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Characterization of an Inwardly Rectifying Chloride Conductance Expressed by Cultured Rat Cortical Astrocytes

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ABSTRACT The biophysical and pharmacological properties of the inwardly rectifying Cl^- conductance (I_{Clh}), expressed in rat type-1 neocortical cultured astrocytes upon a long-term treatment (1–3 weeks) with dibutyryl-cyclic-AMP (dBcAMP), were investigated with the whole-cell patch-clamp technique. Using intra- and extra-cellular solutions with symmetrical high Cl^- content and with the monovalent cations replaced with N-methyl-D-glucamine, time- and voltage-dependent Cl^- currents were elicited in response to hyperpolarizing voltage steps from a holding potential of 0 mV. The inward currents activated slowly and did not display any time-dependent inactivation. The rising phase of the current traces was best fitted with two exponential components whose time constants decreased with larger hyperpolarization. The steady-state activation of I_{Clh} was well described by a single Boltzmann function with a half-maximal activation potential at −62 mV and a slope of 19 mV that yields to an apparent gating charge of 1.3. The anion selectivity sequence was $Cl^- = Br^- = I^- > F^-$ > cyclamate ≥ gluconate. External application of the putative Cl^- channel blockers 4,4 diisothiocyanatostilbene-2,2 disulphonic acid or 4-acetamido-4-isothiocyanatostilbene-2,2-disulphonic acid did not affect I_{Clh}. By contrast, anthracene-9-carboxylic acid, as well as Cd^{2+} and Zn^{2+}, inhibited, albeit with different potencies, the Cl^- current. Taken together, these results indicate that dBcAMP-treated cultured rat cortical astrocytes express a Cl^- inward rectifier, which exhibits similar but not identical features compared with those of the cloned and heterologously expressed hyperpolarization-activated Cl^- channel ClC-2. *GLIA 21:217–227, 1997.*

INTRODUCTION

The transport of Cl^- ions through ion channels in both excitable and non-excitable cells underlies important physiological functions such as stabilizing the cell resting membrane potential (Bretag, 1987), controlling the cell volume (Hoffmann and Simonsen, 1989), and regulating the transepithelial transport of salt (Gogelein, 1988). In astrocytes, chloride channels have been proposed to play an important role in the neuronal-astrocytic interaction, particularly in the control of the extracellular K^+ homeostasis (Barres et al., 1990; Barres, 1991). Rat cortical astrocytes conventionally cultured possess Cl^- channels, which however are visible only after removal of some unknown intracellular factor and are activated in response to transmembrane potentials more positive than −40 mV, values that astrocyte cannot reach under physiological condi-

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tions (Gray and Ritchie, 1986; Sonnhof, 1987). Some of these channels were proposed to serve as an emergency Cl^- conductive pathway (Jalonen, 1993) activated during the large swelling-induced astrocyte depolarization (Kimelberg and O'Connor, 1988). Conversely, Lascola and Kraig (1996) have recently shown that experimental manipulations transforming the cultured rat type-1 neocortical astrocytes from a flat polygonal to a process-bearing morphology, led to the expression of a steady ohmic Cl^- current that is likely involved in the process of extracellular K^+ buffering. Furthermore, in our recent study (Ferroni et al., 1995a), carried out on rat type-1 neocortical astrocytes in culture, we identified a slowly activating, non-inactivating inwardly rectifying Cl^- conductance (I_{Clh}) that was already observable, together with a K^+ inward rectifier, around the resting membrane potential. The two conductances were expressed in process-bearing astrocytes following a long-term treatment (1–3 weeks) of the cultures with 250 μM dibutyryl-cyclic-AMP (dBcAMP). They were not evidenced either in control cultures composed of epitheloid flat astrocytes or in process-bearing astrocytes which have been exposed for a short period (4–24 hours) to dBcAMP.

In the vertebrate central nervous system, Cl^- inward rectifiers, which are involved in the stabilization of the membrane potential and in the control of the intracellular Cl^- homeostasis, have been described in hippocampal neurons both in situ and in vitro (Madison et al., 1986; Mager et al., 1990; Staley, 1994). Recently, a Cl^- channel protein called ClC-2 has been cloned from a rat brain cDNA library and functionally expressed in *Xenopus* oocytes (Thiemann et al., 1992). The ClC-2-induced Cl^- current activated slowly upon hyperpolarization and did not display any time-dependent inactivation. These characteristics, together with the fact that in rat brain ClC-2 is expressed exclusively in neurons (Smith et al., 1995), suggest that the channel underlying I_{Clh} is a structurally related but different channel protein and therefore it may have properties distinguishable from those of ClC-2.

In this study, we have characterized the biophysical and pharmacological properties of I_{Clh} by using the whole-cell patch-clamp technique. The data indicate that I_{Clh} shares many pharmacological similarities with ClC-2 but exhibits some different biophysical features. A preliminary account of this work has been presented elsewhere (Ferroni et al., 1995b).

MATERIALS AND METHODS

Homogeneous primary cultures of rat type-1 cortical astrocytes were obtained from 1–2 day-old pups, as previously described (Ferroni et al., 1995a). Briefly, following the mechanical dissociation of the cerebral cortices, astrocytes were plated in culture flasks containing DMEM medium supplemented with 15% of heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The flasks were maintained for 4–6 weeks in an incubator with humidified atmosphere at 37°C and 5% CO_2. Starting 1–3 weeks before electrophysiological measurements, 250 μM dBcAMP was added to the supplemented medium of the culture flasks. This treatment induced shape changes of the astrocytes which acquired a process-bearing phenotype. Three days before the experiments, astrocytes were replated at a density of $1–3 \times 10^4$ onto Petri dishes (35 mm diameter) containing DMEM supplemented medium and 250 μM dBcAMP. All medium components were from Gibco Brl, Life Technologies (Gaithersburg, MD).

For the electrophysiological recordings, the Petri dish was mounted on the stage of an inverted microscope (Nikon Diaphot-TMD) equipped with phase-contrast optics. Plasma-membrane currents were recorded at room temperature (20–25°C) using the whole-cell configuration of the patch clamp technique (Hamill et al., 1981). Patch pipettes were pulled from borosilicate glass capillaries (Clark Electromedical, Pangbourne, England) and after fire polishing (Narishige, Tokyo, Japan), they had a tip resistance of 3–6 MΩ when filled with the internal solution containing (mM): 144 N-methyl-D-glucamine ($NMDG^+$) chloride, 2 $MgCl_2$, 5 N-tris-hydroxymethyl-2-aminoethanesulphonic acid (TES), and 5 EGTA, titrated to pH 7.3 with $NMDG^+$. In experiments performed by using low intracellular Cl^-, the Cl^- content was decreased to 20 mM replaced with equimolar gluconate. The initial bathing solution was composed of (mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 TES, and 5 glucose, titrated to pH 7.3 with NaOH. In the recording control solution, the monovalent cations were replaced with equimolar $NMDG^+$ and pH was adjusted to 7.3 with $NMDG^+$. When the external solution without Ca^{2+} was employed, $CaCl_2$ was replaced with the same amount of $MgCl_2$, also adding 0.5 mM EGTA. The nominally Mg^{2+}-free internal solution was obtained by removing the intracellular Mg^{2+} and adding 1 mM EDTA. The pharmacological agents were dissolved in $NMDG^+$-containing stock solutions prepared 50 times more concentrated than the final amount used in the perfusing solution. All salts and pharmacological agents were of the highest purity grade (Sigma Chemical Co., St. Louis, MO). The osmolality of the different internal and external solutions, measured with a vapor pressure osmometer (Wescor 5500, Delcon s.r.l., Italy), was 330 ± 10 mOsm kg^{-1}. Currents were recorded with a patch-clamp amplifier (Jens Meyer, Munich, Germany) and were low-pass filtered at 0.2–1 kHz (−3 dB) before acquisition. Both the voltage stimulation and data acquisition were performed with a DMA interface (Axon Instruments, Foster City, CA) and a microcomputer equipped with P-Clamp (5.5.1) software (Axon Instruments, Foster City, CA). An agar bridge filled with 150 mM NaCl was used as a reference electrode. When experiments of anion substitution were performed, the reference electrode was an agar bridge filled with 1 M KCl. The junction potentials were below 4 mV and the applied voltages were not corrected for these values. Both the capacitance transient neutraliza-

