High-Conductance Chloride Channels in BC3H1 Myoblasts

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Abstract. The existence of a high-conductance voltage sensitive chloride channel in BC3H1 myoblasts is documented. The conductance of the channel in symmetrical 150 mmol/l sodium chloride is around 400 pS. The probability of the channel being in the open state decreases with increasing of the imposed voltage from holding potential (0 mV) in both the depolarizing and the hyperpolarizing direction respectively. The bell-shaped open probability plot is asymmetrical, and can be fitted by two Boltzmann equations with different V_h and k_n constants; the fitted values were −53 mV and −8.4 respectively for the negative side and +41 mV and +10.2 for the right side. When the unit Cl currents to rectangular pulses are summated the resulting total ionic Cl current shows relaxation, which increases with the amplitude of the pulse. The activation as well as the shape of the current can be significantly influenced by varying the amplitude and the direction of prepulses or holding potential. The high-conductance Cl channel shows several substates (at least four with amplitudes of around 100 pS); frequently accompanied by flickerings.

Key words: Chloride channel — Large conductance channel - Maxi channel — Anion channel — Muscle cell line — BC3H1 myoblasts — Patch clamp — Voltage dependent channels — Channel open probability

Introduction

The BC3H1 muscle cells, a clonal line (Schubert et al. 1974) have been used extensively as a model system for studies of muscle cell differentiation as well as of reversal of differentiation upon addition of growth stimuli (DeSmedt et al. 1991). The expressions of a number of muscle-specific gene products and their sequences in the development of muscle cell components were thus determined, including ligand gated (Patrick et al. 1977; Olson et al. 1984; Sine and Taylor 1979; Sine and Steinbach 1986) and voltage gated ionic channels (Caffrey et al. 1987; 1989). In the course of an inquiry into the expression of chloride channels in these cells we disclosed the presence of high-conductance voltage gated channel in early stages of development, e.g. in undifferentiated myoblasts. The channels were subsequently

shown to be closely related with maxi-Cl channels which do exist in a number of cells, including myoblasts (for a review see Hurňák and Zachar 1992). In the present study we shortly summarize the basic characteristics and properties of these channels. The remarkable features of the channel are (a) steep voltage dependence of probability of the channel being in the open state, which can be described by two Boltzmann equations and supports a gating model with two contiguous gates, (b) induction of dormant channels by prolonged voltage changes or voltage jumps, (c) complex gating properties with multiplicity of conductance states, (d) asymmetry of channel behavior to positive and negative pulses.

Materials and Methods

Cell culture. The BC3H1 cells were obtained from American Type Culture Collection (A.T.C.C.; Rockville, MD, U.S.A.). Cells for experiments were subcultured at regular time intervals to prevent the cultures to reach confluency. Cells were seeded in plastic or glass dishes at a maximum density of $2 - 5 \times 10^5$ cells/Petri dish (60 mm in diameter) in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and antibiotics: streptomycin, kanamycin, (100 μg/ml each) and penicillin (100 units), and were kept in a humidified atmosphere under 5% CO_2 / 95% O_2 at 37°C. Before the experiments, the culture medium was exchanged for saline of the following composition (in mmol/l): 150 NaCl; 0.5 $CaCl_2$; 20 HEPES; pH 7.4. In most experiments the pipette solution was the same as that in the bath solution.

Patch-clamp. Currents were recorded in the excised (inside-out) configuration of the patch-clamp technique (Hamill et al. 1981). Patch pipettes were pulled (LM-3P-A puller, List, Darmstadt, Germany) from borosilicate glass and fire polished. All recordings were made with an Axopatch 1C patch-clamp amplifier and CV-4 0.1/100 headstage (Axon Instruments, Foster City, CA, USA). Currents were low-pass filtered (1-2 kHz) by an eight pole Bessel filter. Data were acquired and analyzed using an IBM AT compatible computer with an analog-to-digital interface board (Labmaster DMA, Scientific Solutions Inc., Solon, OH, USA) using pClamp 5.5.1 software (Axon Instruments). The ramp-pulse recordings were evaluated using software developed in this laboratory (Stavrovský et al. 1992). Voltage-dependence of channel activation (Fig. 4) was determined by a method similar to that described by Groschner and Kukovetz (1992). Current responses to voltage ramp pulses were averaged, corrected for leak current, and data points were divided by single channel current amplitude at respective potentials by protocols written in Mathematica 2.1 software (Wolfram Research, Champaign, IL, U.S.A).

