Single-channel analysis of a ClC-2-like chloride conductance in cultured rat cortical astrocytes

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Abstract The single-channel behavior of the hyperpolarization-activated, ClC-2-like inwardly rectifying Cl^- current (I_{Clh}), induced by long-term dibutyryl-cyclic-AMP-treated cultured cortical rat astrocytes, was analyzed with the patch-clamp technique. In outside-out patches in symmetrical 144 mM Cl^- solutions, openings of hyperpolarization-activated small-conductance Cl^- channels revealed burst activity of two equidistant conductance levels of 3 and 6 pS. The unitary openings displayed slow activation kinetics. The probabilities of the closed and conducting states were consistent with a double-barrelled structure of the channel protein. These results suggest that the astrocytic ClC-2-like Cl^- current I_{Clh} is mediated by a small-conductance Cl^- channel, which has the same structural motif as the Cl^- channel prototype ClC-0.

Key words: Astroglia; ClC-2 channel; Subconductance; Double barrel

1. Introduction

Voltage-dependent chloride (Cl^-) channel proteins form a single large gene family (ClC). Among others, the ClC-2 channel activated by plasma membrane hyperpolarization and cell swelling was cloned and heterologously expressed in *Xenopus* oocytes [1,2]. Several studies have indicated that ClC-2 or ClC-2-like channels are expressed by various neuronal and non-neuronal cell types [3–10], which suggests that this current may play an important functional role. However, despite the large body of information on the macroscopic behavior of this channel, there are no data on the properties of the single channel underlying ClC-2 or ClC-2-like currents. This differs from the findings on two other members of the ClC gene family, ClC-0 and ClC-1, whose unitary currents have been investigated in detail. It has been proposed that both channels are dimeric proteins with a structure defined as double-barrelled in which two protopores gate independently of each other and both are regulated by a common gate [11–17]. This result has suggested that these ClC channel proteins exhibit important structural similarities, which are likely to be extended to other members of the ClC family.

Recently, a hyperpolarization-activated, inwardly rectifying chloride current (I_{Clh}), similar to the ClC-2-mediated current, has been identified in primary cultured rat neocortical astrocytes long-term treated with dibutyryl-cyclic-AMP (dBcAMP) [18]. This current has biophysical and pharmacological properties resembling those of the heterologously expressed ClC-2, suggesting that it may be either the same channel or a channel characterized by a high structural homology [19]. Here we investigate the unitary properties of the channel underlying I_{Clh}. We show that dBcAMP-treated cultured cortical astrocytes possess a Cl^- channel that is activated upon hyperpolarization and has two equidistant open-conductance levels of 3 and 6 pS. Analyses of open probability and kinetics suggest that the channel behaves as if it comprised two independently gated conductance states regulated in parallel by a common gate. A preliminary account of this work has been presented elsewhere [20].

2. Materials and methods

2.1. Cell cultures

Primary cultures of rat cortical astrocytes were obtained as previously described [18]. Following the mechanical dissociation of the cerebral cortices of 1–2-day-old pups, the cells were plated in 25 cm^2 culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% of heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine, and maintained in an incubator with humidified atmosphere at 37°C and 5% CO_2. The medium was replaced every 3 days. After the first week, before the change of the medium, the flasks were gently shaken for 5 min in order to detach the O-2A progenitor cells and microglia which had seeded on top of the astrocyte layer. More than 90% of the cultured cells were immunologically identified as type-1-like cortical astrocytes [18].

At confluency, after 1–2 weeks of culturing, 250 μM dBcAMP was added to the culture medium for the following 1–2 weeks to induce the astrocyte morphological differentiation. Three days prior to the electrophysiological recordings, astroglial cells were replated in 33 mm Petri dishes at a density of $1–3\times10^4$ per dish and maintained in the DMEM to which 250 μM dBcAMP had been added.

2.2. Materials

For the astrocyte culture preparations, all the materials used were purchased from Gibco-BRL (Life Technologies, Italy). The chemicals of the salt solutions for the electrophysiological measurements were obtained from Sigma.