tion and series resistance compensation (50–80%) were optimized and regularly monitored throughout the experiment. Currents recorded from astrocytes in which the compensated series resistance still introduced an error of more than 5 mV in the command voltages were discarded during the analysis. The different perfusing solutions were locally applied utilizing a gravity-driven, multi-barrelled perfusion system with a flow rate of 30–50 μl min^{-1} that was positioned within 50–100 μm of the recording cell. According to the different experimental protocols the voltage stimulation was performed by applying either families of voltage steps from a holding potential of 0 mV or depolarizing voltage ramps delivered after having activated the conductance with a voltage step to −120 mV for 3 s. In the latter case the current measured was the average of three sweeps. The steady-state activation curve was obtained by the analysis of the tail currents recorded at −50 mV and elicited by stepping back from test potentials ranging from +10 to −130 mV. The normalized currents were fitted with a single Boltzmann isotherm according to the following formalism:

$$G/Gmax = 1/1 + exp\,[(V - V_{1/2})/k]$$

where $G/Gmax$ is the relative conductance, V is the step potential, $V_{1/2}$ is the potential at which the conductance is half-maximally activated, and k is the slope factor that indicates the steepness of the curve and depicts the limiting voltage range inducing an e-fold change in relative conductance. The results were expressed as mean ± standard deviation (SD) and n indicates the number of cells tested under each experimental condition. The statistical analysis was performed with the Student's t test and a P value < 0.05 was taken as a level of significance.

RESULTS

Gating Properties

Whole-cell Cl^- currents were recorded in long-term dBcAMP-treated rat neocortical cultured astrocytes, using intra- and extra-cellular solutions in which K^+ and Na^+ were replaced with equimolar $NMDG^+$ and with symmetrical high Cl^- (n = 191). Under these experimental conditions, the zero-current potential, after the evoked currents had reached their stability, measured −3 ± 2 mV (n = 46). Depolarizing voltage steps up to +50 mV from a holding potential (V_h) of 0 mV elicited negligible outward currents whereas slowly activating, non-inactivating inward currents of increasing amplitude were evoked when hyperpolarizing voltages up to −120 mV were applied (Fig. 1A). The normalized current-voltage relationship (I–V) of the time-dependent current component revealed a steep inwardly rectifying profile (n = 22; Fig. 1B) and a similar current behavior was observed using a slowly depolarizing voltage ramp of 5 s from −120 to +60 mV (see Fig. 5). The instantaneous current jump at the end of each voltage step was larger than that at the beginning of the pulse, indicating that the membrane conductivity increased during the command voltage, and therefore the inward relaxation represented the activation of I_{Clh}. Accordingly, stepping back to −50 mV, inward or outward tail currents denoting, respectively, the deactivation or the activation of the conductance were observed. The correlation between the amplitude of the inward relaxations and the development of the tail currents was confirmed by using an envelope stimulation paradigm showing that the increase in magnitude of the inward relaxation generated by a repetitive pulse of increasing duration to −120 mV was accompanied by the development of a larger deactivating current (n = 7; Fig. 1C).

Usually, under these experimental conditions, I_{Clh} was the only time- and voltage-dependent current activated in dBcAMP-treated astrocytes. However, about 20% of the astrocytes examined displayed, individually or in combination with I_{Clh}, outward-rectifying currents that were clearly visible at potentials above 0 mV and were affected by modifications of the extracellular Cl^- content. This conductance was not investigated in detail but some of its gating properties resemble those of the Cl^- outward rectifier previously observed in cultured rat cortical astrocytes (Gray and Ritchie, 1986).

The amplitude of the inward relaxation measured at −120 mV ranged from 3 to 38 pA/pF, with a mean of 26 ± 5 pA/pF (n = 51). The variability in current density was more pronounced when comparing astrocytes from different cultures rather than astrocytes from the same culture, but it was not always correlated with the time of incubation with dBcAMP. This indicates that the intracellular cAMP elevation is not the only permissive factor necessary to induce the expression of I_{Clh}. Furthermore, though I_{Clh} was usually already visible just after accessing the cell, over the following 10–20 min inward currents increased in magnitude and their activation kinetics became faster, afterwards remaining stable over the residual time of the recordings, which could last up to 45 min. The variable rate for the currents to reach their steady magnitude was not directly correlated to differences in access resistance or cell capacitance but rather to the relative amplitude of the currents few seconds after accessing the cell, with initial larger currents reaching their maximum value more rapidly.

In order to determine the contribution of the intrinsic conduction property of I_{Clh} to the overall current rectification, the I–V plot of the instantaneous currents elicited after having fully activated the conductance was constructed. Astrocytes were clamped at a conditioning voltage of −120 mV for 3 s and then post-stepped to different potentials (n = 5). The amplitude of the instantaneous currents at the end of the conditioning pulse should be due to the Cl^- ions flowing through multiple I_{Clh} channels with a driving force equal to the poststep potential but with a constant single-channel open probability. The I–V curve (Fig. 1D) was also slightly inwardly rectifying and a similar behavior was observed utilizing a fast-depolarizing voltage ramp of 30