Results

The channel activity was less frequent (in about 25% of the excised patches examined) in comparison with the occurrence of channels in the excised patch membrane investigated in L6 myoblasts under identical experimental conditions (Hurňák and Zachar 1992). The channels were silent, and to induce activity, strong continuous membrane displacements (mostly depolarization) had to be applied and/or volt-

age jumps from the holding potential corresponding to the equilibrium potential in symmetrical NaCl solutions (close to 0 mV). If no channel activity appeared in 30 minutes the membrane patch was abandoned. The channel activity started either abruptly or after a transient period (10–100 s) of small amplitude unitary currents; subsequently, they were overrun by large amplitude single channel currents interrupted for few minutes by periods with the channel mainly in the closed state and showing rapid transitions to the open state. The transitions were generally too fast to be resolved and did not clearly attain the open state or one of

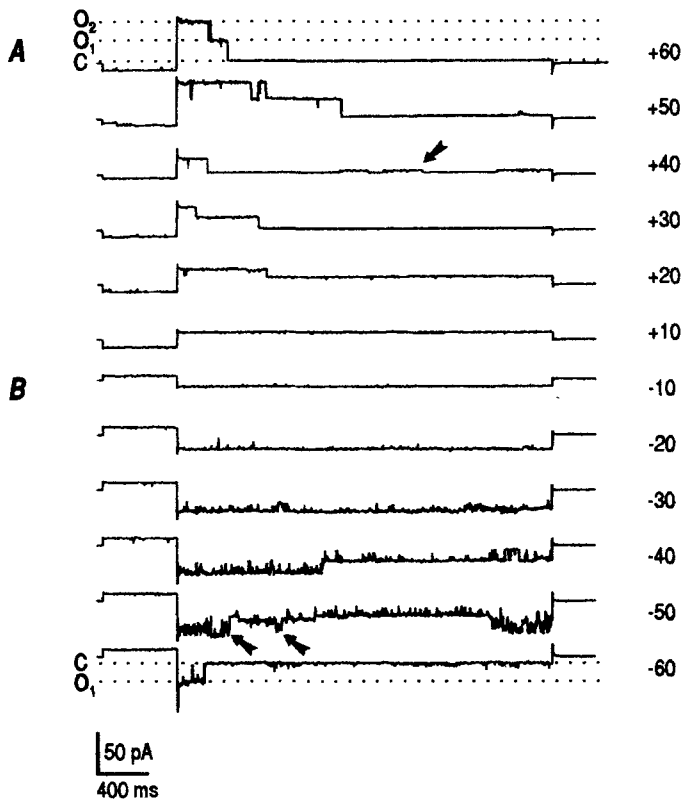

Figure 1. Single-channel current records from an excised patch (inside-out) of a BC3H1 myoblast. The holding potential was held at 0 mV and stepped to −10 mV (*A*) or +10 mV (*B*) respectively for 600 ms before the application of test pulses in 10 mV steps. Duration of test pulses 3000 ms; interpulse interval 10 s; sampling interval 2 ms. C – closed state, O_1, O_2 – open states of two activated channels. The subconductance states are marked by the arrows in *A* (pulse to +40 mV) and in *B* (pulse to −50 mV). Note extensive flickering in substates. Symmetrical solutions: patch pipette // bath (in mmol/l): 150 NaCl; 0.5 $CaCl_2$ // 150 NaCl; 0.5 $CaCl_2$; pH 7.4.

the substates. When the first signs of activation appeared forced depolarization was abandonned and measurements of channel conductance were routinely started using voltage ramp pulses (Stavrovský et al. 1992) and/or voltage steps of varying amplitudes.

