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# PURIFICATION OF A $Cl^-$-CHANNEL PROTEIN OF SARCOPLASMIC RETICULUM BY ASSAYING THE CHANNEL ACTIVITY IN THE PLANAR LIPID BILAYER SYSTEM

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**SUMMARY:** A $Cl^-$ channel protein of 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 CHAPS and fractionated by anion exchange, gel filtration, and affinity chromatography with concanavalin A. All fractions in each step were reconstituted into vesicles with asolectin by dialysis and their channel activities were assayed after the vesicles had been fused into a planar lipid bilayer. A 100-kDa protein, different from $Ca^{2+}$- ATPase, was found to form anion channels. 

Miller and Racker made it possible to measure the current through channels in membrane vesicles by incorporating them into planar lipid bilayers (1). By applying this method to sarcoplasmic reticulum (SR) vesicles, they found a $Cl^-$ channel (2), and we studied their properties in detail on the single channel levels (3). We have found that stilbene derivatives such as SITS or DIDS block the $Cl^-$ permeability of SR vesicles and $H_2$DIDS binds to a 100-kDa protein. The protein was suggested to be different from $Ca^{2+}$-ATPase, although the molecular weights of these proteins are similar (4). However, attempts to purify this proteins were unsuccessful because of the low specificity of DIDS binding.

In the present study, we purified a $Cl^-$ channel from the light fraction of the 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 weight of about 100 kDa and showed the same channel activity as that observed in native SR vesicles in the planar bilayer method. Part of this work has been already reported (5).

Abbreviations: SR, sarcoplasmic reticulum; LSR, light fraction of sarcoplasmic reticulum; SITS, 4-acetoamido-4'-isothiocyanostilbene-2,2'-disulfonic acid; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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## MATERIALS AND METHODS

**Preparation of SR vesicles**: LSR vesicles were prepared from rabbit skeletal muscle by the method of Kasai et al. (6) with slight modifications.

**Solubilization**: LSR vesicles were solubilized by stirring them at 4°C for 2 hr in 10 volumes of 1% CHAPS, 2 mM EGTA, and 20 mM Tris-HCl, pH 7.0, in the presence of a mixture of protease inhibitors (0.5 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin, 1 $\mu$g/ml antipain). The suspension was centrifuged at 100,000 x g for 30 min, and the supernatant was separated from the pellet.

**Anion-exchange chromatography (HPLC)**: The supernatant was applied to an anion-exchange column, COSMOGEL DEAE (Cosmo Bio, Japan), which was equilibrated in 1% CHAPS, 2 mM EGTA, the mixture of protease inhibitors described in the Materials section 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 fraction of the anion-exchanger column 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 x 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 1M $\alpha$-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, Sweden).

**Reconstitution of the vesicles containing the channels**: Before reconstitution, proteins solubilized in CHAPS were supplemented with asolectin solubilized in 0.8-1.0% CHAPS, 0.1 M KCl and 20 mM Tris-HCl to give a final concentration of 2-5 mg/ml and then dialyzed against 0.1 M KCl, 2 mM EGTA, and 20 mM Tris-HCl at pH 7.0 for over 72 h.

**Bilayers and vesicle incorporation**: Planar bilayers were formed by the painting method of Mueller and Rudin (7). The vesicles containing ion channel-forming proteins in their membranes were added to the aqueous solution on one side of the planar lipid bilayer (*cis* side) in the presence of 1 mM $CaCl_2$. 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 (8). The gels were stained with silver.

**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

LSR vesicles (7.5 mg protein) were solubilized in 1% CHAPS and the supernatant obtained after centrifugation was applied to an anion-exchange column. Figure 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. Each fraction was analyzed by SDS gel electrophoresis followed by silver staining as shown in Fig. 1B. Asolectin solubilized in 1% CHAPS was added to all the DEAE

