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Biochimica et Biophysica Acta 1237 (1995) 115–120

BBA Biochimica et Biophysica Acta

An anion channel from transverse tubular membranes incorporated into planar bilayers

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Received 7 October 1994; revised 22 February 1995; accepted 10 March 1995

Abstract

Transverse tubular (TT) vesicles from rabbit skeletal muscle were incorporated into planar lipid bilayers to characterize the chloride channel. The single channel conductance of the channel was 40 pS in choline-Cl solution (*cis*, 300 mM/100 mM, *trans*). The gating rate of the channel does not depend on membrane voltage. The channel was blocked by stilbene derivatives (DIDS and SITS), which are known as inhibitors of voltage-dependent Cl^- channels of the *Torpedo* electric organ, from both sides of the membrane. An inhibitor of voltage-dependent Cl^- channels of skeletal muscles, 9-anthracene carboxylic acid (9-AC) inhibited the channel from the *cis* side of the membranes, which corresponded to the cytoplasmic space. Ethacrynic acid (EA), which is reported to inhibit Cl^- conductance of the kidney and trachea, decreased the open probability of the TT Cl^- channel concentration dependently. Indanyloxyacetic acid (IAA), which is also reported to be an inhibitor of kidney and trachea Cl^- channels, decreased the single channel current without affecting open probability of the TT Cl^- channel.

Keywords: Anion channel; Transverse tubule; Planar bilayer

1. Introduction

Chloride channels are found in the plasma and the intracellular membrane of most cells, where they play a variety of roles, e.g., control of cell volume, intracellular pH, and membrane potential. The importance of Cl^- channels is illustrated by diseases resulting from mutations in the channels. In cystic fibrosis, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel is mutated, and protein kinase A and C are unable to open the channel [1,2]. Genetic alteration of a Cl^- channel of skeletal muscle results in the reduction of chloride conductance, which leads to the disease myotonia [3,4].

Steinmeyer et al. [4] cloned a gene encoded Cl^- channel of skeletal muscle by homology to the structure of a Cl^- channel in the electric organ of the marine ray *Torpedo californica*. Recently, Weber-Schurholz et al. [5] used the blocker indanyloxyacetic acid (IAA) to enrich Cl^- channels from rabbit skeletal muscle sarcolemma. They reported that two specific IAA-binding proteins (110–120k and 60k by reducing SDS-PAGE) were identified. The 110–120k protein has the size of the Cl^- channel subunit deduced from the rat ClC-1 cDNA.

An electrical impulse from the motor nerves is transferred through the muscle fiber surface to the transverse tubules (TT) which is the interface between depolarization of the membrane potential and Ca^{2+} release from the sarcoplasmic reticulum (SR). It has been known that L-type Ca^{2+} channels [6] and Ca^{2+}-activated K^+ channels [7] exist in the TT membrane. In previous studies [8], we found a 'background type' of Cl^- channel in the TT membrane of rabbit skeletal muscle and investigated the basic properties of the channel incorporated into the planar bilayers. In this study, the channel has been further characterized pharmacologically in a planar lipid bilayer system.

2. Materials and methods

2.1. Materials

Asolectin, proteinase inhibitors, and ethacrynic acid (EA) were purchased from Sigma, 9-anthracene carboxylic

Abbreviations: TT, transverse tubule; SR, sarcoplasmic reticulum; 9-AC, 9-anthracene carboxylic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; SITS, 4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid; EA, ethacrynic acid; IAA, indanyloxyacetic acid.

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SSDI 0005-2736(95)00091-7

acid (9-AC) from Aldrich, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) from Pierce, and 4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) from Nacalai Tesque. Indanyloxyacetic acid (IAA) was kindly given by Dr. Landry, Columbia University. All other chemicals were commercial products of analytical grade.

