Low-Conductance Chloride Channel from Crayfish Skeletal Muscle Incorporated into Planar Lipid Bilayers

P.PROKS, O.HURŇÁK and J. ZACHAR[1]

Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava, Czechoslovakia

Abstract. Low-conductance chloride channel from skeletal muscle SR vesicles of the crayfish *Astacus fluviatilis* was incorporated into planar lipid bilayers and its basic characteristics were investigated. The channel has a relatively low unitary conductance of 26 pS in symmetrical 160 mmol/l choline-chloride. The dependence of the channel conductance on Cl^- concentration shows saturating behavior with a maximum conductance of 37 pS and an ionic activity for half-maximum conductance K_m = 75 mmol/l. The channel exhibits a complex kinetics with several modes of activity. Open state probability slightly decreases with the increasing absolute value of voltage. The channel activity does not appear to be dependent on the presence of Ca^{2+} ions. The channel is effectively inhibited by DIDS, a stilbene derivative. The permeability properties of the channel are similar to the specific behavior of the "double-barrelled" channel from *Torpedo* electroplax described by Miller and White (1984).

Key words: Planar lipid bilayer — Chloride channel — Sarcoplasmic reticulum — Crayfish skeletal muscle

Introduction

The incorporation of membrane vesicles from the sarcoplasmic reticulum (SR) membranes into planar lipid bilayers represented a major step in the inquiry into the molecular mechanisms of the SR calcium release process (Miller and Racker 1976). This development culminated in isolation and reconstitution of the main molecule of the Ca release machinery, i.e. the ryanodine - receptor complex in planar bilayers (Lai et al. 1988; Smith et al. 1988). For the elucidation of the

[1] Correspondence to: Dr. Jozef Zachar, Laboratory of Molecular Physiology, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava, Czechoslovakia

physiological release process, however, it is important to consider besides the role of the calcium release channel also the role of additional ion selective pathways. This knowledge is especially important when differences in the basic types of muscle activation mechanisms are to be elucidated. Both K^+ (Miller 1978; Coronado et al. 1980; Coronado and Miller 1982) and Cl^- (Tanifuji et al. 1987; Rousseau et al. 1988; Rousseau 1989) conductances have been identified in SR membranes from skeletal muscle incorporated into planar lipid bilayer.

There are two types of excitation - contraction coupling mechanisms in striated skeletal muscle systems which differ in the role played by external Ca ions in this process (for a review see Zachar 1971; Tanabe et al. 1990). The vertebrate type of the E-C coupling mechanism does work also in the absence of external Ca^{2+} ions (Armstrong et al. 1972). Ca^{2+} ions are, however, required for the normal functioning of the E-C link in invertebrate muscle (Zacharová and Zachar 1967). We have demonstrated recently the existence of the ryanodine-receptor complex in a crustacean skeletal muscle (Formelová et al. 1990); upon its incorporation in a purified form into planar lipid bilayers, this complex showed characteristics similar to those of the Ca release channel complex in vertebrate muscle. During the inquiry into the characteristics of the calcium release channel in crayfish SR membrane vesicles we noticed the presence of several kinds of K^+ and Cl^- channels, most of which might be compared in their characteristics to their companion ionic channels in vertebrate muscle SR membranes. We describe here the functional properties of a low conductance ionic channel present in the crayfish SR membrane fraction, which differs in many respects from the SR chloride channels described so far. Some of these results have been presented previously in abstract form (Proks et al. 1991).

Abbreviations: DIDS: 4,4'-Diisothiocyan-2,2'-stilbenedisulfonic acid; PIPES: Piperazine-N,N'-*bis*[2-ethanesulfonic acid]; TRIS: Tris-(hydroxy-methyl)-aminomethane; HEPES: N-2-Hydroxyethylene- piperazine-N'-2-ethanesulfonic acid

Materials and Methods

Chemicals

Choline chloride and HEPES buffer were obtained from Serva, PIPES from Sigma, asolectin and *n*-decane from Fluka. TRIS buffer was purchased from Loba Feinbiochemie, $CaCl_2$ was purchased from Merck, DIDS from Sigma. All other materials were of reagent grade.