Fig. 1 shows the channel activity to positive (A) or negative (B) voltage steps of 10 mV (lasting 3 s) from a holding potential of 0 mV and a prepulse level ±10 mV to +60 or −60 mV respectively. Two channels were present in this particular patch membrane; the open levels are represented by the dotted lines O_1 and O_2 respectively (C indicates closed state). All individual voltage steps were preceeded by a short prepulse of 10 mV (600 ms in duration) in direction opposite to the membrane potential displacement during the test pulse. Fig. 1 illustrates at least three characteristic properties of the channel. *First,* there were typical current relaxations similar to those seen with membranes patches containing several chan-

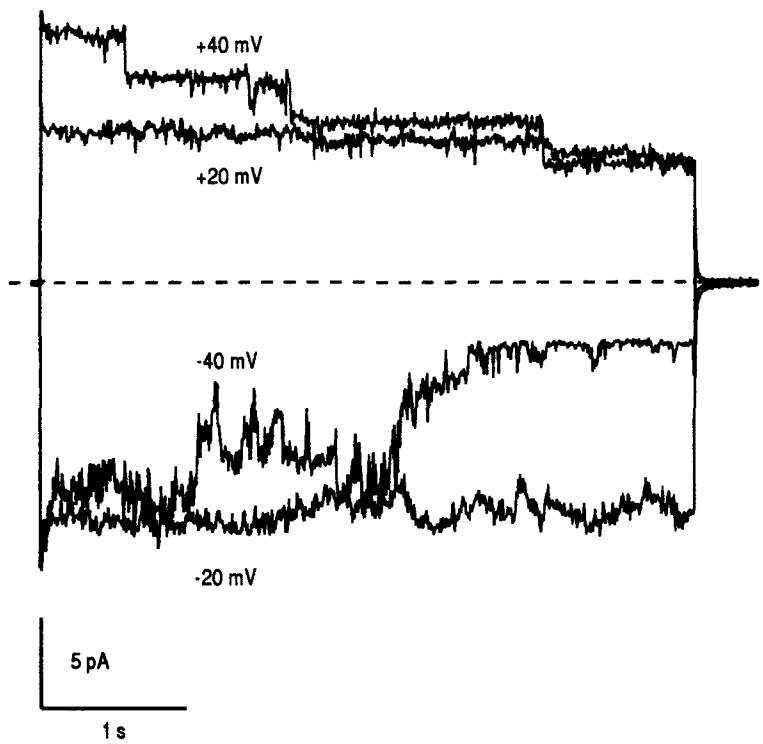

Figure 2. Ensemble averages of single-channel currents at different potential steps. Numbers of current traces averaged were 10 at ±20 and to −40 mV potential steps or 5 at +40 mV steps respectively. The repetition interval was 15 s, the sampling interval 5 ms and the pulse duration 4500 ms. The holding potential was 0 mV. For solutions see legend to Fig. 1.

nels. Voltage steps from 0 mV to ±10 mV and those from 0 mV to −20 mV and −30 mV respectively resulted in channels being open all the time during the pulse. Following voltage steps to higher displacements of membrane potential the channel closed in stepwise fashion. *Second,* several substates and current transitions (represented by arrows) between fully open and fully closed states occur (see also Fig. 5). *Third,* the recording shows that responses to negative and positive membrane potential displacements might not be symmetrical. Prepulses were used since they have been shown to facilitate the activation of the high-conductance channels to test pulses of opposing polarity. Fig. 2 shows ensemble average current relaxations without prepulses in response to 5–10 consecutive test pulses. The channels were open much of the time when ±20 mV pulses were applied, but relaxed in a stepwise manner to ±40 mV pulses. The relaxation was, however, steeper to negative than to positive pulses.

