moreover, it was usual that several channels were simultaneously incorporated into the bilayer. On the contrary, in spite of several tens of bilayer experiments for each fraction, no cationic channel event was observed when proteins from the other fractions were incorporated into the lipid bilayers. The fraction eluted with 0.5 M KCl (Lane 0.5) contains a protein which was about 100 kDa as a major component.

Fractionation by anion-exchange chromatography was followed by further purification using a gel exclusion column, TSK-G4000SW (elution profile shown in Fig. 2A). The anion-exchange fraction eluted with 0.5 M KCl was transferred directly to the gel exclusion column, which had been equilibrated in 0.8% CHAPS, 0.1 M KCl, 2 mM EGTA, and 20 mM Tris-HCl (pH 7.0), and elution was accomplished with the same solution at a flow rate of 3 ml/min. SDS-PAGE analysis of the TSK-G4000SW fractions (Fig. 2B) indicate that most contain protein of about 100-kDa molecular mass.

To all the fractions, asolectin solubilized in 0.8% CHAPS was added to a final concentration of 2 mg/ml,

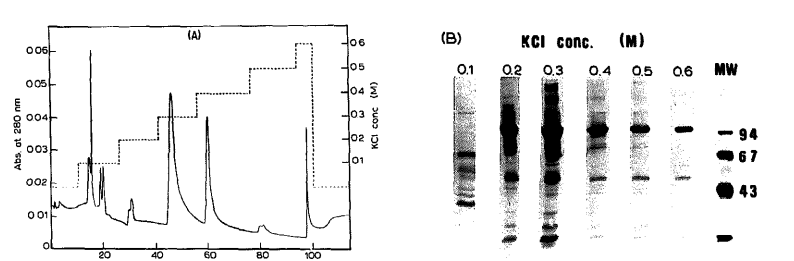

Fig. 1. (A) DEAE column elution profile with a discontinuous KCl gradient. 7.5 mg of SR proteins solubilized in 1% CHAPS, 2 mM EGTA, and 20 mM Tris-HCl (pH 7.0), in the presence of a mixture of proteinase inhibitors was applied to the DEAE column, and eluted with a discontinuous gradient of KCl from 0 to 0.6 M in the same buffer at the flow rate of 0.75 ml/min. A_{280} (——) and KCl concentration (- - - - -) are shown in figure. (B) SDS-PAGE (10% acrylamide gels) analysis of DEAE column fractions. Peptides were stained by silver.

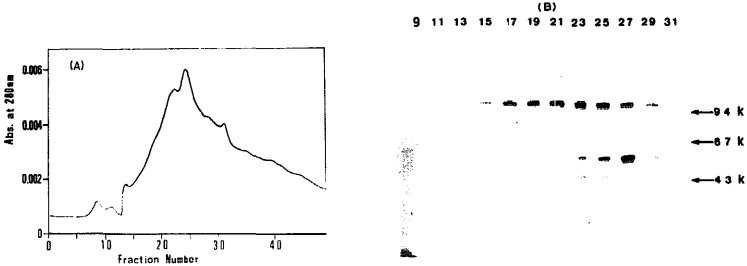

Fig. 2. Gel exclusion column elution profile. The DEAE fraction eluted with 0.5 M KCl was transferred directly to the gel exclusion column, TSK-G4000SW. The sample was eluted with 0.1 M KCl, 0.8% CHAPS, 2 mM EGTA, and 20 mM Tris-HCl (pH 7.0), at the flow rate of 3 ml/min. (A) A_{280} is shown in the figure. (B) SDS-PAGE of the fractions from TSK-G4000SW. Proteins were stained by silver.

and vesicles were formed by dialysis. Only when the vesicles reconstituted from the fraction No. 17, which contained a 100-kDa protein and a very small amount of 120-kDa protein, were incorporated into the lipid bilayers, cationic channel events were observed. No other channels, anionic or non-selective channels, were observed in the same vesicles. Other fractions exhibited no cationic channel event as far as we could determine.