Isolation of SR membranes

Sarcoplasmic reticulum membrane fractions derived from crayfish tail muscle were prepared as follows: 50g of tail muscle tissue was homogenized in 250 ml of 0.1 mol/l NaCl, 5 mmol/l TRIS maleate, pH = 7.4 at 4 °C for 60 s with a Polytron homogenizer. The homogenate was centrifuged for 40 min at $2600 \times g$. The heavy SR was isolated as a pellet after centrifugation for 1 h at $36,000 \times g$. The pellet was resuspended in a solution containing

10 mmol/l PIPES, 0.1 mol/l NaCl, 70 μmol/l $CaCl_2$, pH 7.4, and stored frozen in liquid nitrogen.

Planar lipid bilayer measurements

Bilayers were formed at room temperature (20 °C) from asolectin dissolved in *n*-decane (concentration 40 mg/ml) across an aperture (0.6 mm in diameter) in the septum of a teflon cup according to Mueller et al. (1962). Both compartments of the teflon cup contained approximately 5.5 ml of electrolyte. In view of the presence of several kinds of channels in the SR vesicle preparations (Formelová et al. 1990; Hurňák and Zachar 1990), choline chloride solutions were chosen for our purposes. Choline is practically impermeant for K^+ channels of SR vesicles (Coronado and Miller 1982). Also, 5 mmol/l $CaCl_2$ was used to detect the presence of the calcium-release channel which was found permeable for choline (Smith et al. 1988), and to explore the influence of $CaCl_2$ on the channel kinetics. All the solutions were buffered with HEPES (concentration 10 mmol/l) and were adjusted to pH 7.4 with NaOH. Suspension of SR vesicles (50 μl) was added to the *cis* side of the chamber to a final concentration of 10-100 μg/ml. (The *cis* side was connected to a voltage command signal, and the *trans* side was connected to a current-voltage converter circuit). Fusion was either spontaneous or induced by stirring or applying a potential across the bilayer. Data were recorded and analyzed on an IBM PC.

Results

Basic characteristics of the channel

Practically immediately after addition of SR vesicles into the *cis* chamber, activity of one to four channels appeared. The channel activity often oscillated between periods of low probability of open state (1–5 percents) - with short flickerings - and periods with relatively high probability of open state ($P_0 \approx 0.3-0.6$) (Fig. 1). This behavior is sometimes referred to as "gear shifting" or "mode" activity; for instance, it has been reported in a chloride channel from lobster walking leg nerves by Lukács and Moczydlowski (1990), K^+ channels by Moczydlowski and Latorre (1983), and Na^+ channels (Moczydlowski et al. (1984). As the underlying mechanism of this phenomenon have generally been considered: 1. slow conformational transitions of the channel molecule between several states with quasi-stationary kinetics; 2. presence of some channel ligands in the preparation, 3. non-stationary kinetics due to conformational changes of the BLM.

The channel mostly flickered between single open and closed level, although some minor substates were also present (see e.g. the control trace in Fig. 5). This property were shown to exhibit SR chloride channels from skeletal (Rousseau et al. 1988) and cardiac muscles (Rousseau 1989), as well as neuronal channels of the lobster peripheral nerves (Lukács and Moczydlowski 1990).

To check the anion selectivity, the current-voltage dependence was determined in asymmetrical conditions (Fig. 2*A*). The $I-V$ curve shows marked rectification, which is also slightly recognizable in symmetrical conditions (Fig. 2*B*).