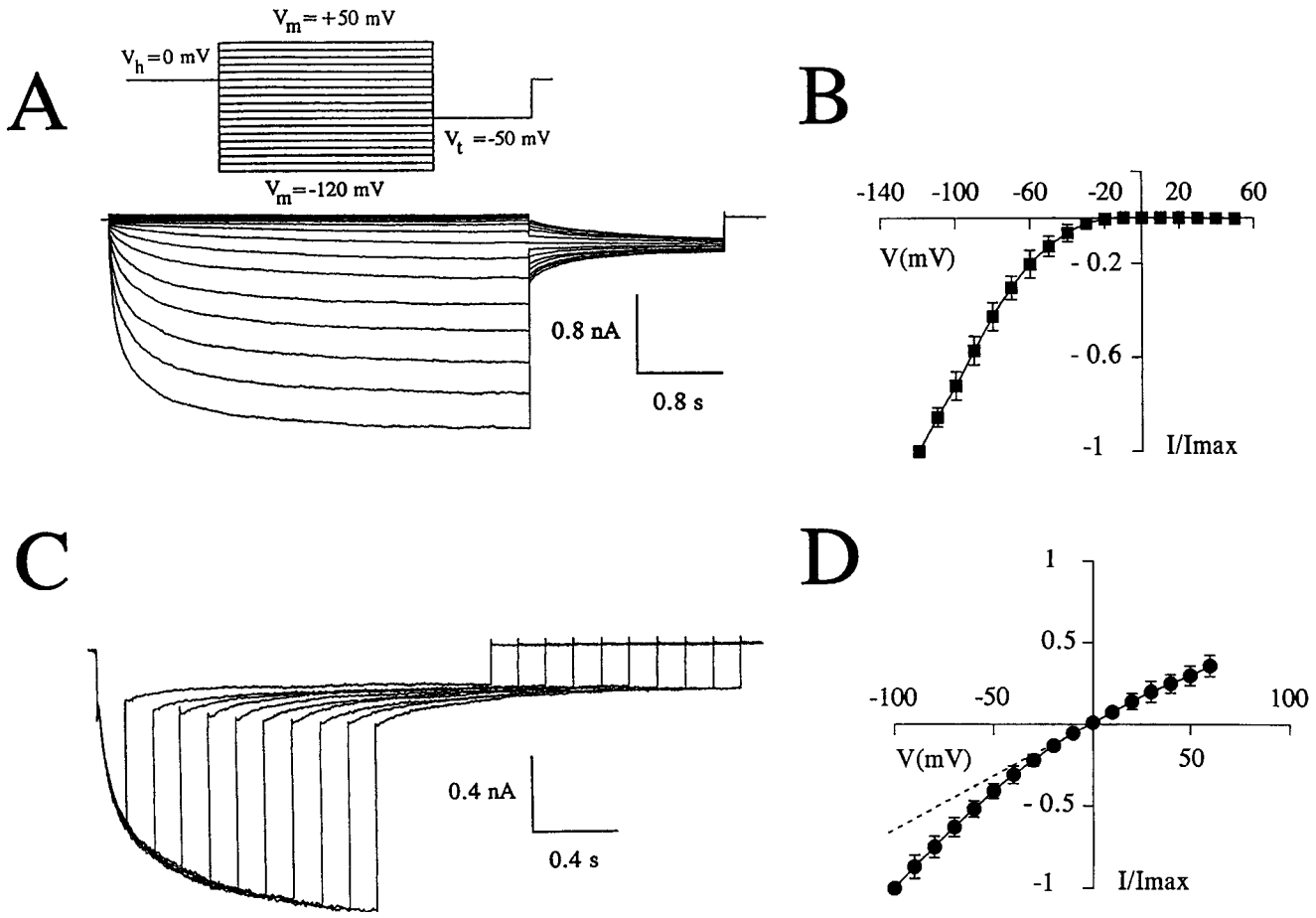

Fig. 1. Voltage sensitivity of the Cl^- conductance. **A**: Representative family of Cl^- currents activated from a holding potential (V_h) of 0 mV in response to a stimulation paradigm (inset) consisting of 4 s voltage jumps (V_m) ranging from +50 to −120 mV in 10 mV steps delivered every 25 s; the returning potential (V_t) was −50 mV. **B**: The I–V plot relative to the time-dependent component of the currents normalized (I/Imax) to the current at −120 mV shows a pronounced inwardly rectifying profile (n = 22) **C**: Representative currents activated from a V_h of 0 mV with an envelope stimulation consisting of repetitive jumps of increasing duration to −120 mV and then stepping back to −50 mV. Note that in relation to the larger inward relaxations elicited by longer steps, a concomitant rise of the outward relaxations was observed. **D**: The I–V plot of the instantaneous currents normalized to the value at −100 mV (n=5) denotes also a slight inwardly rectifying profile, as shown by the deviation from the linear extrapolation (dashed line). Solutions were without K^+ and Na^+, replaced by $NMDG^+$, and with symmetrical high Cl^-. Currents were not leak-subtracted. In B and D, in some cases, the standard deviation bars were smaller than the symbols. Lines in B and D are spline curves through the data points.

ms from −120 to +60 mV which minimized the effect of the current deactivation at depolarized potentials (see Fig. 4). Inward currents were not significantly modified upon removal of extracellular Ca^{2+} indicating that Ca^{2+} is not required for the activation of I_{Clh} (n = 6). An identical result was obtained by replacing the extracellular Mg^{2+} with Ca^{2+} (n = 4). Furthermore, the inwardly rectifying profile of the conductance was unchanged when Mg^{2+} was omitted in the intracellular solution (n = 5), thereby indicating that the rectification is not attributable to a modulating effect of intracellular Mg^{2+}, as it has been described for different kinds of inwardly rectifying K^+ channels (Hille, 1992). Thus, all together these data indicate that the rectifying profile of I_{Clh} is mainly due to its voltage-dependent gating properties.

The activation kinetics of I_{Clh} was voltage-dependent with inward currents reaching their steady-state level more rapidly with larger hyperpolarizations. The time-dependent component of the current traces was best fitted with a double exponential function (Fig. 2A). Both time constants of activation τ_1 and τ_2 were voltage-dependent (Fig. 2B) and decreased with more negative potentials; at −70 mV τ_1 was 174 ± 36 ms and τ_2 = 1,016 ± 130 ms, whereas at −120 mV τ_1 was 66 ± 14 ms and τ_2 = 489 ± 56 ms (n = 16). Moreover, the tail currents recorded at different potentials and elicited following a test potential at −120 mV were also fitted with two exponential components (n = 5; data not shown). The voltage dependence of I_{Clh} was determined by the steady-state activation curve derived from the analysis of the tail currents recorded at −50 mV (Fig. 2C). The normalized currents were plotted against the different test potentials and fitted with a single Boltzmann isotherm (n = 14; Fig. 2D). The Boltzmann parameters indicate that the conductance was half-maximally activated ($V_{1/2}$) at −61.7 mV and the slope factor (k) was 18.8 mV yielding to an apparent gating charge of 1.3.