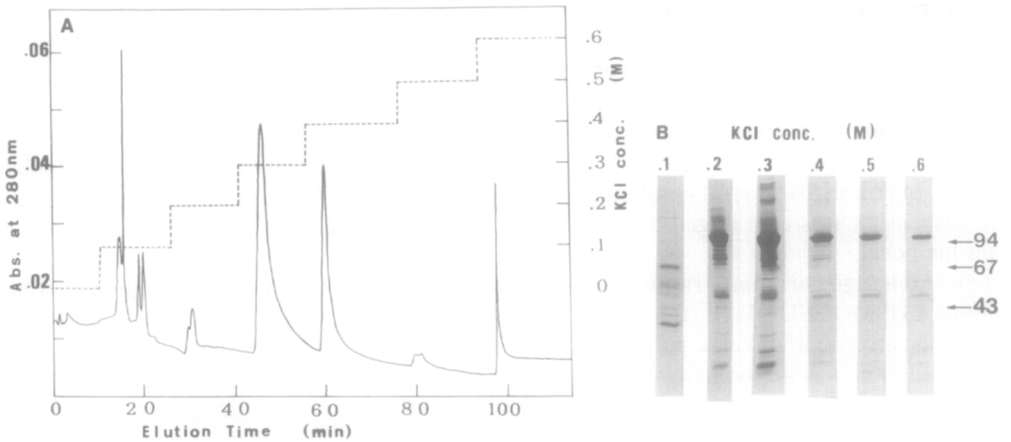


**Fig. 1.** (A) DEAE column elution profile with a discontinuous KCl gradient. 7.5 mg of SR proteins solubilized in 1% CHAPS, 2 mM EGTA and 20 mM Tris-HCl, pH 7.0, in the presence of a mixture of protease inhibitors was applied to the DEAE column (7.5 x 75 mm) and eluted with a discontinuous gradient of KCl from 0 to 0.6 M in 1% CHAPS, 2 mM EGTA, 20 mM Tris-HCl, pH 7.0, at the flow rate of 0.75 ml/min. $OD_{280}$ (———) and KCl concentration (- - - - - -) are shown in the figure. (B) SDS-PAGE (10 % acrylamide gels) analysis of DEAE column fractions. Peptides were stained by silver.

fractions to a final concentration of 5 mg/ml, and proteins were reconstituted into vesicles by dialysis as described in Materials and Methods section. Each fraction reconstituted into vesicles was added to the *cis* side of a planar bilayer to determine the fraction containing anion channels. Current fluctuations were observed when protein eluted with 0.5 M KCl was reconstituted into the planar bilayer by fusion of the vesicles. The frequency distribution of current fluctuations indicated the presence of an anion channel with a large single channel conductance. On the contrary, no anionic channel event was observed when proteins from the other fractions were incorporated into the lipid bilayers .

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 (about 400 $\mu$g protein) was transferred directly to the gel exclusion column. SDS-PAGE analysis of the TSK-G4000SW fractions (Fig. 2B) indicate that most contain proteins of about 100-kDa molecular mass. Lanes 17-23 contained 120-kDa proteins as a minor components and Lanes 23-29 contained proteins with low molecular weight. To all the fractions, asolectin solubilized in 0.8% CHAPS was added to the final concentration of 2 mg/ml, and vesicles were formed by dialysis. Only when vesicles reconstituted from fractions No. 20-22 were incorporated into the lipid bilayers were anionic channel events observed. No other channels, cationic or non-selective channels, were observed. Furthermore, the other fractions exhibited no anionic channel event as far as we could determine.

We applied the gel filtration fractions No. 20-22 (about 30 $\mu$g protein), which contained the $Cl^-$-channel protein, to a Con A-Sepharose column, and the protein was eluted with 1 M