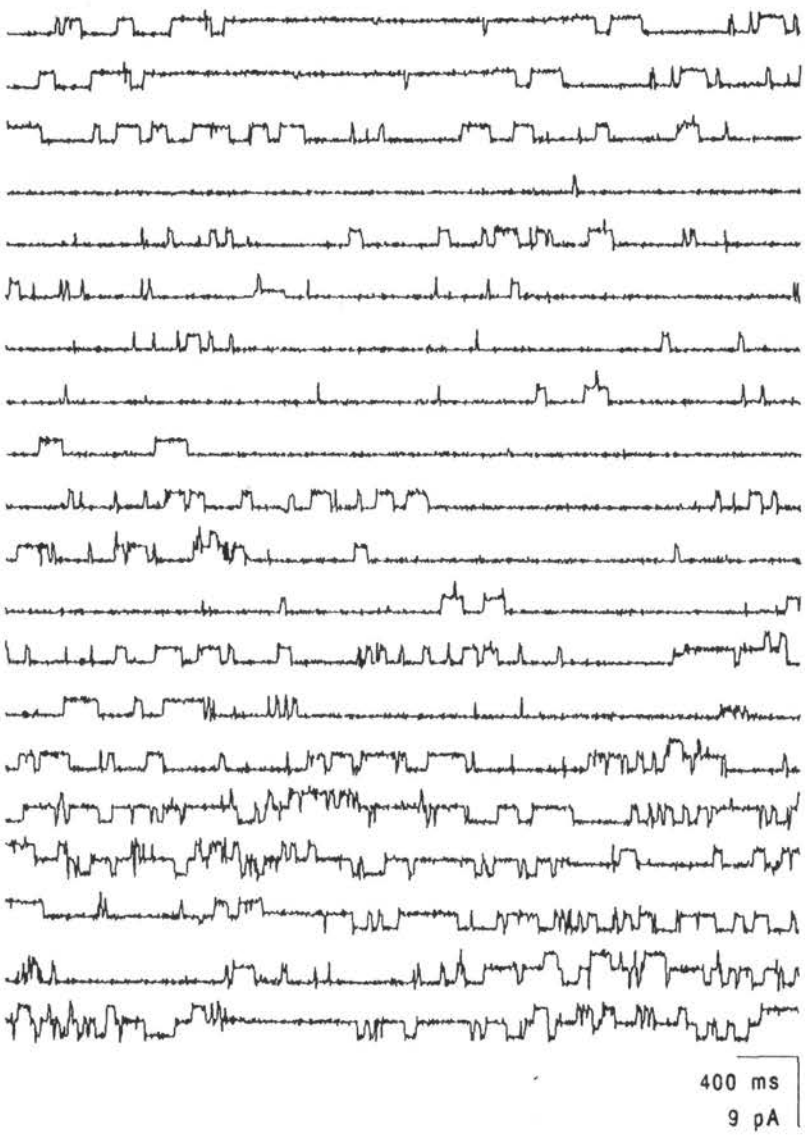

Figure 1. A record of single channel activity of Cl^- channel in asymmetrical 250 mmol/l choline chloride (*cis*) and 50 mmol/l choline chloride (*trans*) solutions at −20 mV.

This behavior is also characteristic for the neuronal chloride channels of the lobster (Lukács and Moczydlowski 1990), and rabbit white dorsal and leg muscle SR anion channel (Tanifuji et al. 1987). The channel has a relatively low unitary conductance (26 pS in symmetrical 160 mmol/l choline, Fig. 2B), which is slightly larger than that of the Cl^- channel of the lobster walking leg nerves ($\approx 15-20$ pS;

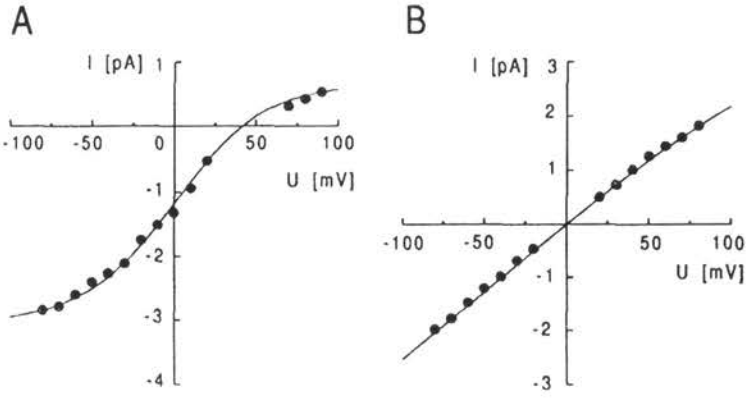

Figure 2. *A.* $I-V$ relationship in asymmetrical 250 mmol/l choline chloride + 5 mmol/l $CaCl_2$ (*cis*) and 50 mmol/l choline chloride + 5 mmol/l $CaCl_2$ (*trans*) (10 mmol/l HEPES, pH 7.4). *B.* Example of $I-V$ relationship in symmetrical conditions 160 mmol/l of choline chloride, 5 mmol/l $CaCl_2$ and 10 mmol/l HEPES, pH 7.4 on both sides of the membrane. Mean values from 3 independent measurements. Value of unit conductance 26 pS.