2.3. Electrophysiological recordings

The electrophysiological studies were performed at room temperature (20–23°C) by using the cell-attached and excised (outside-out) configurations of the patch-clamp technique [21]. Patch pipettes were pulled from thick borosilicate glass (Clark Electromedical, UK), sylgard-coated and heat-polished to obtain a resistance of 7–10 MΩ when filled with the aforesaid solutions. The external solution was composed of (mM): 144 *N*-methyl-D-glucamine ($NMDG^+$)-Cl, 1 $CaCl_2$, 2 $MgCl_2$, 5 TES, 5 glucose, adjusted with $NMDG^+$ to pH 7.3. As there is evidence that I_{Clh} is insensitive to extracellular applications of the putative Cl^- channels blocker diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), whereas other types of Cl^- currents are

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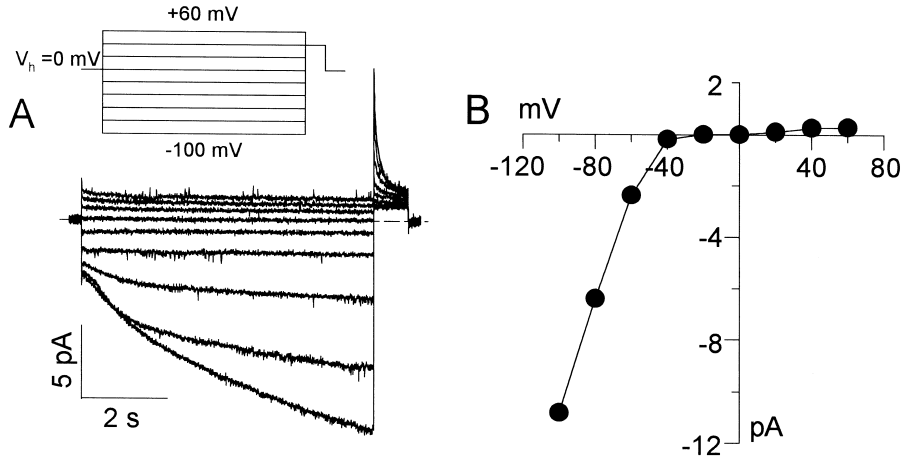

Fig. 1. A: Hyperpolarization-activated Cl^- currents (I_{Clh}) in a cell-attached patch. Family of I_{Clh} obtained from a dBcAMP-treated cultured rat astrocyte by using the stimulation protocol shown in the inset. Currents were filtered at 1 kHz. Note the inwardly rectifying current behavior and the fact that no single-channel transitions are clearly distinguishable. The tails at +40 mV show the current deactivation. B: *I–V* relationship relative to the time-dependent component of the currents. In order to depolarize the cell and to move the transmembrane potential close to the pipette potential, the solutions in the bath and the pipette contained 140 mM KCl.

inhibited, in excised patches, DIDS (200 μM) was added to the bath [19,22–25]. In cell-attached experiments, the NMDG-Cl in bath and pipette solutions was replaced with KCl to depolarize the recorded cell. The activity of K^+ channels was abolished by adding 0.5 mM Ba^{2+} and 10 mM tetraethylammonium (TEA) to the pipette solution. In excised patches, the pipette solution contained (mM): 144 NMDG-Cl, 2 $MgCl_2$, 5 TES, 5 EGTA, 5 glucose adjusted with $NMDG^+$ to pH 7.3. Osmolarity was set to 320 ± 5 mOsmol with mannitol. Membrane currents were recorded using an Axopatch 200A amplifier (Axon Instruments Inc., Foster City, CA, USA), and were low-pass-filtered at one third of the sample frequency. Both voltage stimulation and data acquisition were performed using a 12-bit interface (Axon Instruments) and a microcomputer equipped with pClamp (5.5.1) software (Axon Instruments). Current traces, obtained by 6- or 30-s-long voltage pulses, were not corrected for leakage. Data analysis was performed as previously described by Saviane et al. [17].

3. Results

Plasma membrane ionic currents were recorded from cell-attached patches of long-term dBcAMP-treated cultured type-1 cortical astrocytes, using 140 mM KCl solution in bath and pipette. Barium ions and TEA were included in the pipette solution to block potassium currents (Fig. 1A). Under these experimental conditions, depolarizing voltage steps up to +60 mV from a holding potential (V_h) of 0 mV elicited negligible outward currents, whereas slowly activating, non-inactivating inward currents of increasing amplitudes were evoked when hyperpolarizing voltages up to −100 mV were applied. The current–voltage relationship (*I–V*) of the current measured at the end of the voltage pulses revealed a steep inwardly rectifying profile ($n = 7$; Fig. 1B). Both the voltage and time dependences are similar to those of the previously characterized whole-cell, inwardly rectifying whole-cell Cl^- current (I_{Clh}) expressed by cultured type-1 cortical astrocytes following a long-term treatment with dBcAMP [19]. However, because under these experimental conditions we did not succeed in resolving the single-channel transitions, further experiments were carried out using the outside-out configuration in symmetrical 144 mM NMDG-Cl salt solutions and in the presence of 200 μM DIDS. In 56 of such astrocytes, we obtained recording conditions with high seal resistance, and low background noise, which allowed us to resolve the single-channel events clearly. However, among these, only in a few patches ($n = 12$) sufficiently long recordings were performed to allow a quantitative analysis. Even though the whole-cell I_{Clh} currents did not show any rundown, even during long (> 30 min) recording periods [19], in the majority of excised patches, channel activity started to decrease quickly following patch excision (onset < 30 s) and disappeared completely within 3–5 min. A possible explanation for such a behavior is that,