2.2. Preparation of transverse tubular (TT) vesicles

TT vesicles were prepared from rabbit skeletal muscle as follows. Dorsal and leg muscles were homogenized with 4 volumes of 0.1 M NaCl, 2.5 mM EGTA, and 5 mM Tris-maleate (pH 7.0) using a Waring blender for 2 min. The homogenate was centrifuged at $4000 \times g$ for 30 min. The supernatant was centrifuged at $10\,000 \times g$ for 30 min to remove the mitochondria. The pellet was homogenized with 0.6 M KCl and 5 mM Tris-maleate (pH 7.0) using a glass-Teflon homogenizer and then centrifuged at $10\,000 \times g$ for 30 min. The supernatant was centrifuged at $150\,000 \times g$ for 60 min. The pellet was homogenized with 0.1 M KCl, 10% sucrose, and 5 mM Tris-maleate (pH 7.0) and layered on the top of a discontinuous sucrose density gradient. The density gradients were prepared using the method of Rosemblatt et al. [9]. This was centrifuged at $60\,000 \times g$ for 12 h. TT vesicles were collected at the interphase of the 26% and the 15% sucrose layers. The suspension was centrifuged for 60 min at $150\,000 \times g$ after dilution with 0.1 M KCl and 5 mM Tris-maleate (pH 7.0). The pellet was resuspended with 0.1 M KCl, 10% sucrose, and 5 mM Tris-maleate (pH 7.0) and stored at −80 C. The proteinase inhibitors (0.5 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 mg/ml antipain) were included in all solutions used throughout the preparation.

2.3. Bilayers and vesicle incorporation

Planer bilayers of the Mueller-Rudin type [10] were formed across a 0.5 mm hole in a polypropylene cup. In all experiments, the *cis* chamber is defined as the side to which the TT vesicles are added, and the opposite side is referred to as the *trans* chamber. Recordings were made in the presence of 300 mM choline-Cl, 5 mM Hepes-Tris (pH 7.1), *cis*, and 100 mM choline-Cl, 5 mM Hepes-Tris (pH 7.1), *trans*. Applied voltages are defined with respect to the *trans* chamber held at ground. Within a few minutes after the addition of the vesicles, a step-like vesicle-bilayer fusion event was observed. TT vesicles were found to insert into the bilayers in oriented fashion such that the *cis* chamber corresponded to the cytoplasmic space [11]. Current fluctuations were recorded on a VTR tape recorder. For analysis, the records were taken from a VTR tape, filtered at 300-1kHz, and digitized by a 12 bit A/D converter at an appropriate sampling rate. Analysis was carried out using a microcomputer (PC-9801V, NEC).

3. Results

In previous experiments, we have found that the Ca^{2+} activating site of the TT K^+ channels reconstituted into the bilayers is always at the *cis* side of the membrane. This indicates that the TT vesicles insert into the bilayers in oriented fashion such that the *cis* chamber corresponded to the cytoplasmic space and the *trans* to the extracellular