Lukács and Moczydlowski 1990). Nevertheless, its value is smaller than that of the chloride channels derived from SR preparations (rabbit dorsal and leg muscle: Tanifuji et al. 1987 (200 pS in 100 mmol/l Cl^-); rabbit and trout skeletal muscle: Rousseau et al. 1988 (65 pS in 100 mmol/l Cl^-); canine cardiac SR: Rousseau 1989 (38 pS in 60 mmol/l Cl^-)).

Channel kinetics

Since most experiments yielded records of two or more channels, it was difficult to estimate channel gating kinetics. In several cases of multi-channel recordings, the distribution of open and closed states was not binomial; therefore, some kind of cooperation of channels might be expected. This behavior of chloride channels is well documented (for a review see Francolini and Petris 1990). Generally, we found a weak voltage dependence of gating, with the probability of open state slightly decreasing with the absolute value of voltage. E.g., in symmetrical 260 mmol/l choline-Cl^- the open state probability decreased from 0.2 to 0.05 in the -30 mV

to −70 mV range, and from 0.4 to 0.15 between 20 mV and 60 mV. A similar behavior was reported for Cl^- channels in SR preparations by Rousseau et al. (1988), Rousseau (1989) and Lukács and Moczydlowski (1990). With the increasing Cl^- concentration, the channel shows more apparent flickering behavior combined with long-lasting openings and closings (Fig. 3). The weak voltage dependence raises the question of a possible ligand regulation of this channel.

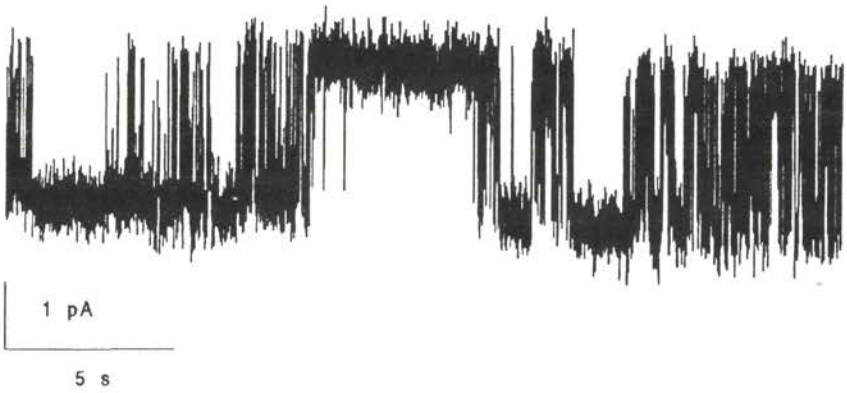

Figure 3. Example of kinetics behavior of the channel at higher Cl^- concentrations (symmetrical 500 mmol/l choline chloride, −70 mV): long periods of the channel openings and closings combine with flickering behavior.

Franke et al. (1986) recorded glutamate and GABA-activated single channel currents from 5 different muscles of the crayfish. The opening probability of these channels increased with the increasing concentration of the agent. To check whether the activity of our channel could be modulated by these compounds, we inspected the effect of glutamate or GABA (final concentration of 0.5 mmol/l and 0.1 mmol/l respectively) into the chamber; the concentrations used were in the range of saturating effects of these compounds. We did not observe, however, any marked effect of the compounds on the channel kinetics (not shown).

Concentration dependence of the channel conductance

Fig. 4 summarizes the current-voltage relationship for various chloride concentrations measured in symmetrical conditions. The conductance (γ) v.s. concentration (c) plot was fitted with the hyperbolic relation:

$$\gamma = \gamma_{max} \frac{c}{1 + K_m c} \tag{1}$$

where γ_{max} stands for maximal conductance and K_m is ionic activity for half-maximal conductance. Values of γ were estimated as $(\partial I/\partial V)$ at $V = 0$ mV. The

best fit curve gives $\gamma_{max} = 37$ pS and $K_m = 75$ mmol/l. This apparent Michaelis-Menten behavior implies that the channel contains at least one Cl^- binding site. We failed to record any channel activity below 70 mmol/l choline chloride. Probably, this has been due to the vesicle - membrane fusion mechanism.