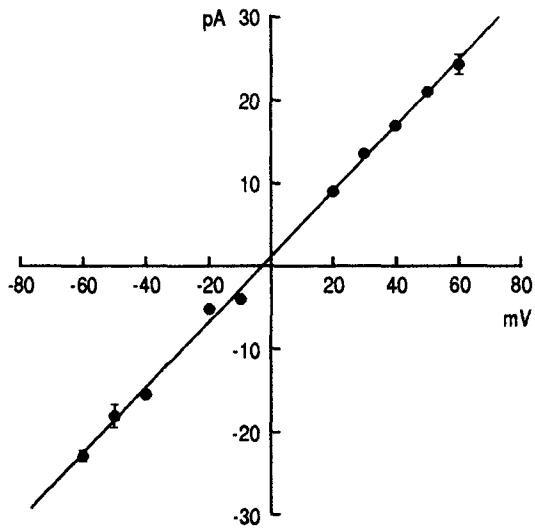

Figure 3. Current-voltage relation of the main conductance level. The filled circles represent means ± S.E.M. (n = 3) from records such as shown in Fig. 1. The slope of the regression line is 395±6 pS. The reversal potential is not significantly different from 0 mV. Symmetrical 150 mmol/l NaCl (with 0.5 mmol/l of $CaCl_2$ on both sides of the membrane).

The conductance of the channel (Fig. 3) evaluated from the records shown in Fig. 1 and from other runs was 395 ± 6 pS in symmetrical 150 NaCl (mmol/l). Also the channel conductance was evaluated by means of ramp pulses (see Fig. 5) as described elsewhere (Stavrovský et al. 1992) and it averaged around 400 pS. In the solution used the equilibrium potential for an Na^+, Cl^-, or a nonselective channel would be ≈ 0 mV.

Fig. 4 shows voltage dependence of the probability (P_0) of the channel in the main open state; it was computed from 16 summated records of the channel activity to voltage ramp pulses from +10 to −60 mV and −10 to +60 mV respectively,

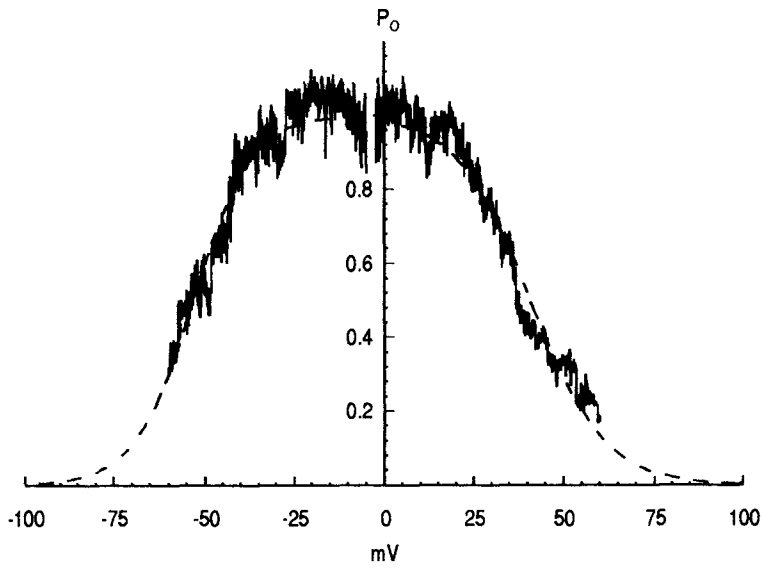

Figure 4. Voltage dependence of the steady-state probability (P_0) of the channel being in the open state. The bell-shaped relation represents ensemble averaged current from 16 ramp responses. Two series of ramp pulses (0.02 V/s) were used, e.g. from −10 mV to 60 mV and from +10 mV to −60 mV; interpulse interval 20 s. The dashed curves were fitted separately for two Boltzmann equations (given in the text) with coefficients $V_h = -53$ mV and $k = -8.4$ for the negative side, and +41 mV and +10.2 for the right side.