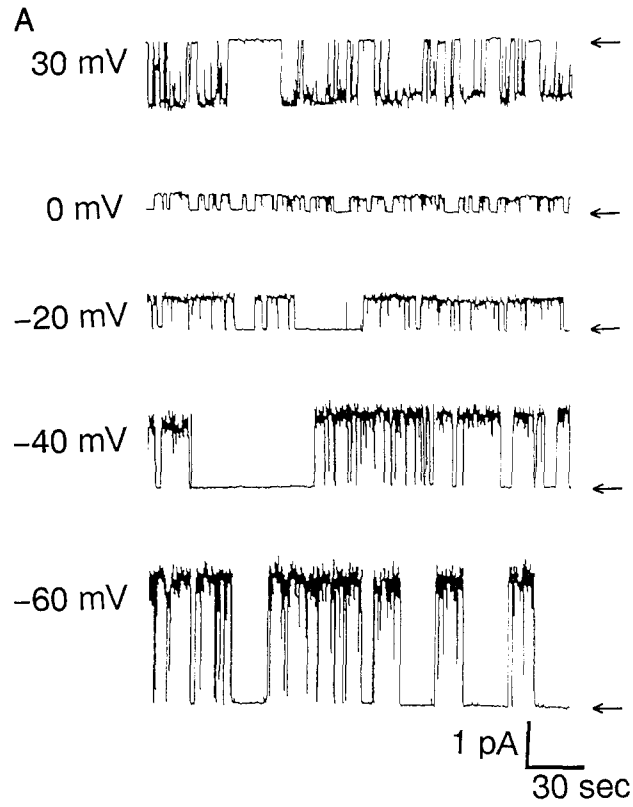


Fig. 1. (A) Single channel recordings of the TT Cl^- channel. Applied voltages are indicated at the left of each trace. Arrows show the baseline current. (B) Relationship between applied voltage and single channel current. The slope of the line is the single channel conductance, γ, of 40 pS. The equilibrium reversal potential is 10 mV, corresponding to the permeability ratio (P_{Cl} / $P_{choline}$) of 2.5.

space; i.e., the Cl^- channel transferred to the planar bilayer from the TT membrane always has the same polarity.

Fig. 1A shows current fluctuations recorded when the channel was present in the bilayers at the indicated voltages. The channel was slightly voltage-dependent. In the trace, the open probability, P_0, increased from 0.48 to 0.66 when the membrane voltage was raised from −60 mV to 30 mV. From the relationship between membrane voltage and single-channel current, the single-channel conductance, γ, of the TT Cl^- channel was determined to be 40 pS (Fig. 1B).

The current traces in Fig. 2 show an effect of 9-AC, which is known as an inhibitor of the voltage-gated Cl^- channel of the skeletal surface membrane [12], on the TT Cl^- channel. In the trace 2A, 200 μM 9-AC was added to the *cis* chamber. 10 min after the addition, the channel was completely inhibited and no resolvable open channel event was observed. The trace in Fig. 2B is a single-channel current record taken in the presence of 550 μM *trans* 9-AC. The gating behavior of the channel was not affected at this concentration as long as we observed. However, *trans* 9-AC inhibited the channel at a much higher concentration, 10 mM (data not shown).

Stilbene derivatives, DIDS and SITS, are known as inhibitors of Cl^- channels [13,14]. Fig. 3A shows the effect of *cis* DIDS on the single channel current. DIDS was added to the *cis* chamber to a final concentration of 50 μM. In this trace, 6 min after the addition of DIDS, the channel activities were completely inhibited. In Fig. 3B, 50 μM DIDS was added to the *trans* chamber. 20 min after the addition, the channel was blocked and no resolvable open event was observed after that. The effect of DIDS was irreversible. The channel activity was not restored by perfusion with fresh buffer. Fig. 4A represents the gating behavior in the presence of SITS. Addition of SITS produced channel flickering and a decrease in the single channel current. In Fig. 4B, a plot of the single channel current vs. membrane voltage shows that the effect of SITS was not noticeably voltage dependent and the single