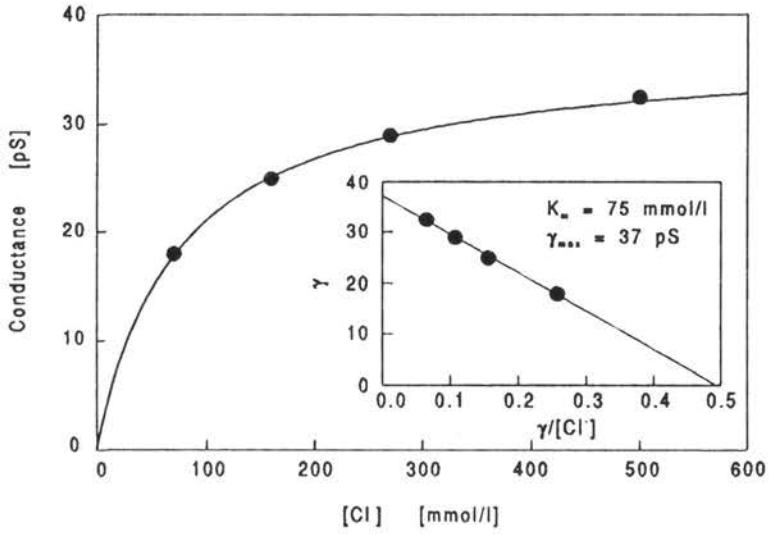

Figure 4. Conductance - Cl^- concentration dependence of a low- conductance SR chloride channel. Data are fitted by equation (1). *Inset*: linearized plot of this relationship; it shows a very good agreement between the experimental data and theory. Each point represents a result of fitting $I - V$ characteristics in symmetrical solutions with SEM $<$ 2%.

Insensitivity to Ca^{2+} concentration

The possible influence of Ca^{2+} on the channel kinetics was checked by comparing the channel activity in the presence and in the absence of Ca^{2+} ions in solutions with identical overall chloride concentrations. No changes in channel unit current amplitude, conductance, contribution of subconducting states or opening probabilities could be detected in Ca^{2+} free as compared to 5 mmol/l Ca^{2+} containing solutions (data not shown). Similar insensitivity to Ca^{2+} has been previously reported for chloride channels from skeletal (Rousseau et al. 1988) and cardiac (Rousseau 1989) SR membranes, and from neuronal membranes of the lobster (Lukács and Moczydlowski 1990).

Inhibition by DIDS.

Stilbene derivatives are known to inhibit the anion permeability of skeletal SR vesicles (Kasai and Kometani 1979). Kasai and Tagushi (1981) found half maximum inhibition of sulfate permeability in the presence of 0.06 mmol/l DIDS. DIDS was also used at millimolar concentrations to abolish Cl^- channel activity from electric organ of *Torpedo* electroplax (Miller and White 1984). Rousseau (1989) found marked blocking effect of DIDS on cardiac SR Cl^- channel at a concentration of 10 mmol/l. The channel activity displayed a period of low amplitude flickering behavior before complete and irreversible inhibition. We observed the same effect with DIDS applied at 5 mmol/l to the *cis* side of the chamber (Fig. 5).

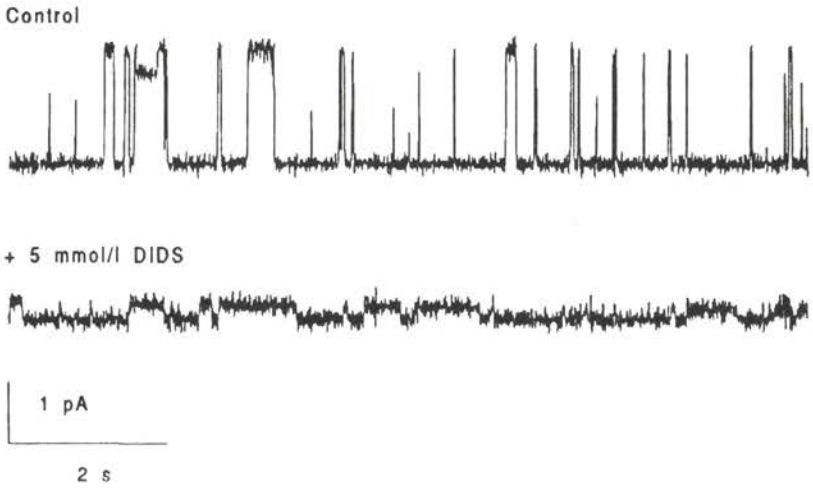

Figure 5. Blocking effect of DIDS on the single-channel behavior. Upper trace: control, bottom trace: after addition and stirring (about 5s) of 5 mmol/l DIDS on the (*cis*) side of the membrane. Measured in asymmetrical conditions: 250 mmol/l choline chloride (*cis*) and 50 mmol/l choline chloride (*trans*); applied voltage - 20 mV.