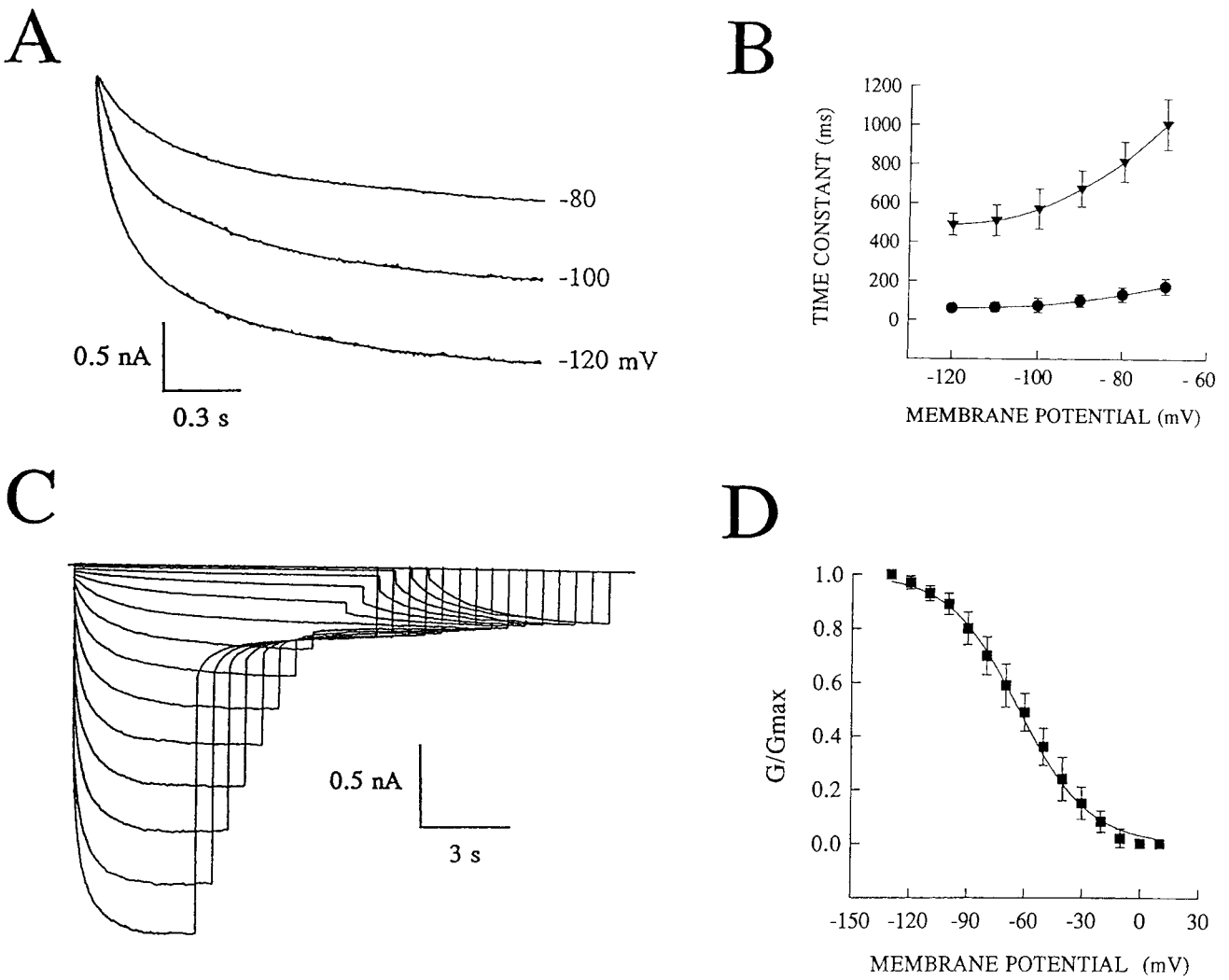

Fig. 2. Gating properties of I_{Clh}. **A**: Activation time course of the inward Cl^- currents. Representative current traces evoked at the indicated membrane potentials from a V_h of 0 mV are shown. For each current only the time-dependent component is displayed and the onset of the inward relaxations has been offset at the same level for all traces. The relaxations were best fitted (least squares residual > 0.99) by using a polynomial function with two exponential terms. **B**: Plots against the different potentials of both the slow (black triangle) and fast (black circle) time constants resulting from the current fits (n = 16). Lines are spline curves through the data points. **C**: Determination of the steady-state activation. Representative family of currents elicited from a V_h of 0 mV in response to conditioning test pulses, delivered every 25 s, ranging from −130 to +10 mV in 10 mV steps and then clamped back to −50 mV. The pulse duration was increased in order to fully activate the conductance. The tail currents measured at −50 mV were recorded as inward and outward current relaxations and were due to the activation and deactivation of I_{Clh}, respectively. **D**: The steady-state activation curve was obtained by the analysis of the normalized tails measured as follows:

$$I_t(V)/I_t(V_{max}) = [I_t(V) - I_t(V_{min})]/[I_t(V_{max}) - I_t(V_{min})]$$

where the tail current (I_t)relative to each test potential (V) is the difference between the current value at the onset of the tail and that at the steady-state; V_{max} and V_{min} are the voltages corresponding to maximum and zero activation of I_{Clh}. The normalized currents, expressing the relative conductance (G/Gmax), were plotted against the different test potentials and fitted with a single Boltzmann isotherm. The best fit depicts the potential at which I_{Clh} was half maximally activated equal to −61.7 mV and a slope factor of 18.8 mV (n = 14). In B and D, in some cases, the standard deviation bars were smaller than the symbols. Solutions as in Figure 1.

Selectivity

When the external Cl^- content ($[Cl^-]_o$) was reduced from 152 to 23 mM, replacing Cl^- with the larger anion gluconate, the reversal potential of the tail currents, obtained by returning from a test potential at −100 mV to different membrane voltages, shifted to a more depolarized value. With 152 mM $[Cl^-]_o$ the potential at which the tail currents reversed was −4 ± 1 mV (n = 8; Fig. 3A), whereas with 23 mM $[Cl^-]_o$ the reversal potential shifted to +30 ± 3 mV (n = 6; Fig. 3B), which does not correspond to the theoretical Nerst potential for a channel selectively permeable to Cl^-. The permeability ratio $P_{gluc} : P_{Cl}$ obtained by applying the Goldman-Hodgkin-Katz equation (Hodgkin, 1951) under the constraint of a pure anion-selective conductance, was 0.13 ± 0.02. The change in reversal potential at several $[Cl^-]_o$ confirmed the partial Cl^- selectivity of the current