as described in Methods. The two dashed curves (for the negative and the positive voltage abscissa respectively) represent the best fit of the experimental points according to two Boltzmann equations in the form:

$$P_0 = P_{\max}/[1 + \exp(V - V_h)/k_n],$$

where P_0 is the steady-state probability of the channel being in the open state; $P_{\max}$ is the maximum probability; V_h is the voltage at $P_0 = 0.5$, and k_n is the slope factor representing the voltage sensitivity of activation. The fitted values of V_h and k_n were −53 mV and −8.4 respectively for the negative side, and +41 mV and +10.2 for the right side.

The channel shows multiple conductance levels. Fig. 5 shows two such levels (S_1, S_s) that are obvious in superimposed records to five ramp pulses from −60 to 60 mV (Fig. 5*A*), but are also apparent in one of the individual records (*B*). S_1 substate represents approx. 1/4 (≈ 100 pS) of the fully open state. S_s substate is much smaller (≈ 30 pS). As follows from the records relaxation of S_1 conductance to positive voltages was slower than relaxation of the main open conductance

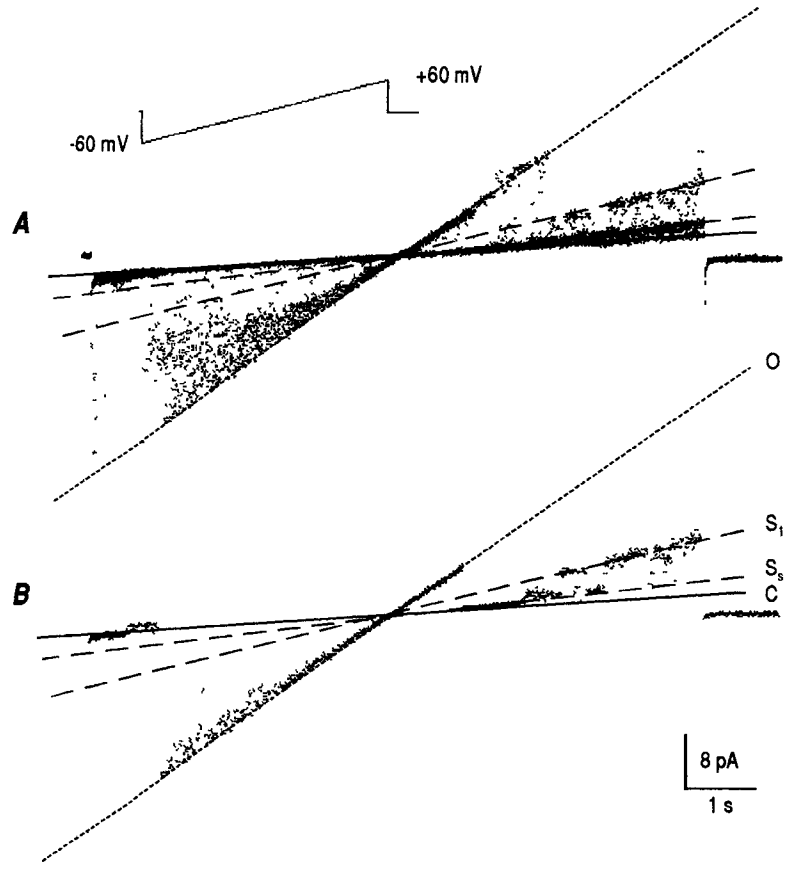

Figure 5. A) Superimposed successive records to 5 ramp pulses from −60 to +60 mV in a run from an excised membrane patch to show a fully open state (O) and two substates (S_1, S_s); C indicates the closed state. An individual record from this series is shown in *B*.