Immediately after adding and stirring (5s), the channel conductance decreased (bottom trace), and after several seconds the channel was completely inhibited.

Channel selectivity for halides

Most of the chloride channels described so far exhibit permeability sequence of a weak cationic binding site (Francolini and Petris 1990), where the permeability ratio decreases in the sequence $I^- > Br^- > Cl^- > F^-$.

We found, however, that the low-conductance Cl^- channel from the crayfish SR is permeable only for Cl^- and Br^- with $P_{Br}/P_{Cl} = 0.25$. All experiments were

done in 250 mmol/l choline- chloride at the *cis* and 250 mmol/l KX^- or NaX^- at the *trans* side. (The permeability ratio for K^+ and Na^+, which was below 0.04, was neglected in these measurements). We found further that addition of I^- (which is not permeant by itself) to the *cis* chamber blocked the channel activity (Fig. 6). This is a quite specific behavior, similar only to that found in the "double-barelled"

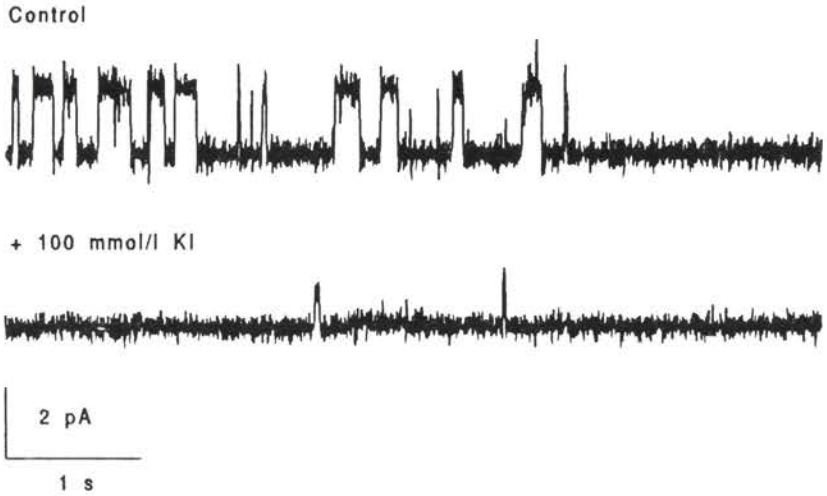

Figure 6. Blocking effect of I^- ions after addition to the *cis* side of the chamber (final concentration: 100 mmol/l). Upper trace - control; bottom trace - after adition and stirring (about 5s). Other conditions (solutions and voltage) were the same as described in legend to Fig. 5.

chloride channel from *Torpedo* electroplax membrane (Miller and White 1984). It can be assumed that I^- can enter the channel and bind to the selectivity filter, but it cannot pass further through the channel.

The effect of H^+

H^+ ions are known to block activity of various chloride channels (see Bretag 1987); e.g., in crustacean neuronal membrane decreasing the pH value to 6.0 led to the closing of the chloride channel (Lukács and Moczydlowski 1990). The low- conductance SR chloride channel of the crayfish was, however, active even at pH 5.0 on both sides of the chamber with a probability of the open state between 0.02 and 0.05. Channel conductance remained practically unchanged (28 pS in 250 mmol/l choline chloride).