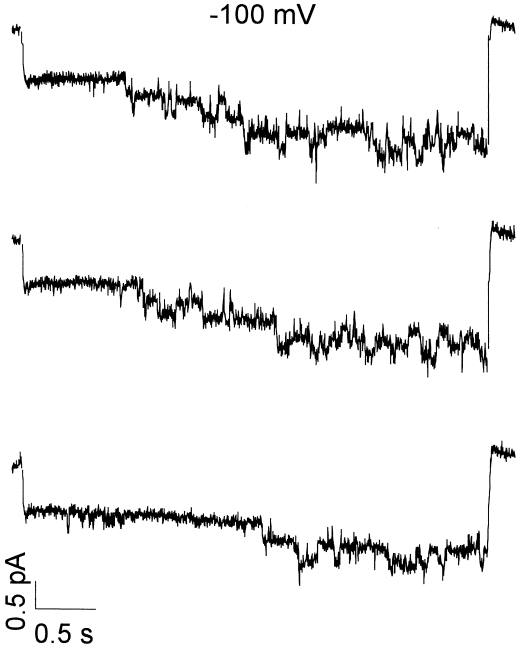

Fig. 2. Time dependence of the activation of single Cl^- channels in dBcAMP-treated cultured astrocytes. Latency of activation is shown in traces from an outside-out patch in which several I_{Clh} channels were active. Current traces were obtained by repetitive hyperpolarizing voltage steps to −100 mV, 5-s-long, from a V_h of 0 mV and filtered at 300 Hz. The unitary current was about 300 fA. The unitary channel currents were recorded in symmetrical 144 mM NMDG-Cl solutions with 200 μM DIDS in the bath.

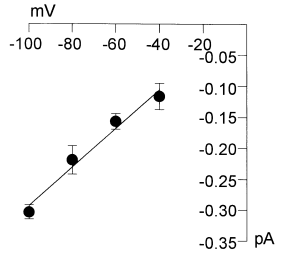

Fig. 3. *I–V* relationship of the astrocytic hyperpolarization-activated Cl^- channel. Data points at each membrane potential represent the averages over 4–10 astrocytes. Mean values ± S.D. are shown. The slope conductance was 3 ± 0.2 pS. For the analysis, data were filtered at 300 Hz.

upon an excised patch, some regulatory molecules, which are associated with the cell cytoskeleton and are essential for the channel activity, are gradually lost. Instead, in long recordings without a decrease in channel activity, it may occur that part of the cytoskeletal matrix remains anchored to the membrane patch and the regulatory factors are preserved. In these patches, using symmetrical NMDG-Cl saline, recordings showed activity of multiple Cl^- channels that at −100 mV had a unitary current of ~300 fA and displayed a latency of activation larger than 0.5 s ($n = 5$; Fig. 2). In two experiments, this channel activity could be blocked by patch exposure to cadmium (500 μM), which we previously demonstrated to be a potent inhibitor of I_{Clh} (data not shown). Fig. 3 shows the current values of the single open-state level at membrane potentials between −40 and −100 mV. The linear fit of the unitary amplitudes gave a slope conductance of 3 ± 0.2 pS. Channel openings were never detected at positive membrane potentials up to +100 mV (data not shown), which points out the strong inwardly rectifying behavior of the channel activity. At all the voltages tested, the current traces consistently showed at least two approximately equidistant open-conductance levels that were distinguishable from background noise. With larger hyperpolarization, the number of openings within the channel burst increased as a multiple of two. Another important qualitative feature can be deduced from stretches of longer traces: long closing periods separated bursts of openings that almost invariably contained both conductance levels (Fig. 4). Amplitude histograms of long bursts of channel activity confirmed these findings, as the histograms obtained at −80 mV were well fitted by the sum of three Gaussian distributions ($n = 4$; Fig. 5A). Accordingly, the relative areas of the three components yielded the probabilities P_0, P_1 and P_2 of the closed and the two open levels, respectively (Fig. 5B, bars). A simple analysis shows that the probability values are different from those expected from a simple binomial superposition of two independently gating channels, each with open probability P (Fig. 5B, circles). Thus, it can be reasonably excluded that the two open-conductance levels are due to the presence of two identical and independent channels. Furthermore, the consistent presence of both levels in all the patches supports the assumption that the current traces of the type shown in Fig. 4 represent a single channel that has two equidistant open states. Experiments with long bursts at a membrane potential of −80 mV showed that dwell-time histograms of the closed and open times of the single and double openings could be fitted by double exponential functions (Fig. 6). The histograms are well fitted by the rate constants given in the legend to Fig. 6. However, it is worth noting that the fast time constants τ_{c1} and τ_{o1} are at the limit of the filter resolution, thus making difficult the fine dissection of the gating processes. In addition, a further slow time constant could be obtained from the closed periods between bursts.