level. This also applies to S_s conductance. The conductances activated by negative voltages could not be analyzed in a similar way; there was a multiplicity of subconductance states in this region as evident from the continuous record (to a negative pulse of −20 mV) in Fig 6. The record in the upper part of the Figure shows that most of the time during this period the channel was at four substate levels (S_1, S_2, S_s, S_3) or closed (C), but not fully open. The lower part of the Figure shows a segment of the above record on an expanded time scale. The small S_s substate is similar in amplitude to that shown in Fig. 5. The abrupt transition from the closed level to S_3 level and probably open level respectively supports

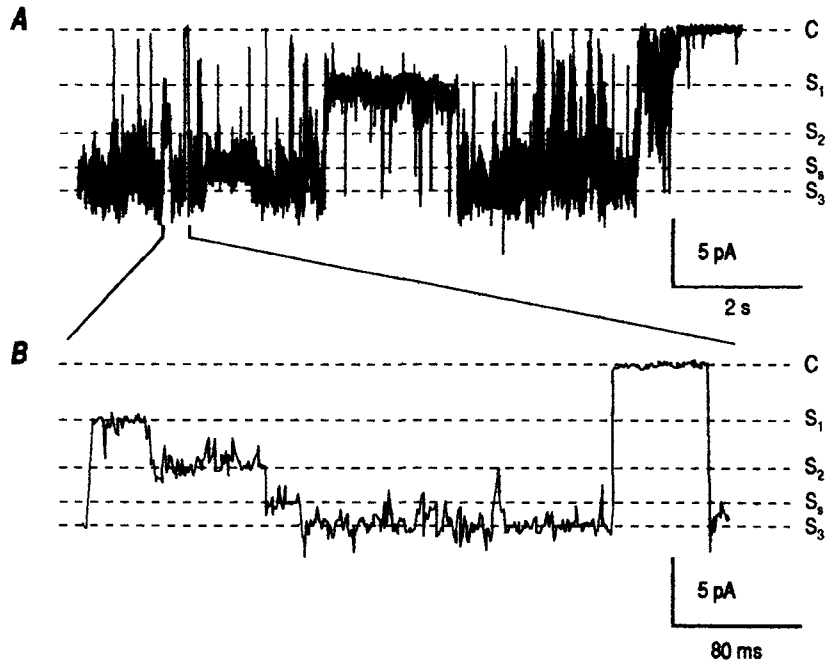

Figure 6. (*A*) Segment of a continuous single-channel record from an excised patch at the start of maxi-Cl channel activation. Expanded part of the record is shown in (*B*). At least four substates (S_1, S_2, S_3, S_s) can be recognized in addition to the full open state; C indicates closed state. The holding potential was −20 mV. Symmetrical 150 NaCl.

the claim that the substates are not individual channels but rather substates of a channel conductance. The record was taken at the start of channel activation and suggests that the channel passes through a series of conductance steps to reach full open state. It happens that in the early stages of the channel build-up the chanel remains in a uniform subconductance state for several tens of seconds, thus imitating a low-conductance channel behavior, especially when the channel activation in the membrane patch dies out before the development of full conductance state. However, this kind of activity – although frequent – was not present for sufficient time intervals to permit complete characterization.

Discussion

The high-conductance channel shares many properties with the high-conductance anion channel in other cell types as reviewed in our previous paper (Hurňák and Zachar 1992) that described the properties of the maxi-Cl channel in L6 myoblasts.

This concerns not only the magnitude of channel conductance but also the other characteristic properties of this type of channel, which was discovered by Blatz and Magleby (1983). The remarkable features of the channel are (a) steep voltage dependence of probability of the channel being in open state, (b) induction of dormant channels by prolonged voltage changes or voltage jumps, (c) complex gating properties with multiplicity of conductance states, (d) asymmetry of channel behavior to positive and negative pulses.