Discussion

The low-conductance Cl^- channel showed several properties in common with Cl^- channels from the sarcoplasmic reticulum studied in planar lipid bilayers by Rousseau et al. (1988) in mammalian skeletal muscles, and by Rousseau (1989) in cardiac muscle, as well as in excised patches from collagenase treated tonic muscle fibres of the crayfish *Procambarus clarkii* (Bishop et al. 1991). These include blocking effect of DIDS, weak voltage dependence of channel kinetics, insensitivity of channel activity to Ca^{2+} ions, and presence of minor substates. On the other hand, the low-conductance channel of the crayfish had a conductance lower than those of the vertebrate SR Cl^- channels reported. The channel conductance and its peculiar properties - unidirectional rectification and several modes of activity - were quite similar to low-conductance Cl^- channels in neuronal membranes of the lobster (Lukács and Moczydlowski 1990). Rectification has also been well documented for various kinds of Cl^- channels, including those of cultured human colon tumor cells (Frizzell et al. 1986), apical membrane of rat colon epithelia (Halm et al. 1988), and human lymphocytes (Chen et al. 1989). The channel exhibited rare selectivity properties, which resembled to those of the *Torpedo* electroplax channel described by Miller and White (1980). On the other hand, the new channel had approximately twice as high conductance as did the latter channel, and there has been no marked evidence for its "double- barrelled" structure. The effect of H^+ was less pronounced than the effect of pH on the low-conductance Cl^- channel in the nerve membrane described by Lukács and Moczydlowski (1990). Data from similar SR preparations have, however, been rare; Rousseau et al. (1988) reported no marked changes of SR Cl^- channel in the range of pH 6.8–8.0.

We assume that the channel is localized in the sarcoplasmic reticulum membranes. Bishop et al. (1991), however, found in the surface muscle membranes of the crayfish *Procambarus clarkii* a low-conductance channel with a similar conductance (25 pS in 280 mm Cl^-). Even if no other characteristics of the channel have been reported, the question is to be asked whether the chloride channel in our preparation was not of surface membrane origin as well. The heavy SR fraction containing mainly SR membranes might have been contaminated with the surface membrane fraction. At least two facts make this supposition improbable. First, we did not found any low-conductance channel in the light membrane fraction originating from the tubular membrane system, which is in direct continuation with the surface membranes of the muscle fibres. Second, the high incidence of activity in each trial to incorporate the channel from the SR membrane fraction to planar lipid bilayers favors the assumption that the channel was localized in the SR membranes.

In contrast to the sarcoplasmic reticulum Ca^{2+} release channel, physiological function of SR monovalent ion channels is less clear. They have been suggested

to probably permit rapid movements of K^+, Na^+, H^+ and Cl^- across the SR membrane against the electrogenic Ca^{2+} fluxes during Ca^{2+} release and uptake (Meissner 1983; Garcia and Miller 1984).

Acknowledgements. The authors wish to thank Dr. L. Varečka for the generous gift of DIDS.

References

Armstrong C.M., Bezanilla F.M., Horowicz P. (1972): Twitches in the presence of ethylene glycol bis(β-aminoethyl ether)-N,N'- tetraacetic acid. Biochim. Biophys. Acta **267**, 605—608

Bishop C.A., Krouse M.E., Wine J.J. (1991): Peptide cotransmitter potentiates calcium channel activity in crayfish skeletal muscle. J. Neurosci. **11**, 269—276

Bretag A.M. (1987): Muscle chloride channels. Physiol. Rev. **67**, 618—723

Chen J.H., Schulman H., Gardner P. (1989): A cAMP-regulated chloride channel in lymphocytes that is affected in cystic fibrosis. Science **243**, 657—660

Coronado R., Rosenberg R.L., Miller C. (1980): Ionic selectivity, saturation, and block in a K^+ - selective channel from sarcoplasmic reticulum. J. Gen. Physiol. **76**, 425—446

Coronado R., Miller C. (1982): Conduction and block by organic cations in a K^+-selective channel from sarcoplasmic reticulum incorporated into planar phospholipid bilayers. J. Gen. Physiol. **79**, 529—547

Formelová J., Hurňák O., Novotová M., Zachar J. (1990): Ryanodine receptor purified from crayfish skeletal muscle. Gen. Physiol. Biophys. **9**, 445—453

Francolini F., Petris A. (1990): Chloride channels of biological membranes. Biochim. Biophys. Acta **1031**, 247—259

Franke C., Hatt H., Dudel J. (1986): The inhibitory chloride channel activated by glutamate as well as gamma-amino-butyric acid (GABA). J. Comp. Physiol. A **159**, 591—609

Frizzel R.A., Halm D.R., Rechkemmer G., Shoemaker R.L. (1986): Chloride channel regulation in secretory epithelia. Fed. Proc. **45**, 2727—2731

Garcia A.M., Miller C. (1984): Channel mediated monovalent cation fluxes in isolated sarcoplasmic reticulum vesicles. J. Gen. Physiol. **85**, 247—289