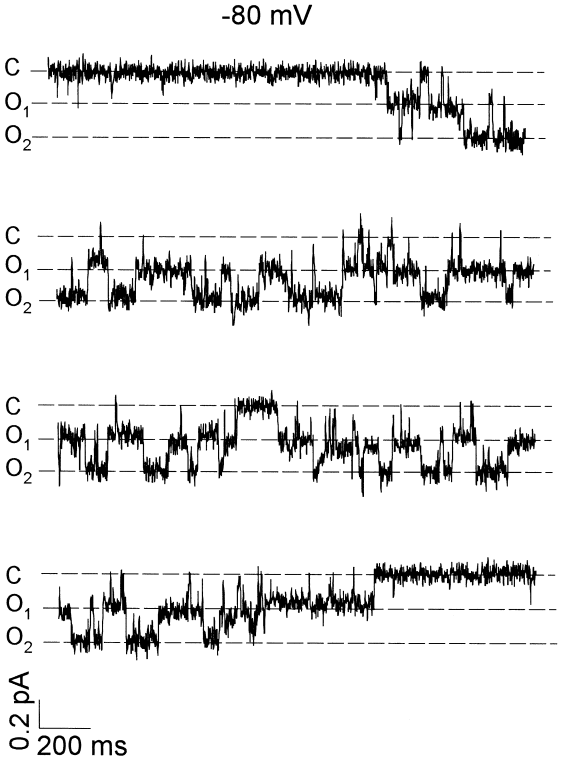

Fig. 4. Burst activity of the astrocytic Cl^- channels. Single-channel traces obtained from an outside-out patch held at −80 mV. Subsequent short segments of longer registration are shown. Current traces were filtered at 300 Hz. Note the burst activity of the channel openings characterized by two open-conductance levels. Solutions as in Fig. 2.

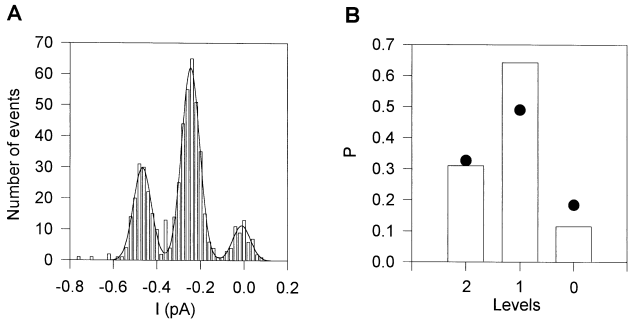

Fig. 5. Amplitude histogram and open probability of the conductance states of the astrocytic Cl^- channel. A: Amplitude histogram of a recording at −80 mV fitted by the sum of three Gaussian distributions. Each open-state level is at 240 ± 41 fA. B: Stationary probabilities for the three conductance levels obtained from the Gaussian fit (boxes). Filled circles indicate the best fit assuming the binomial superposition of two independent channels, each with open probability $P = 0.57$.