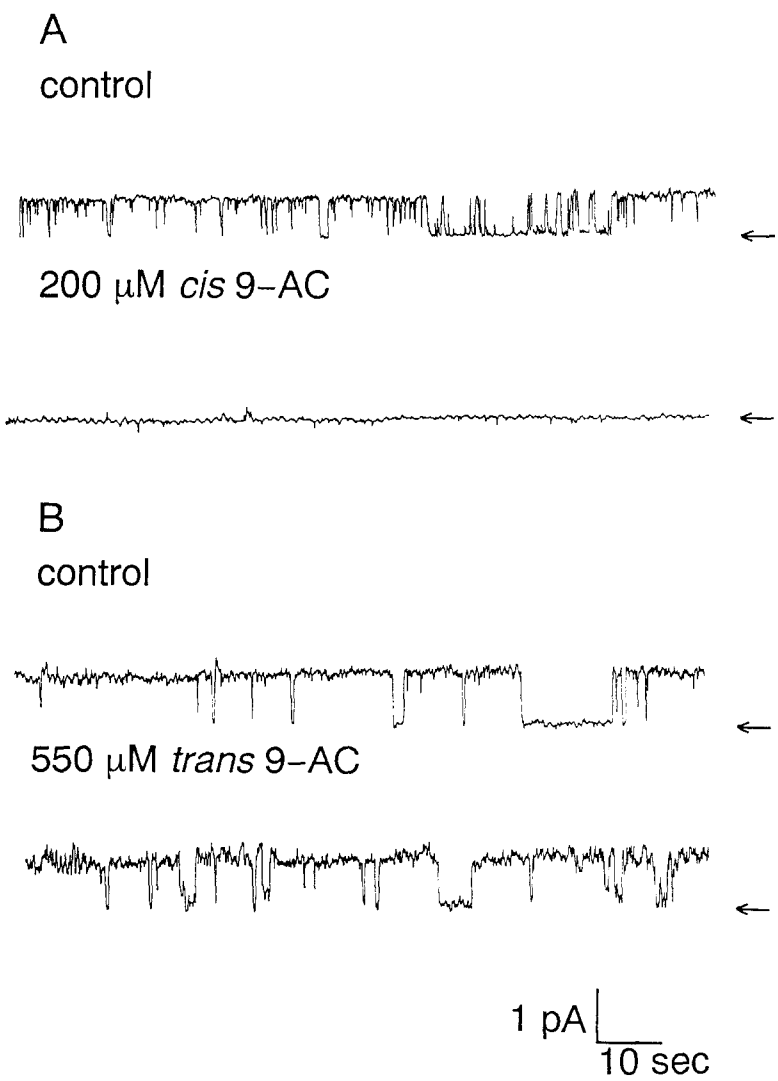


Fig. 2. Effect of 9-anthracene carboxylic acid (9-AC) on the TT Cl^- channel. Current records were made at −10 mV.

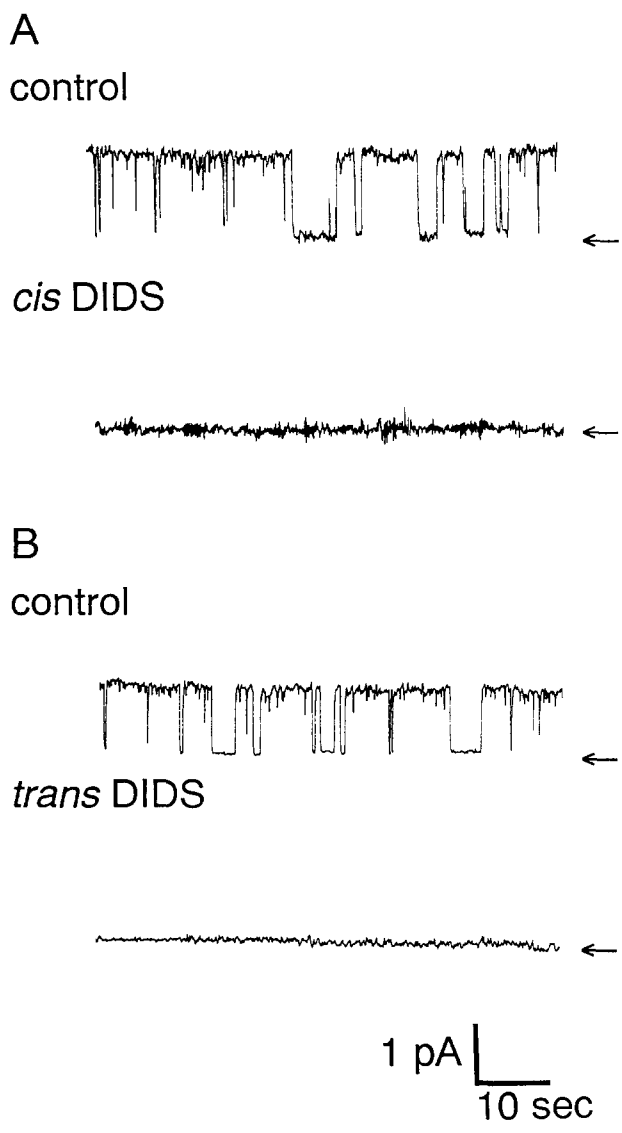
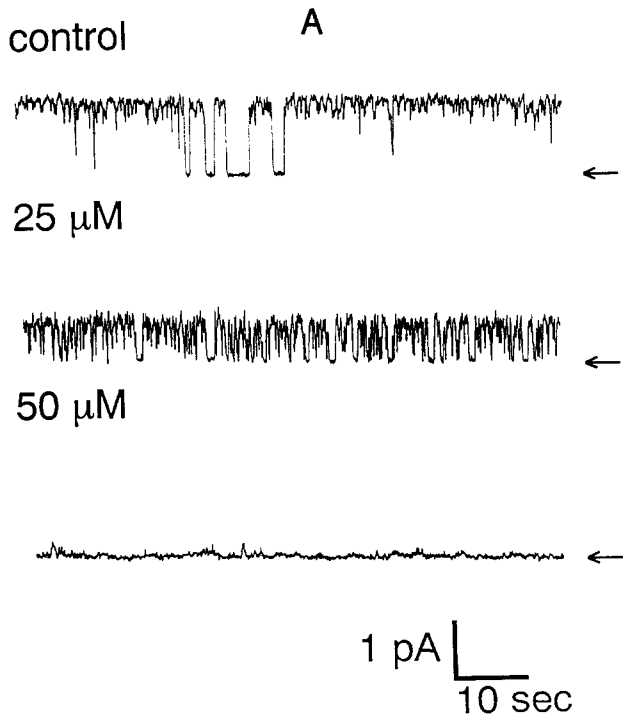