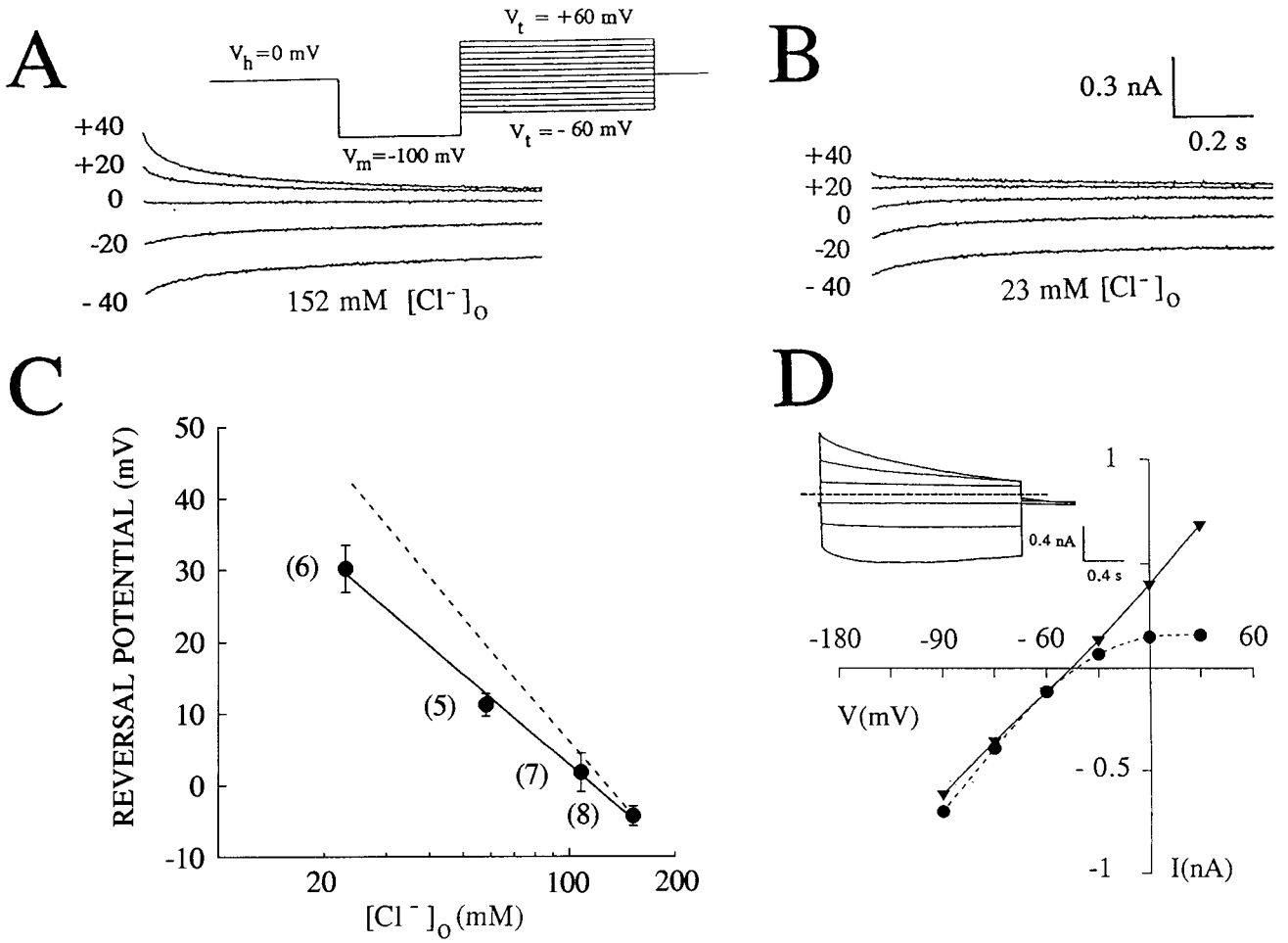

Fig. 3. Chloride dependence of I_{Clh}. **A**: Tail currents were measured at different membrane potentials (V_t) stepping back from a conditioning potential jump (V_m) of 3 s at −100 mV (inset). With 152 mM Cl^- in the extracellular solution ($[Cl^-]_o$), tail currents changed polarity at ~0 mV. **B**: When $[Cl^-]_o$ was lowered to 23 mM, replaced with equimolar gluconate, the reversal potential shifted to ~+30 mV. **C**: Semilogarithmic plot of the tail current reversal potential in different $[Cl^-]_o$ (152, 108, 58, and 23 mM) with Cl^- substituted by gluconate. Next to the data points, the number of cells tested in each condition are shown. The linear regression fit (r = −0.99) depicts a shift of 42 mV in reversal potential per 10-fold change in $[Cl^-]_o$, which deviates from the Nerstian potential for a pure Cl^--selective conductance (dashed line). No significantly different results were obtained by using a fast-depolarizing ramp stimulation (180 mV/30 ms) from −120 to +60 mV. **D**: Representative I–V plots of the instantaneous (black triangle) and the steady-state (black circle) component of a current family (inset) recorded by using low intracellular Cl^- (20 mM) and elicited from a V_h of −60 mV in response to voltage jumps from −120 to +30 mV in 30 mV steps delivered every 25 s. The voltages applied were corrected for the liquid junction potential at the pipette tip. The plot shows the linear behavior of the instantaneous current and the marked current decay at potentials positive to −30 mV. Lines are spline curves through the data points. The dashed line in the inset indicates the zero-current level.

(Fig. 3C). When the intracellular Cl^- content ($[Cl^-]_i$) was decreased to 20 mM, smaller inward and outward currents, in relation to the Cl^- driving force, were evoked from a V_h of −60 mV (n = 6; Fig. 3D, inset). Under these experimental conditions, the instantaneous current had an ohmic behavior, and, for potentials positive to −30 mV, it was larger than the current at the end of the pulses as expected if the current relaxation represented the deactivation of I_{Clh} activated at the V_h held (Fig. 3D). In addition, compared with those obtained with high $[Cl^-]_i$, the inward currents activated more slowly and displayed a less pronounced time-dependent component.

The halide selectivity was investigated by partially replacing $[Cl^-]_o$ with Br^-, F^-, or I^-. The I–V plots of the instantaneous currents, elicited by a fast-depolarizing ramp and obtained by substituting 144 mM Cl^- with an equimolar amount of these anions, revealed a profile of selectivity $Cl^- = Br^- = I^- > F^-$ (n = 6; Fig. 4A). The permeability ratios of Br^-, I^-, and F^- over Cl^- were 0.95 ± 0.04, 1.01 ± 0.02, and 0.34 ± 0.03, respectively. Moreover, while Br^- and F^- did not change the slope conductance of the instantaneous current measured at −120 mV, I^- decreased the overall slope conductance, indicating that it partially blocks the Cl^- channel. The partial equimolar replacement of $[Cl^-]_o$ with the anionic molecules cyclamate and gluconate indicated that these two large anions were less permeable with both anions also decreasing the slope conductance of the instantaneous current (n = 5; Fig. 4B).