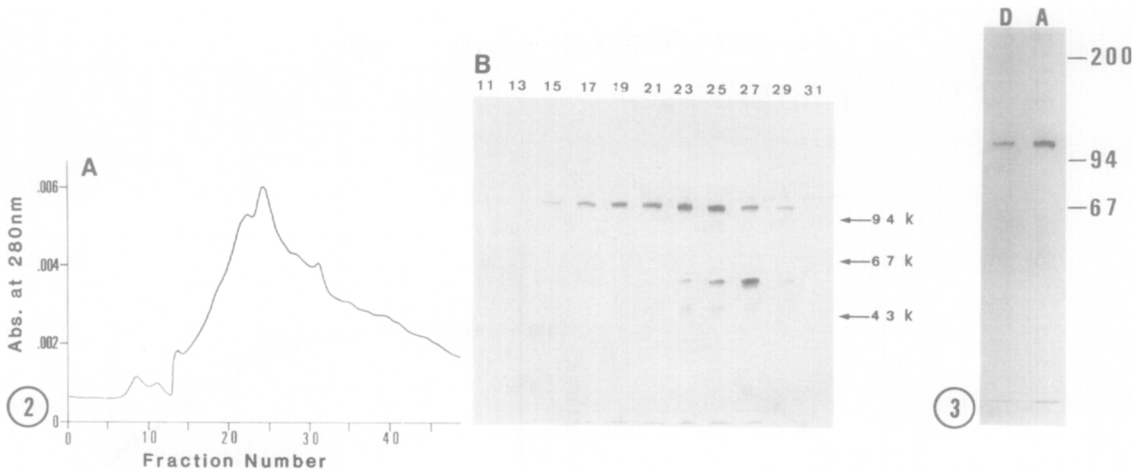


**Fig. 2.** Gel exclusion column elution profile. The DEAE fraction eluted with 0.5 M KCl was transferred directly to the gel exclusion column, TSK-G4000SW (2.15 x 60 cm). The sample was eluted with 0.1 M KCl, 0.8% CHAPS, 2 mM EGTA, and 20 mM Tris-HCl, pH 7.0, at the flow rate of 3 ml/min. Fraction size was 6 ml. (A) $OD_{280}$ is shown in the figure. (B) SDS PAGE of the fractions from TSK-G4000SW. Proteins were stained by silver.

**Fig. 3.** Affinity chromatography on a Con A-Sepharose column. TSK-G4000SW fractions (No. 20-22 in Fig. 2) were applied to a Con A-Sepharose column and eluted with 1M $\alpha$-methyl-D-mannoside. Before transfer to the column, EGTA in the sample was removed by a gel exclusion column, PD-10. Unbound and absorbed proteins were analyzed by SDS-PAGE (10% acrylamide gel).

$\alpha$-methyl-D-mannoside. The Con A-Sepharose adsorbed proteins consisted of approximately 100-kDa proteins and a very small amount of 120 kDa proteins (Fig. 3). The finally obtained protein was about 10 $\mu$g. The pooled proteins eluted from Con A-Sepharose was supplemented with asolectin (2 mg/ml), and the proteins were reconstituted into vesicles by dialysis. Incorporation of the proteins into a planar lipid bilayer enabled them to exhibit anionic channel events. The open probability of the purified channels was decreased when the applied voltage was maintained at a level higher than 50 mV. This result suggests that the purified $Cl^-$-channels are oriented in the right direction in the reconstituted vesicles. No cation-selective or non-selective channels were observed in the same vesicles.

Figure 4A shows current fluctuations in the purified channel. The *cis* solution contained 0.3 M KCl, 1 mM $CaCl_2$, and 20 mM Tris-HCl, pH 7.0; and the *trans* solution was the same buffer with 0.1 M KCl. Single channel current was measured as a function of the transmembrane potential, and it indicated the presence of an anion selective channel of 115 pS (Fig. 4B). The measured reversal potential was 20 mV, corresponding to a permeability ratio ($P_{Cl}/P_K$) of about 7. Furthermore, Fig. 5 shows that the purified channel was blocked by $SO_4^{2-}$ in the same manner as a native anion-selective channel from LSR (3). Tris-$SO_4$ was added to the *cis* side at an applied voltage of 0 mV. Relative conductance decreased with increasing $SO_4^{2-}$ concentration, falling to 0.5 at 110 mM. The $Cl^-$ channel was also blocked by DIDS (data not shown),