Fig. 3. Effect of DIDS on the channel. 50 μM DIDS was added to the *cis* (A) or the *trans* (B) chamber. Single channel records were made at −10 mV.

channel conductance decreased from 40 pS to 20 pS when 25 μM SITS was added to the *trans* chamber. As shown in the figure, because the equilibrium reversal potential was not changed by the addition of SITS, the ionic selectivity, $P_{Cl}/P_{choline}$, was not changed. *cis* SITS inhibited the channel in the same manner as *trans* (data not shown).

Ethacrynic acid is known as a diuretic agent that inhibits chloride conductance of the kidney and trachea [15]. The current traces in Fig. 5A illustrate the channel gating behavior when various concentrations of EA were added to the *cis* chamber. In each case, the membrane potential was held at −15 mV. The TT Cl^- channel in the bilayers is inhibited by increasing *cis* EA. In the figure, when EA was raised from 0 to 100, the open probability, P_0, decreased from 0.57 to 0.01. The half inhibition constant, IC_{50}, was determined to be 13 ± 3.4 μM ($n = 3 \pm$ S.D.).

It is reported that several IAA derivatives inhibited Cl^- transport of the membrane vesicles from the kidney cortex and trachea [15]. In this study, we investigated the effects of the IAA 94/95 racemate on the TT Cl^- channel. Fig. 6 illustrates effect of *cis* IAA on the TT Cl^- channel. The control record in Fig. 6A shows a single channel current fluctuation before the addition of the drug. 50 μM IAA was added to the *cis* chamber and the solution was stirred. About 5 min after the addition, the single open current decreased to 50% of that determined before addition as shown in the middle trace. When the *cis* IAA concentra-