The bell-shaped steady state channel conductance-voltage relationship was fitted by two Boltzmann equations in several other cells, not only for the high-conductance anion channel of this type (Schlichter et al. 1990; Pahapill and Schlichter 1992; Groschner and Kukowetz 1992; McGill et al. 1992) but also for other high-conductance channels, e.g. gap junction channels (Wang et al. 1992) and porins (Benz et al. 1992). This behavior can be interpreted as being due to two gates in series with each other. Harris et al. (1981) developed alternative models for the gating of this type, i.e. an idependent gating model and a contiguous model. The contingent model assumes that the closed gate of the polarized channel has to open before the other gate in series can open. Although not systematically followed it seems evident from the presented results (Fig. 1) that the gates are reciprocally related. From the maximum slopes of P_0 versus V curve (8.4 mV or -10.2 mV) an estimate can be made of the charges for the gates that close at negative or positive potentials respectively (Almers 1978; Schlichter et al. 1990).

The high-conductance channel in BC3H1 myoblasts required rather prolonged voltage changes (sustained depolarization or voltage jumps) for induction of activation. This is in contrast with activation of high-conductance channels in L6 myoblasts (Hurňák and Zachar 1992), which also were spontaneously active, and the incidence of finding the channel in excised patch membranes was approximately twice as high as in BC3H1 cells. The activation process in BC3H1 cells was also slower and showed a stepwise development. Small amplitude unit currents resembling a separate channel could be finally recognized as components of the high conductance channel. As shown in Fig. 6, up to four equally spaced steps could be recognized in addition to a small substate representing about 9% of the main open state. There are several indications that these (around 100 pS) substates represent subconductance levels rather than a separate channel: a) the 25% level was present in almost all of the recordings where the high-conductance anion channel was present; b) there were frequent transitions between the fully open or closed level and the 25% level of the kind illustrated in Fig. 6B, that are hardly to reconcile with the existence of separate channels. The multiplicity of substates represents a standard finding in maxi-Cl channels; one to 16 substates have been shown to exist in this type of channels (Gray et. al. 1984; Geletyuk and Kazachenko 1985; Krouse et al. 1986; Bolotina et al. 1987; Woll et al. 1987; Schlichter et al. 1990; Becq et al. 1992; Groschner and Kukovetz 1992; Hurňák and Zachar 1992; McGill

et al. 1992; Olesen and Bundgaard 1992). If we follow the reasoning of Krouse et al. (1986) the high-conductance anion channel in BC3H1 myoblasts may be a co-channel formed by the association of four channels with a unitary conductance of about 100 pS.

The physiological significance of the maxi chloride channel is still obscure. The channel does not appear to be specific to any particular cell type (see Hurňák and Zachar 1992 for a review). The presence of these channels in non diferentiated myoblasts with a rather scant channel set-up in the membranes of these cells (L6) has led to the suggestion that high-conductance anion channels contribute to some basic function including regulation of cell volume or communication with other cells (Hurňák and Zachar 1992), and this was also one of the reasons for the inquiry into their presence in BC3H1 myoblasts. There are some indications that this idea is worth to follow. McGill et al. (1992) have shown that in bile duct epithelial cells an anion channel can be activated by negative pipette pressure, which has been used as a marker for stretch activated ion channels in other cell types (Christensen 1987). Kubo and Okada (1992) have demonstrated recently in cultured human small intestinal epithelial cells, during osmotic swelling under the patch-clamp whole cell configuration, activation of large Cl^- currents, which showed several characteristics similar to those of high-conductance channels. Rugolo et al. (1992) reported the existence of volume-sensitive Cl^- currents in human keratocytes as revealed by ^{36}Cl fluxes and whole cell current recordings. On the other hand, Velasco et al. (1989) were not able to induce by hypoosmotic shock in cell attached patches from GBK cells of bovine kidney cortex the activation of channels, which subsequently appeared in excised configuration. Cellular shrinking was reported to activate chloride-dependent cation conductance (Chen and Nelson 1992). Even if channel activation by volume changes can be proven in cell attached patch-clamp configuration the mechanism of maxi-Cl channel activation in excised patches will still remain to be elucidated.

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