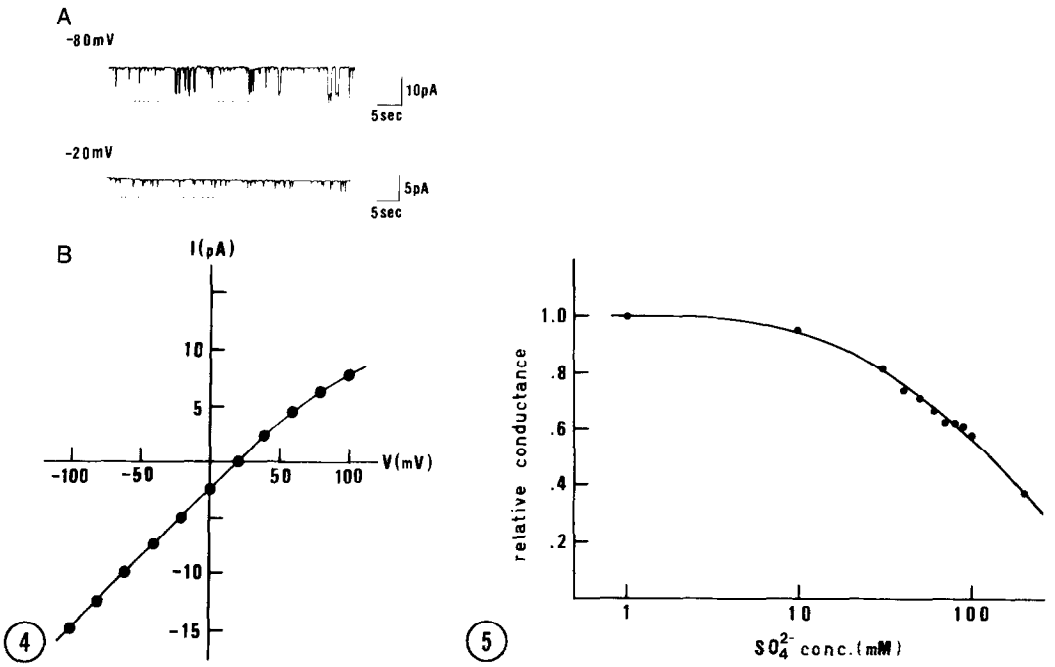


Fig. 4. (A) Current records from purified anion-selective channels incorporated into a lipid bilayer. Con A absorption pool was added with asolectin to give a final concentration of 2 mg/ml and vesicles were then formed by dialysis. The reconstituted vesicle suspension (4-10 $\mu$l) was added to the *cis* side of the bilayers. Current fluctuations were recorded in 0.3 M KCl, 1 mM $CaCl_2$, 20 mM Hepes-Tris, pH 7.2 (*cis*), and 0.1 M KCl, 20 mM Hepes-Tris, pH 7.2 (trans). (B) $Cl^-$ versus $K^+$ selectivity of the purified anion channel from SR. The slope of the line is the single channel conductance, $g_{Cl}$ of 115 pS. The reversal potential was 20 mV, corresponding to a permeability ratio ($P_{Cl}/P_K$) of 7.

Fig. 5. The purified channels were blocked by $SO_4^{2-}$. Relative $Cl^-$ conductances, normalized with the value obtained without $SO_4^{2-}$, were plotted against $SO_4^{2-}$ concentrations. Tris-$SO_4^{2-}$ was added to the *cis* side. *Cis* solution: 0.3 M KCl, 1 mM $CaCl_2$, 20 mM Hepes-Tris, pH 7.2. *Trans* solution: 0.1 M KCl, 20 mM Hepes-Tris, pH 7.2.

suggesting that the channel protein was the same one as that originally reported (4). Since the molecular mass of the $Cl^-$ 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.

## DISCUSSION

A number of ion channel proteins were purified using specific markers. For example, STX or TTX was used for purification of $Na^+$-selective channels from several sources(9-11) and DIDS, for the $Cl^-$-channel from an electric organ (12). However, no specific marker for the anion channel from SR has been found, although DIDS inhibits the anion permeability (4). For

an ion channel protein that has no specific ligand, a 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.

We fractionated proteins from the LSR of rabbit skeletal muscle by anion exchange chromatography, gel filtration chromatography, and affinity chromatography with concanavalin A. All these fractions were assayed by planar bilayer experiments. Consequently, we found that the anion-selective channel from SR is formed by 100-kDa proteins.

It is reported that $Ca^{2+}$-ATPase, which has a molecular mass of about 100 kDa, forms 60-70% of the total amount of protein from SR (13) and that its fragment forms a divalent cation ionophore in the membrane (14). The purified anion-selective channel also has a molecular mass of about 100 kDa; therefore, it cannot be distinguished from $Ca^{2+}$-ATPase by SDS gels alone. However, because the anti-ATPase anti-sera did not cross-react with the purified proteins, the purified protein was different from $Ca^{2+}$-ATPase.

It was found that the purified channels were anion-selective and could be blocked by $SO_4^{2-}$. Although the gating kinetics of the channel were slightly different from that of the native one, the channel protein purified in the present study is considered to be identical to the anion-selective channel in LSR. It is probable that the voltage sensor of the channel was modified during the purification procedures. Further studies of the molecular properties of the purified channels are now in progress.

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