Since small amount of 120-kDa protein was observed in fraction No. 17 the fraction was further purified by affinity chromatography with Con A-Sepharose. The 120-kDa protein was adsorbed to the column and the non-adsorbed fraction showed K^+ channel activity (Fig. 3). On the contrary, the adsorbed

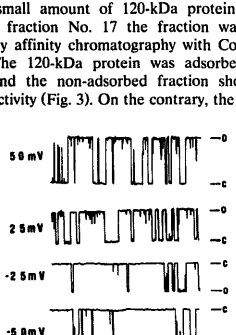

Fig. 3. Single-channel fluctuations in the purified channel. The proteins which did not absorbed to Con A-Sepharose column were reconstituted into vesicles, and was incorporated into a lipid bilayer by vesicle fusion. The current records were taken in the symmetrical solution of 0.1 M KCl and 10 mM Hepes-Tris (pH 7.2) at the indicated voltages.

fraction did not show K^+ channel activity. Thus, we concluded that the 100-kDa protein can form K^+ channel without 120-kDa peptide. Since the molecular mass of K^+ channel protein is very close to that of Ca^{2+}-ATPase, cross reactivity with anti-ATPase antibody was studied. An anti-sera against the Ca^{2+}-ATPase of scallop SR, which could cross-react with rabbit Ca^{2+}-ATPase, did not cross-react with the purified proteins (data not shown), indicating that the purified protein was different from Ca^{2+}-ATPase.

Properties of purified K^+ channel

Since the yield of the purified protein after Con A chromatography was largely decreased, we used fraction No 17 for further analysis of the properties of the purified K^+ channel protein. Fig. 4A shows current fluctuations of the purified channels. The *cis* solution contained 0.25 M KCl, 1 mM $CaCl_2$, and 10 mM Hepes-Tris (pH 7.2), and the *trans* solution was the same buffer with 0.1 M KCl. The frequency distribution of the current fluctuations indicated the presence of a cation-selective channel with a 2/3-subconductance state. Single-channel current was measured as a function of transmembrane potential, and the current-voltage relationships shows that the single-channel conductance was about 120 pS (Fig. 4B). The measured reversal potential was -17 mV, corresponding to a permeability ratio (P_K/P_{Cl}) of about 7.

Under the same condition as in Fig. 4, positive current fluctuations were observed when the voltage was held at 0 mV. This indicated that K^+ ions moved from the *cis* side to the *trans* side in the purified channel. We perfused the *cis* chamber with the buffer containing 0.1 M NaCl instead of KCl, and observed that the amplitude of current fluctuations began to decrease gradually and finally the direction of the current reversed. This indicated that the purified chan-

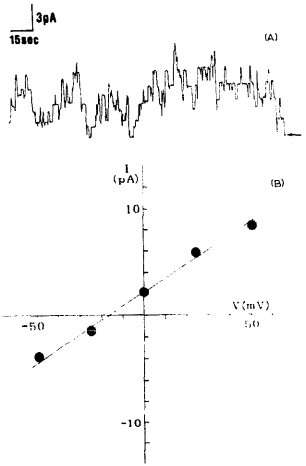

Fig. 4. Current records following addition of vesicles reconstituted from fraction No. 17 in Fig. 2B to the *cis* side of a bilayer. (A) Current was recorded in 0.25 M KCl, 1 mM $CaCl_2$, 10 mM Hepes-Tris (pH 7.2) (*cis*), and 0.1 M KCl, 10 mM Hepes-Tris (pH 7.2) (*trans*). The applied voltage was held at 0 mV. The arrow denotes the baseline. (B) Current-voltage relationship for the channel.

nel is selective for K^+ over Na^+. Analysis of current-voltage relationship yielded a reversal potential of 17 mV (Fig. 5). The equation

$$E_{rev} = \frac{RT}{F} \ln \frac{P_K[K]_{trans} + P_{Na}[Na]_{trans}}{P_K[K]_{cis} + P_{Na}[Na]_{cis}}$$

was used to calculate a permeability ratio, P_K/P_{Na} of 1.9.