Halm D.R., Rechkemmer G.R., Schoumacher R.A., Frizell R.A. (1988): Apical membrane chloride channels in a colonic cell line activated by secretory agonists. Amer. J. Physiol. **254**, C505—C511

Hurňák O., Zachar J. (1990): Two types of potassium channels from sarcoplasmic reticulum of crayfish skeletal muscle incorporated into planar lipid bilayers. Gen. Physiol. Biophys. **9**, 635—641

Kasai M., Kometani T. (1979): Inhibition of anion permeability of sarcoplasmic reticulum vesicles by 4-acetoamido- 4'-isothiocyano-stilbene-2,2'-disulfonate. Biochim. Biophys. Acta **557**, 243—247

Kasai M., Tagushi T. (1981): Inhibition of anion permeability of sarcoplasmic reticulum vesicles by stilbene derivatives and the identification of an inhibitor-binding protein. Biochim.Biophys. Acta. **643**, 213—219

Lai F.A., Erickson H.P., Rousseau E., Liu A.-Y., Meissner G. (1988): Purification and reconstitution of the calcium release channel from skeletal muscle. Nature **331**, 315—319

Lukács G.L., Moczydlowski E. (1990): A chloride channel from lobster walking leg nerves. Characterization of single-channel properties in planar bilayers. J.Gen. Physiol. **96,** 707—733

Meissner G. (1983): Monovalent ion and calcium ion fluxes in sarcoplasmic reticulum. Moll. Cell Biochem. **55,** 65—82

Miller C. (1978): Voltage gated cation conductance channel from fragmented sarcoplasmic reticulum; steady state electrical properties. J. Membrane Biol. **40,** 1—23

Miller C., Racker E. (1976): Ca^{++}-induced fusion of fragmented sarcoplasmic reticulum with artificial planar bilayers. J. Membrane Biol. **30,** 283—300

Miller C., White M.M. (1980): A voltage-dependent chloride conductance channel from *Torpedo* electroplax membrane. Anna. NY Acad. Sci. **341,** 534—551

Miller C., White M.W. (1984): Dimeric structure of single chloride channels from *Torpedo* electroplax. Proc. Nat. Acad. Sci. USA **81,** 2772—2775

Moczydlowski E., Latorre R., (1983): Gating kinetics of Ca^{2+}- activated K^+ from rat muscle incorporated into planar lipid bilayers. J. Gen. Physiol. **82,** 511—542

Moczydlowski E., Gerber S.S., Miller C. (1984): Batrachotoxin- activated Na^+ channels in planar lipid bilayers. Competition of tetrodotoxin in block by Na^+. J. Gen. Physiol. **84,** 665—686

Mueller P., Rudin D.O., Tien H.T., Wescott W.C. (1962): Reconstitution of cell membrane structure *in vitro* and its transformation into an excitable system. Nature **194,** 979—980

Proks P., Hurňák O., Zachar J. (1991): Chloride channels in crayfish skeletal muscle. Abstracts of the IUPS Regional Meeting in Prague 1991 (Eds. S. Tuček, D. Marešová and F. Šťastný), PH18.

Rousseau E. (1989): Single chloride-selective channel from cardiac reticulum studied in planar lipid bilayers. J. Membrane Biol. **110,** 39—47

Rousseau E., Robertson M., Meissner G. (1988): Properties of single chloride selective channel from sarcoplasmic reticulum. Eur. Biophys. J. **16,** 143—151

Smith J.S., Imagawa T., Ma J., Fill M., Campbell K.P., Coronado R. (1988): Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. J. Gen. Physiol. **92,** 1—26

Tanabe T., Beam K.G., Adams B.A., Nadome T., Numa S.(1990): Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. Nature **346,** 567—569

Tanifuji, M., Sokabe M., Kasai M. (1987): An anion channel of sarcoplasmic reticulum incorporated into planar lipid bilayers: Single channel behavior and conductance properties. J. Membrane Biol. **99,** 103—111

Zachar J. (1971) : Electrogenesis and Contractility in Skeletal Muscle Cells. University Park Press, Baltimore and London

Zacharová D., Zachar J. (1967): The effect of external calcium ions on the excitation - contraction coupling in single muscle fibres of the crayfish. Physiol. Bohemoslov. **16,** 191—207

Final version accepted October 29, 1991