4. Discussion

The data presented here demonstrate that primary cultured

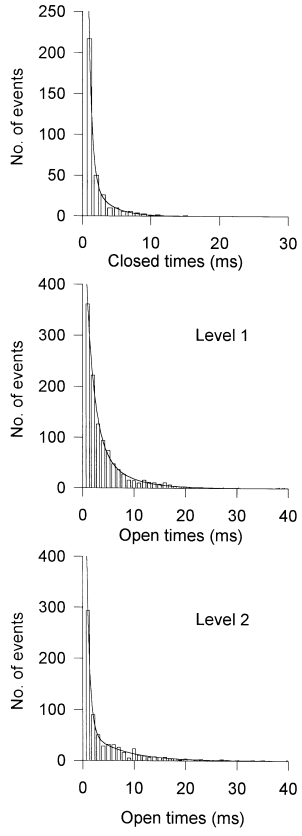

Fig. 6. Kinetic analysis of the astrocytic Cl^- channel. Dwell-time histograms for the three conductance levels. The closed- and open-time distributions were fitted by the sum of two exponential functions, with $\tau_{c1} = 0.47$ ms and $\tau_{c2} = 2.64$ ms, $\tau_{o1} = 1.47$ ms and $\tau_{o2} = 5.04$ ms (level 1), and $\tau_{o1} = 0.59$ ms and $\tau_{o2} = 6.49$ ms (level 2), respectively. The data shown are representative of four experiments.

rat neocortical astrocytes that have been morphologically differentiated by a long-term treatment (1–2 weeks) with dBcAMP exhibit a hyperpolarization-activated, small-conductance Cl^- channel with two equidistant open-conductance levels of 3 and 6 pS.

Previous works have shown that rat neocortical astrocytes in untreated culture, which have a flat, polygonal morphological phenotype, possess both large- and intermediate-conductance Cl^- channels with gating kinetics that markedly differ from those of the channel described here [23,25]. Interestingly, these channels were seldom observed under our experimental conditions, suggesting that the state of differentiation of the cultured astrocytes may play an important role in the functional expression of the different types of Cl^- channels. A small-conductance (5 pS), hyperpolarization-activated Cl^- channel with a half-conductance substate was previously identified, but not characterized, in mouse cortical astrocytes [26].

The gating properties of the single Cl^- channel described here (e.g. opening by membrane hyperpolarization, slow activation kinetics) are similar to those of the previously identified hyperpolarization-activated, inwardly rectifying macroscopic Cl^- conductance, I_{Clh}, identified in dBcAMP-treated rat neocortical astrocytes [19]. Consistently with the slow activation kinetics observed for I_{Clh}, the latency of first openings indicates that also the hyperpolarization-activated single Cl^- channels activated slowly. Moreover, small-conductance channel activity was observed only in the astrocytes from which significant I_{Clh} could be elicited. The result that the activation and deactivation of I_{Clh} were well described by double exponential functions showed that the channel underlying I_{Clh} has several kinetic states [19]. Our data are in agreement with that observation because the dwell-time analyses of the open and closed states of the small-conductance Cl^- channel were best fitted by biexponential functions. On the whole, these results suggest that the single channel studied here underlies the astrocytic I_{Clh} current.

Voltage-gated Cl^- channels are encoded by the large family of ClC genes (for a review, see [27]). The biophysical and pharmacological properties of I_{Clh} strongly resemble those of the ubiquitously distributed hyperpolarization-activated ClC-2 channel, which was heterologously expressed in *Xenopus* oocytes [1]. This supports the hypothesis that the astrocytic inward rectifier Cl^- channel represents ClC-2. In this context, it is worth noting that it has recently been demonstrated that, in situ, ClC-2 is highly expressed at the end-feet of process-bearing hippocampal astrocytes [28]. Also, by noise analysis, heterologously expressed ClC-2 was found to have a single-channel conductance of 3–5 pS [29], a result that further stresses the remarkable homology of ClC-2 with the astrocytic small-conductance Cl^- channel.

A detailed characterization of the single-channel properties of a member of the ClC family has been performed on the *Torpedo* channel ClC-0 [11,14,15,30]. A kinetic analysis revealed that ClC-0 has a double-barrelled behavior, as if it comprised two protochannels that can gate independently of each other and a common gate that regulates both protochannels. Such a behavior has recently been demonstrated also for the muscle ClC-1 protein, although it should be pointed out that another report postulated that ClC-1 has a single pore [17,31]. The fact that the open probabilities of the two conductance states of the Cl^- channel described here were not fitted by a simple binomial distribution for two independently gating channels strongly suggests that the astrocytic ClC-2-like Cl^- channel has also a double-barreled structure.

Thus, these data show that the Cl^- channel that underlies the astrocytic I_{Clh} has a structural motif similar to that of ClC-0 and possibly of ClC-1. This further supports the view that a double-barrelled structure may be a common feature of many of the Cl^- channels belonging to the large family of ClC channel proteins.

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