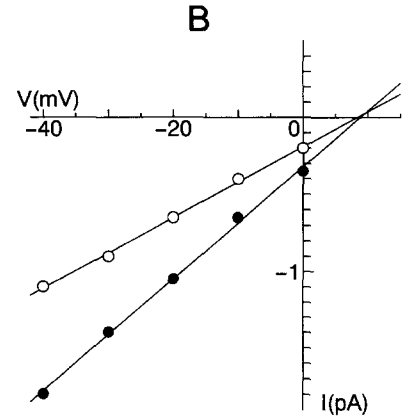
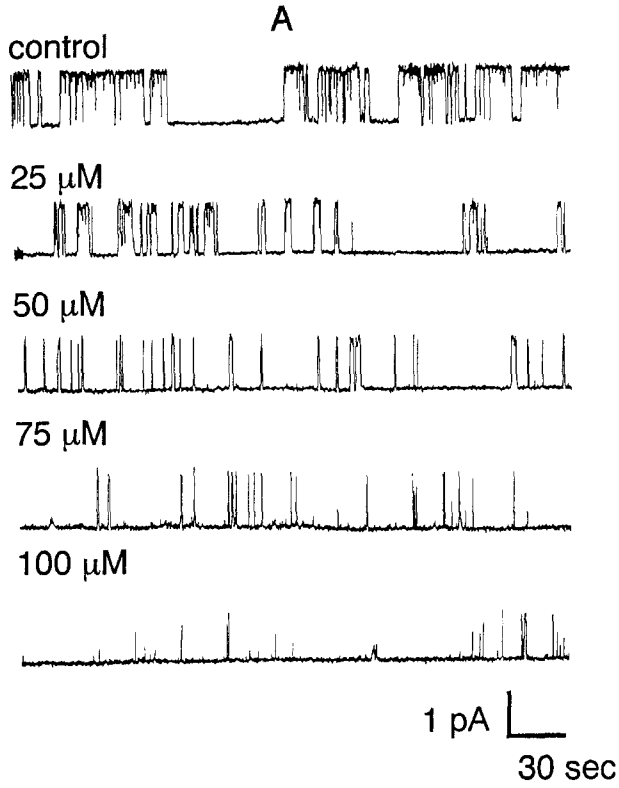


Fig. 4. Effect of SITS on the TT Cl^- channel. (A) SITS was added to the *trans* chamber. Membrane potential was held at −15 mV. (B) Relationship between single channel current and membrane voltage in the absence (●) or the presence (○) of *trans* 25 μM SITS.

tion was raised to 100 μM, the open current decreased to 40%. The single-channel conductance, γ, was decreased by *cis* IAA, whereas the open probability, P_0, was not noticeably affected. Fig. 6B is a blocker-titration curve constructed from current traces in Fig. 6A. Hill plots were generated from which a Hill coefficient of 0.80 was determined at -15 mV. Given one site for IAA binding,



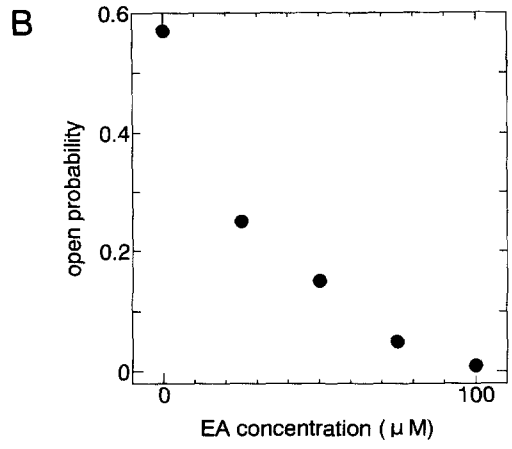
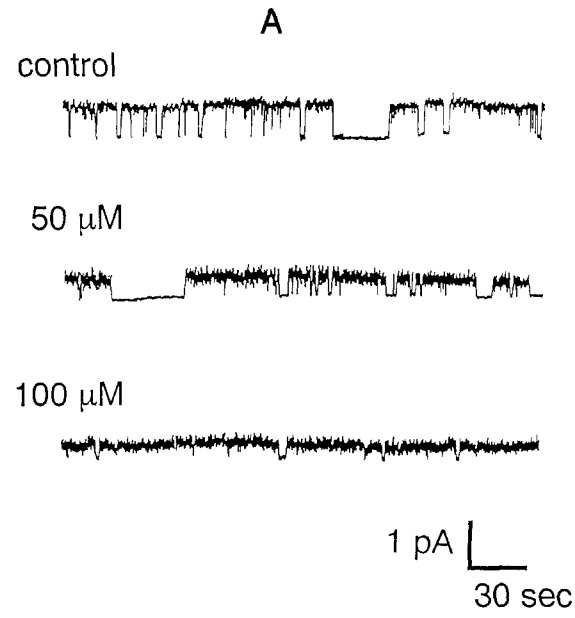


Fig. 5. (A) Single channel records in the presence of ethacrynic acid (EA). EA was added to the *cis* chamber. Displayed recordings were made at -15 mV and were from the same bilayer. (B) Effect of EA concentration on the channel open probability (P_0). The entire plot is constructed from current recordings shown in (A).