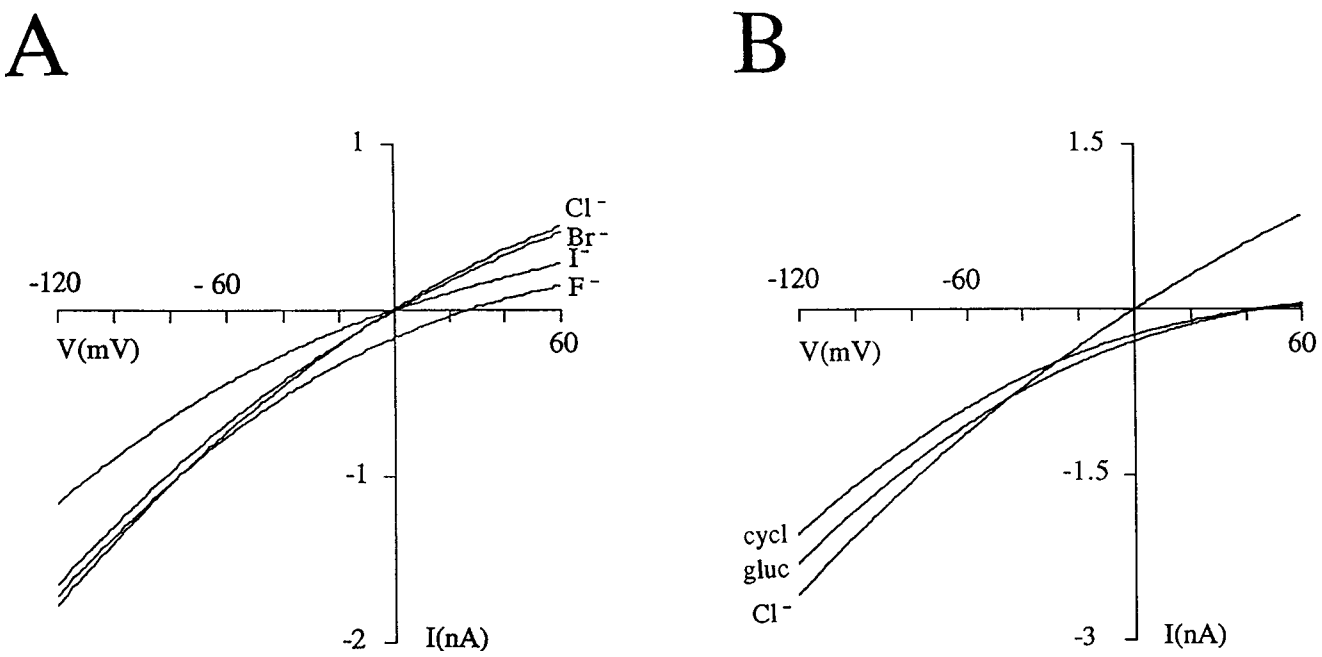

Fig. 4. Anionic selectivity of I_{Clh}. **A**: Representative instantaneous currents activated by a fast-depolarizing voltage ramp (180 mV/30 ms) from −120 to +60 mV and recorded either in symmetrical high Cl^- or partially replacing extracellular Cl^- with bromide (Br^-), iodide (I^-), and fluoride (F^-). **B**: The partial substitution of $[Cl^-]_o$ with cyclamate or gluconate led to the largest shift in reversal potential and decreased the slope conductance of the instantaneous current.

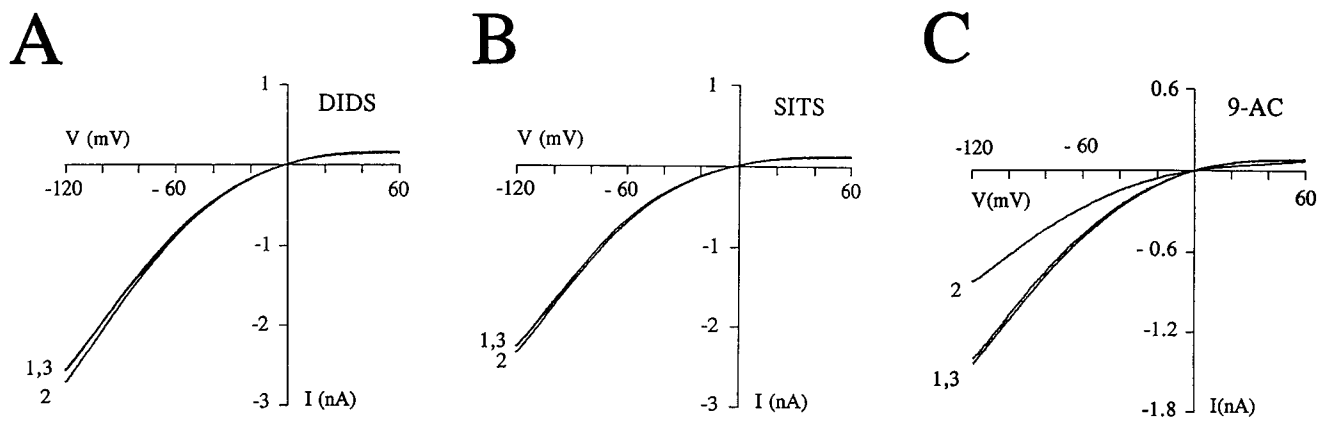

Fig. 5. Pharmacological sensitivity of I_{Clh}. **A,B**: The Cl^- currents elicited in response to a slowly depolarizing (180 mV/5s) ramp in control condition (1) were not modified after external application (2) of 1 mM DIDS (A) or SITS (B) and wash-out (3). **C**: The ramp-evoked current (1) was decreased (2) reversibly (3) by 50% following exposure to 1 mM 9-AC. Solutions as in Figure 1.

Pharmacology

In order to determine the pharmacological sensitivity of I_{Clh}, inward currents were challenged with the putative Cl^- channel blockers 4,4 diisothiocyanatostilbene-2,2-disulphonic acid (DIDS), 4-acetamido-4-isothiocyanatostilbene-2,2-disulphonic acid (SITS), and anthracene-9-carboxylic acid (9-AC) (Palade and Barchi, 1977; Inoue, 1985). Extracellular application of 1 mM DIDS (n = 6) or SITS (n = 7) for 2 min did not affect significantly I_{Clh} (Fig. 5A, B), whereas the same concentration of 9-AC (n = 9) partially and reversibly inhibited the steady-state current activated by the slowly depolarizing voltage ramp (Fig. 5C). Several Cl^- inward rectifiers have been described to be also affected by some divalent but not by monovalent cations (Chesnoy-Marchais, 1983; Selyanko, 1984; Madison et al., 1986; Moorman et al., 1992; Chesnoy-Marchais and Fritsch, 1994; Kowdley et al., 1994; Staley, 1994; Fritsch and Edelman, 1996) and therefore, Ba^{2+}, Cd^{2+}, Zn^{2+}, and Cs^+ were tested on I_{Clh}. Astrocytes were exposed to the different cations for at least 3 min starting 15 s before the first voltage step stimulation. While external application of 2 mM of Ba^{2+} (n = 6) or Cs^+ (n = 5) did not modify I_{Clh} both Cd^{2+} and Zn^{2+} reduced strongly and reversibly the inward currents (Fig. 6). A current