Fig. 6 shows the relationship between single-channel current and the applied voltage of a bilayer containing a purified K^+ channel. The currents were recorded in 0.1 M symmetrical solutions of KCl, NaCl, and CsCl. The current-voltage relation indicates that the slope conductance of the channel was 120, 70 pS and small conductance less than 1 pS, respectively. These values agree with those of native K^+-channel of SR [13].

Fig. 7 shows the voltage dependency of opening and closing of the purified channel. Single-channel current fluctuations and corresponding amplitude histograms are shown in the figure. Increase of applied voltage shifts the channel from the closed (c) to the open (o) state. This result indicates that the purified K^+-channels are oriented to its natural direction in the reconstituted vesicles. Furthermore, Fig. 8 shows that the purified channel was blocked by neomycin B in the same manner as a native K^+-selective channel from LSR [21]. 200 μM neomycin B was added to the *cis* side, and resulted that the channel was blocked voltage dependently. Single-channel conductance was decreased with the increase of the applied voltage.

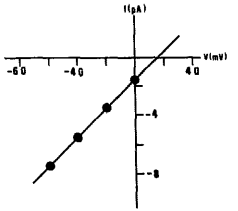

Fig. 5. The purified channel was selective for K^+ over Na^+. The purified protein was incorporated into a lipid bilayer in symmetrical solutions of 0.1 M KCl, 10 mM Hepes-Tris (pH 7.2). Then, the *cis* chamber was perfused with 0.1 M NaCl, 10 mM Hepes-Tris (pH 7.2), and the current fluctuations were measured as a function of applied voltage.

All these characteristics of the purified channel agree with those of a native K^+-selective channel from LSR.

Discussion

A number of ion channel proteins were purified by using specific markers. For example, STX or TTX was used for purification of Na^+-selective channels from brain [22], from electric organ [23], and from skeletal muscle [24], DIDS for anion-selective channel of electric organ [25], and ryanodine for Ca^{2+} release channel from SR [26–28]. However, no specific marker for the

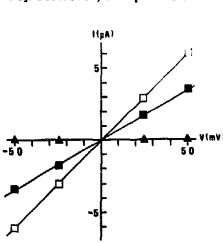

Fig. 6. Current-voltage relation of records from a purified K^+-channel. The purified channel was reconstituted into a lipid bilayer, and current fluctuations were observed as a function of applied voltage in symmetrical solutions of 0.1 M KCl (□), 0.1 M NaCl (■), 0.1 M CsCl (▲), and 10 mM Hepes-Tris (pH 7.2)

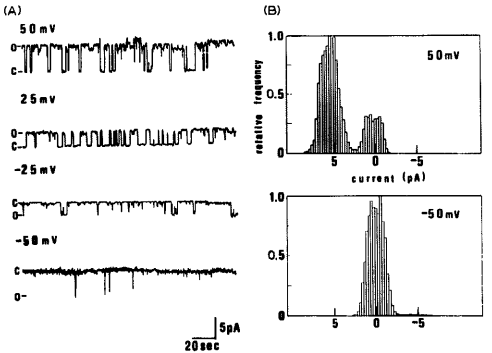

Fig. 7. Single-channel fluctuations following reconstitution of the channel into a lipid bilayer. 1/100 volumes of the reconstituted vesicle suspension was added to the *cis* solution, and current fluctuations were recorded in a symmetrical solution of 0.1 M KCl, 1 mM $CaCl_2$, and 10 mM Hepes-Tris (pH 7.2). Amplitude histogram and corresponding current records from the channel at an indicated voltage, illustrating the voltage dependence of channel gating.