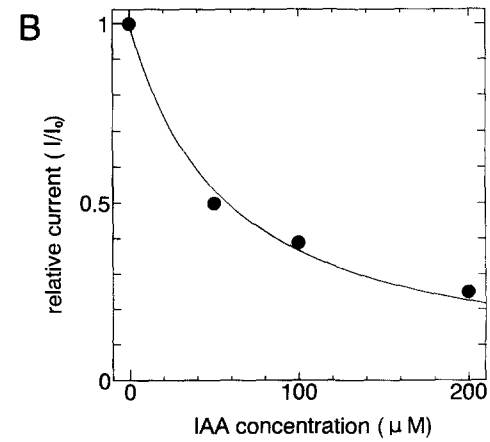


Fig. 6. (A) Effect of indanyloxyacetic acid (IAA) on the TT Cl^- channel. IAA was added to the *cis* chamber. The holding potential was -15 mV. Base-line current is indicated to the right of each trace. (B) Relationship between relative single-channel current and IAA concentration. IAA inhibition fit to a single-site titration curve of the form $I/I_0 = (1 + cis\ \mathrm{IAA}/K_d)^{-1}$ with $K_d = 58$ μM.

dissociation constant, K_d of 58 μM was determined from the single-channel current vs. concentration curve.

4. Discussion

We did not completely eliminate contamination of non-TT vesicle fragments, e.g., sarcoplasmic reticulum membranes. However, when we use TT vesicle suspension, channel activity characteristic of sarcoplasmic reticulum have not been observed, while the Cl^- channel studied in this paper was frequently observed. This shows that the Cl^- channel in this study was not originated from sarcoplasmic reticulum. Further, it was often observed that the

Ca^{2+}-dependent K^+ channel, which is known to present in the membrane of TT [16], and the Cl^- channel studied in this paper were incorporated into a lipid bilayer at the same time. This shows that these channels coexisted in the membrane of a TT vesicle and the vesicle-bilayer fusion resulted in two distinct conductances. Accordingly, it is probable that the Cl^- channel studied in the paper was originated from TT membrane.

Blatz et al. [17,18] reported that there are two types of Cl^- channel on the at skeletal surface membrane and that their single channel conductances are 45 pS and 61 pS. The value of the former is close to that of the Cl^- channel determined in this study. However, the channel of the surface membrane is strongly voltage-dependent and is blocked by 9-AC from the extracellular side of the membrane, while the TT Cl^--channel is weakly voltage-dependent and not affected by 9-AC from the *trans* chamber, which corresponds to the extracellular space. Therefore, the TT Cl^--channel is thought to be a different type of channel from that already reported. The channel is classified as a 'background type' of channel that show a significant probability of being open at resting potential [19]. It is supposed that it contributes to the stabilization of the membrane potential and the rapid recovery of the membrane from the depolarized state to the polarized state.

DIDS, which is known as a blocker of an electric organ Cl^- channel [14], inhibited the TT Cl^--channel from both sides of the membrane. It suddenly closed the channel gate without a preceding change in the gating rate, and it usually exerted its effect 5–20 min after the addition. On the other hand, SITS, a structure close to DIDS, increased the gating rate concentration-dependently immediately after addition to the chamber. It also reduced a single channel conductance, γ, as shown in Fig. 4. At 25 μM, SITS decreased the single-channel open peak current to one-half of its value measured without the drug. A Cl^--channel of an electric organ, a so called double-barreled Cl^--channel [20], is reported to be reduced in single-channel conductance to $1/2$ by DIDS. It is thought that this effect results from blocking of one of the two 'proto-channels' which open and close independently. However, the TT Cl^--channel is not double barreled judging from its single-channel current records; therefore, the reduction of γ of the channel by SITS is thought to occur in a distinctly different way from that of the electric organ channel. It is presumed that the gating rate was increased much faster than the resolution rate of our recording system, and the resulting current was thus apparently reduced.