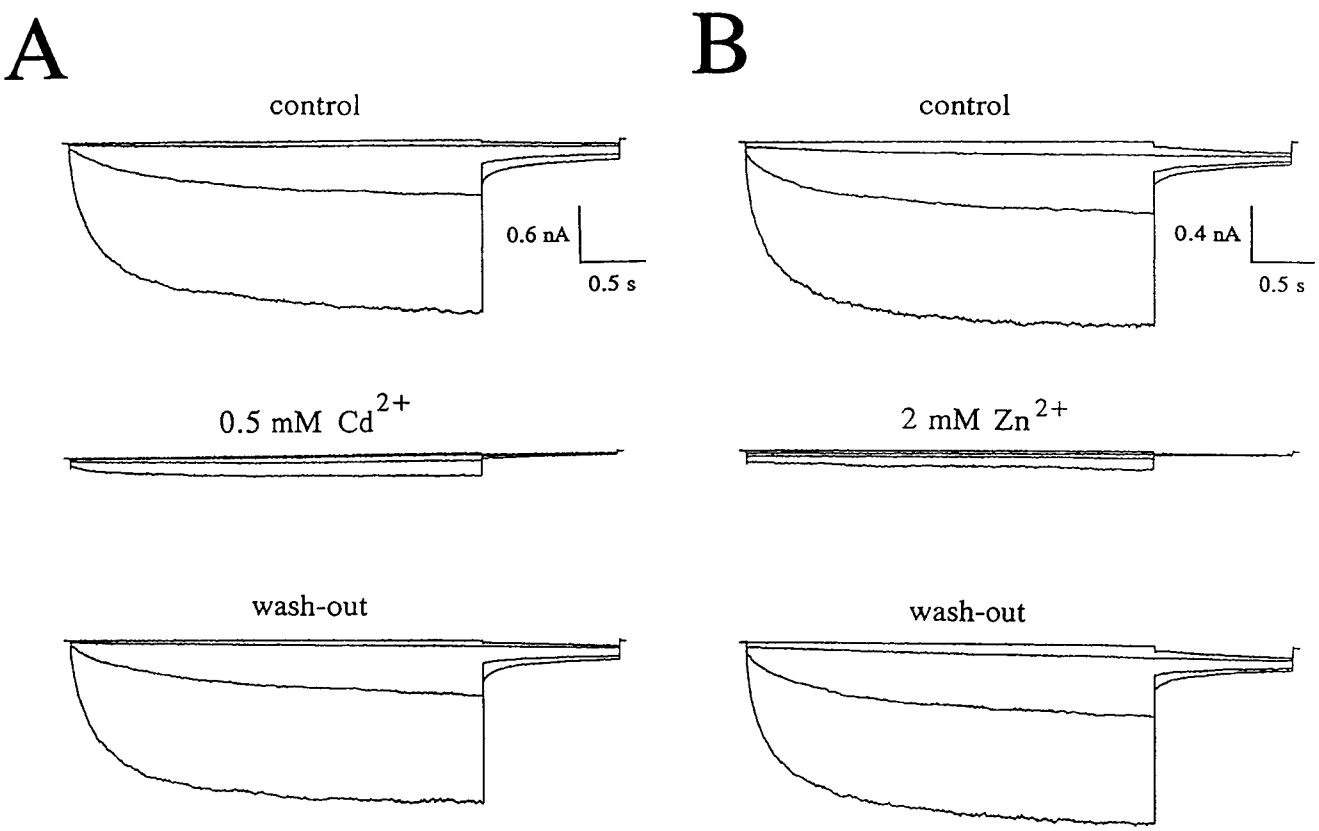

Fig. 6. Blockade of I_{Clh} by divalent cations. **A,B**: Representative families of Cl^- currents activated from a V_h of 0 mV by voltage jumps up to −90 mV in 30 mV steps and then returning to −30 mV (upper panels). The inward Cl^- currents were almost completely inhibited (middle panels) following extracellular application of 0.5 mM Cd^{2+} (A) or 2 mM Zn^{2+} (B) and recovered up to 90% within 2 min after starting wash-out (lower panels). Solutions as in Figure 1.

decrease was already observable in response to the first voltage pulse and the inhibition was maximal within 2 min from the beginning of the stimulation. At −90 mV, the time-dependent component of the inward current activated by a 4 s step was decreased by 89 ± 6% in the presence of 0.5 mM Cd^{2+} (n = 8) and 92 ± 4% by 2 mM Zn^{2+} (n = 9). Interestingly, both cations also diminished the time-independent component of the currents, suggesting either a more complex gating of the channel or the presence of a small time-independent leakage conductance also carried by Cl^- ions and sensitive to Cd^{2+} and Zn^{2+}.

DISCUSSION

In this study, we have characterized both the biophysical and pharmacological properties of I_{Clh} that has previously been described to be expressed, together with a K^+ inward rectifier, by cultured neocortical type-1 astrocytes only following a long-term treatment (1–3 weeks) with dBcAMP, which had converted the astrocytes from a flat epitheloid to a process-bearing phenotype (Ferroni et al., 1995a). Using intra- and extra-cellular solutions with symmetrical high Cl^-, I_{Clh} activates slowly upon hyperpolarization beginning at membrane potentials negative to −10 mV. The I_{Clh} differs clearly from the Cl^- conductance previously described in cultured rat neocortical astrocytes, which were not treated with dBcAMP (Gray and Ritchie, 1986). That conductance was an outward rectifier which activated rapidly at membrane potentials above −40 mV and was strongly blocked by exposure to submillimolar concentrations of DIDS or SITS. Under our experimental conditions, only a small proportion of the astrocytes studied displayed outward currents with gating properties resembling those of the outwardly rectifying conductance. They were mostly observed either in cultures in which the astrocytes, despite the long-term treatment with dBcAMP, did not express large inward Cl^- currents or in old cultures in which astrocytes had a hypertrophic phenotype. An astrocytic Cl^- current resembling the previously described outward rectifier was recently shown to be upregulated by experimental manipulations which transformed the astrocyte morphology from flat polygonal to process-bearing, and by the depolymerization of the actin cytoskeleton (Lascola and Kraig, 1996). Conversely, despite the fact that cell shape changes induced by the conditioning of cultured astrocytes with cAMP analogues are associated with the rapid depolymerization and breakdown of actin network (Goldman and Abram-

son, 1990; Baorto et al., 1992), our previous study, indicating that a short-term treatment with dBcAMP (4–24 h) did not lead to the expression of I_{Clh} (Ferroni et al. 1995a), suggests that these endocellular processes cannot account for its expression in long-term treated astrocytes. It remains to be explained, however, why long-term dBcAMP-treated astrocytes do not express the Cl^- outward rectifier consistently.

The slow rise in cytoplasmic Cl^-, replacing the large, relatively indiffusible, intracellular anions after accessing the cell, can be only partly responsible for the increase in I_{Clh} current magnitude observed over a period of 10–20 min, because the reversal potential of the Cl^- currents matched experimental the Nerst equilibrium potential for Cl^- within 10 min of forming the whole-cell clamp. Therefore, the current upregulation may be attributable either to the slow removal of some inhibitory molecule or to the destabilization of other cytoskeletal structures following the intracellular dialysis.

Comparison of I_{Clh} With Inwardly Rectifying Cl^- Currents in Other Preparations

Inwardly rectifying Cl^- conductances, which slowly activate in the negative range of membrane potentials, have been identified in neurons from Aplysia (Chesnoy-Marchais, 1983; Lotshaw and Levitan, 1987), rat hippocampus (Madison et al., 1986; Mager et al., 1990; Staley, 1994), and also in many cell types of other tissues (Parker and Miledi, 1988; Block and Moody, 1990; Moorman et al., 1992; Noulin and Joffre, 1993; Komwatana et al., 1994; Kowdley et al., 1994; Chesnoy-Marchais and Fritsch, 1994; Fritsch and Edelman, 1996; Arreola et al., 1996). In some cases, their activation has been reported to be strongly dependent on the intracellular Cl^- concentration (Chesnoy-Marchais, 1983; Dinudom et al., 1993; Fritsch and Edelman, 1996). Our study suggests that this might also occur for I_{Clh} because significantly smaller inward currents, compared with those measured with high $[Cl^-]_i$, were recorded when the Cl^- in the pipette was decreased to 20 mM and the currents then normalized with respect to the Cl^- driving force; in addition, lowering $[Cl^-]_i$ to 8 mM did not lead to the generation of any Cl^- current (unpublished results). While the activation as well as the deactivation kinetics of I_{Clh} are best fitted with a double exponential function whose time constants are both voltage-dependent, in Aplysia neurons the activation of the Cl^- inward rectifier was best described by a single exponential (Chesnoy-Marchais, 1983; Lotshaw and Levitan, 1987) and no data are available for the inward Cl^- current from hippocampal neurons. The multiexponential kinetics of both the activation and deactivation indicate that the channel underlying I_{Clh} could have multiple open or closed states, or that two different conductances are activated by the voltage steps. However, the result that the steady-state activation curve can be well fitted with a single Boltzmann function makes the latter hypothesis unlikely, unless it is postulated that the two conductances have overlapping voltage dependency and slopes. The slope of the steady-state activation curve ($k = 18.8$ mV) depicts a low value of the apparent gating charge (1.3), thereby indicating a weak voltage dependence of I_{Clh} similarly to other Cl^- inward rectifiers in different preparations (Kowdley et al., 1994; Staley, 1994). A peculiar feature of I_{Clh} is that the instantaneous current is also slightly inwardly rectifying, indicating that the single-channel conduction might contribute to the overall rectification. This kind of behavior was reported in a Cl^- single-channel study carried out on cultured hippocampal neurons (Mager at al., 1995) and also for Cl^- inward rectifiers in other non-excitable cells (Komwatana et al., 1994; Fritsch and Edelman, 1996).