K^+-channel from SR has been found. For an ion channel protein that has no specific ligand, new technique to assay the protein is required. In this paper, we employed the planar lipid bilayer method to ascertain the existence of channel proteins. We may be the first to employ this technique for the purification of a channel protein, as well as our initial report about the purification of Cl^- channel [29].

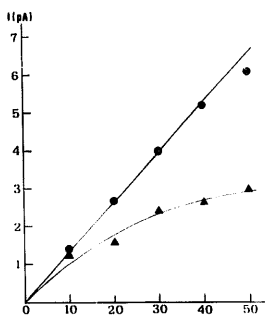

Fig. 8. Effect of neomycin B on the single-channel conductance. Current fluctuations were recorded in a symmetrical solution of 0.1 M KCl, and 10 mM Hepes-Tris (pH 7.2), and the maximum currents were determined in the absence (●) and in the presence (▲) of neomycin B (200 μM *cis* addition).

As described in the above, we fractionated proteins from the LSR of rabbit skeletal muscle by anion exchange chromatography, gel filtration chromatography and Con A affinity chromatography, and assayed all these fractions by the planar bilayer experiments. Consequently, we found that the K^+-selective channel from SR is formed by 100-kDa proteins.

In order to select a detergent, we preliminary tested some detergents, including CHAPS, Triton X-100, NP-40, octyl glucoside and Lubrol PX. These detergents have no net charge themselves, facilitating subsequent purification procedures. With all of these detergents, we have succeeded in reconstituting the channels into vesicles. However, nonionic detergents often produced artifactual current records resembling those expected from ion channels [30], so that we decided to use CHAPS, which is zwitter-ionic and reported to be very effective with solubilization efficiencies and stability characteristics.

Further, we tested some reconstitution methods, dialysis, dilution, gel filtration, salting out, and polystyrene resin (Bio-Beads SM-2). Consequently, we have found that the combination of the CHAPS solubilization and the reconstitution by dialysis is the most effective one. We also attempted to incorporate channel proteins which were solubilized in detergent directly into planar lipid bilayers without a procedure of reconstitution into vesicles. However, success rate of incorporation was much lower than by the vesicle fusion method. Thus, finally we employed the vesicle fusion method for purification of ion channels.

It is reported that Ca^{2+}-ATPase, which has a molecular mass of about 100 kDa, forms for 60–70% of total amount of protein from SR [3] and that its fragment forms a divalent cation ionophore in membrane [32]. The purified anion-selective channel also has the molecular mass of about 100 kDa [29]; therefore it can not be distinguished from Ca^{2+}-ATPase by the assay on SDS gels alone. However, because the purified channel did not conduct divalent cations and the anti-ATPase anti-sera did not cross-react with the purified proteins, the purified protein was different from Ca^{2+}-ATPase.

Recently, Zaidi et al. reported that a 106-kDa protein from SR forms a cationic channel in lipid bilayer [33]. SDS-PAGE assay can not distinguish the purified channel from 106-kDa protein. However, according to Zaidi, the 106-kDa channel is cation-selective and has a high single-channel conductance in NaCl (375 pS) and $CaCl_2$ (107 pS), whereas our purified channels had rather low conductance in NaCl and did not conduct divalent cations. These results indicate that the K^+-channel purified is not the same channel as the 106-kDa channel.

In the last few years, several cDNA clones encoding K^+ channels were isolated, and expression of these cDNAs in *Xenopus* oocytes yielded K^+ currents with properties of A-type K^+ channels [324–36] and delayed rectifier channels [37]. The K^+ channel isolated in the present study belong to neither the A-type nor the delayed rectifier class of channels. It is different from both types of channel in the channel activities. We succeeded in isolating a novel K^+ channel protein.

In the present study, it was found that the K^+-channel of SR is formed of 100-kDa proteins alone. However, since the frequency that the channel events was observed is much lower after removal of a 120-kDa protein, the possibility that the 120-kDa protein is a regulatory subunit of the channel cannot be eliminated. Further characterization of the molecular properties of the purified channel is now in progress.

Acknowledgements

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