Landry et al. [15] reported that several IAA derivatives inhibited the Cl^--channel activity of the kidney and trachea, and they succeeded in purifying the channel proteins by affinity chromatography on Sepharose 4B coupled to IAA [21]. Recently, Weber-Schurholz et al. [5] found that specific binding proteins of IAA exist in the skeletal outer membrane. IAA has a structure analogous to that of EA. It is interesting that an addition of these reagents results in a different type of effect on the channel despite the structural resemblance. EA decreased the open probability, P_0, without affecting the single channel conductance, γ, whereas IAA reduced γ without affecting P_0. A common structural part of these reagents may bind to the channel, and different parts contribute to the different effects. IAA and EA are thought to be effective in identifying the TT channel protein.

Acknowledgements

The authors would like to thank Dr. Donald W. Landry for providing us with IAA. This work is supported in part by a Grant-in-Aid for Scientific Research to M.K. (04454620) and to T.I. (06858071) from the Ministry of Education, Science and Culture of Japan.

References

[1] Riordan, J.R., Rommens, J.M., Keren, B.S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S. and Tsui, L.-C. (1989) Science 245, 1066–1073.
[2] Li, M., McCann, J.D., Anderson, M.P., Clancy, J.P., Liedtke, C.M., Nairn, A.C., Greengard, P. and Welsh, M.J. (1989) Science 244, 1353–1356.
[3] Rudel, R. and Lehmann-Horn, F. (1985) Physiol. Rev. 65, 310–356.
[4] Steinmeyer, K., Ortland, C. and Jentsch, TJ. (1991) Nature 354, 301–304.
[5] Weber-Schurholz, S., Wischmeyer, E., Laurien, M., Jockusch, H., Schurholz, T., Landry, D.W. and Al-Awqati, Q. (1993) J. Biol. Chem. 268, 547–551.
[6] Fosset, M., Jaimovich, E., Delopont, E. and Lazdunski, M. (1983) J. Biol. Chem. 258, 6086–6092.
[7] Latorre, R., Vergara, C. and Hidalgo, C. (1982) Proc. Natl. Acad. Sci. USA 77, 7484–7486.
[8] Hidaka, J., Ide, T., Kawasaki, T., Taguchi, T. and Kasai, M. (1993) Biochem. Biophys. Res. Commun. 191(3), 977–982.
[9] Rosemblatt, M., Hidalgo, C., Vergara, C. and Ikemoto, N. (1981) J. Biol. Chem. 256, 8140–8148.
[10] Mueller, P. and Rudin, D.O. (1969) In Laboratory Techniques in Membrane Biophysics (Passow, H. and Stepfli, R., eds.), pp. 141–156, Springer-Verlag, Berlin.
[11] Latorre, R. (1986) In Ion Channel Reconstitution (Miller, C., eds.), pp. 431–467, Plenum Press, New York.
[12] Bryant, S.H. and Morales-Aguilera, A. (1971) J. Physiol. 219, 369–383.
[13] Kasai, M. (1981) J. Biochem. 89, 943–953.
[14] Miller, C. and White, M.M. (1984) Proc. Natl. Acad. Sci. USA 81, 2772–2775.
[15] Landry, D.W., Reitman, M., Cragoe, E.J., Jr. and Al-Awqati, Q. (1987) J. Gen. Physiol. 90, 779–798.
[16] Vergara, C., Moczydlowski, E. and Latorre, R. (1984) Biophys. J. 45, 73–76.
[17] Blatz, A.L. and Magleby, K.L. (1986) J. Physiol. 378, 141–174.
[18] Blatz, A.L. and Magleby, K.L. (1985) Biophys. J. 47, 119–123.
[19] Franciolini, F. and Petris, A. (1990) Biochim. Biophys. Acta 1031, 247–259.
[20] Miller, C. (1982) Phil. Trans. R. Soc. Lond. B 299, 401–411.
[21] Landry, D.W., Akabas, M.H., Redhead, C., Edelman, A., Cragoe, E.J., Jr. and Al-Awqati, Q. (1989) Science 244, 1469–1472.