The selectivity profile of I_{Clh} is comparable to that of some anion-selective, inwardly rectifying currents observed in different cell types (Dinudom et al., 1993; Arreola et al., 1996). Despite the fact that I_{Clh} exhibits an identical permeability to Cl^-, Br^-, and I^-, the channel activity was partially blocked by the latter ion. This result is not surprising since a similar behavior was described for some Cl^- channels expressed in different tissues although they showed a higher permeability to I^- than to Cl^- (Tabcharani et al., 1992; Guinamard et al., 1995). In addition, in a recent study on a hyperpolarization-activated Cl^- channel it was reported that the instantaneous current decreased upon substitution of $[Cl^-]_o$ with I^- (Fritsch and Edelman, 1996).

For its insensitivity to extracellular applications of DIDS or SITS I_{Clh} resembles some Cl^- inward rectifiers (Chesnoys-Marchais 1983; Lotshaw and Levitan, 1987; Komwatana et al., 1994; Kowdley et al., 1994) but differs from others (Noulin and Joffre, 1993; Chesnoy-Marchais and Fritsch, 1994). The current depression by 9-AC was not unexpected because aromatic carboxylic acids have been described to affect other inward Cl^- conductances (Thiemann et al., 1992; Chesnoy-Marchais and Fritsch, 1994; Fritsch and Edelman, 1996). The inhibition of I_{Clh} by Cd^{2+} and Zn^{2+} is similar to that observed for some Cl^- inward rectifiers (Selyanko, 1984; Madison et al., 1986; Noulin and Joffre, 1993; Staley, 1994; Fritsch and Edelman, 1996). However, whereas in pyramidal neurons of hippocampal slice the binding site for Zn^{2+} has been proposed to be located intracellularly because of the slow development of the inhibition (Staley, 1994), in astrocytes the fast onsets of both the block and recovery (within 15 s) make this possibility unlikely. The inhibition of I_{Clh} by Cd^{2+} cannot be related to a dependence of the current activation on the intracellular Ca^{2+} elevation through Cd^{2+}-sensitive, voltage-gated Ca^{2+} channels (Barres et al., 1989) because inward currents were not modified upon removal of extracellular Ca^{2+}. The sensitivity of I_{Clh} to Zn^{2+} is particularly notable in light of the neuromodulatory action of this ion (Xie and Smart, 1991). There is evidence that, as a consequence of a sustained neuronal activity, Zn^{2+} is released at a synaptic level at submillimolar concentrations (Assaf and Chung 1984; Howell

et al., 1984), which, as we observed, affect significantly I_{Clh} (unpublished results).

Several types of Cl^- channel proteins identified in the ClC family have been cloned and functionally expressed (for a review, see Pusch and Jentsch, 1994). The overall properties of I_{Clh} are similar but not identical with those of the ubiquitously distributed ClC-2 channel which was cloned from a rat brain cDNA library and expressed in *Xenopus* oocytes (Thiemann et al., 1992). I_{Clh} is equally permeable to Cl^-, Br^-, and I^-, whereas ClC-2 conducts better Cl^- than Br^- and I^-. Kinetically, the most striking difference is that the ClC-2-mediated currents activate more slowly upon hyperpolarization starting from membrane potentials negative to −90 mV and the relative conductance does not saturate. Interestingly, an in situ hybridization study has shown that in rat brain ClC-2 channel is expressed exclusively in neurons (Smith et al., 1995), thereby strengthening the possibility that the channel protein carrying the inward Cl^- current in astrocyte is a different one. It remains to be clarified if the channel protein underlying I_{Clh} is encoded by a different gene from that of ClC-2, or if it is a splice variant of the same gene, as can be envisaged from the fact that the Northern blot analysis of rat brain revealed the presence of various messenger RNAs positive for the ClC-2 probe (Thiemann et al., 1992). It should be pointed out, however, that it cannot be *a priori* excluded that in cultured cortical astrocytes the chronic elevation of the intracellular cAMP induces the expression of ClC-2-mediated Cl^- currents and that the different biophysical properties identified are solely due to the different cellular environment.

Possible Functional Significance of I_{Clh}

Evidence exists that, in cultured cortical astrocytes, the value of the Cl^- equilibrium potential, according to the reversal potential of the GABA-activated Cl^- currents, is 30–40 mV above their resting membrane potential (Kettenmann et al., 1984; Kettenmann and Schachner, 1985; Kettenmann et al., 1987), thereby indicating that in these astrocytes the resting Cl^- permeability is not significant, if any. Recently, in our and other laboratories it was demonstrated that indeed, flat, polygonally shaped neocortical type-1 astrocytes conventionally cultured, do not express any whole-cell Cl^- current (Ferroni et al., 1995a; Lascola and Kraig, 1996). When the same cultures are exposed to specific experimental manipulations (e.g., warm serum-free incubation medium, cytoskeletal alterations, and dBcAMP treatment), astrocytes undergo shape changes with development of cytoplasmic processes. The change in cell morphology is accompanied by the expression of I_{Clh} (Ferroni et al., 1995a) and/or of the ohmic conductance (Lascola and Kraig, 1996) both activated in a physiological range of membrane potentials but which, because of their properties, might have different functional roles. The steady ohmic conductance was proposed to be the pathway of the passive Cl^- entry that follows as countercharge the K^+ influx during the astrocyte-mediated process of extracellular K^+ buffering, which occurs according to the postulated mechanism of local accumulation (Gardner-Medwin, 1980). Owing to its characteristics, I_{Clh} may be implicated in cell volume regulation (Hoffmann and Simonsen, 1989). In this respect, a Cl^- efflux has been indicated, together with a parallel outward flux of K^+, to be partially responsible for the regulatory volume decrease (RVD), which follows the astrocyte swelling caused by exposure to hypotonic medium (Kimelberg and Frangakis, 1986). However against this view, it was recently found that in cultured astrocytes, RVD can be inhibited by DIDS but not by 9-AC, suggesting that a pharmacologically different conductive pathway underlies this cellular reaction (Pasantes-Morales et al., 1994). In this context the functional role of I_{Clh} remains elusive and therefore specifically focused experiments utilizing non-invasive techniques are necessary to clarify this issue.

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