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Osmosensitivity of an inwardly rectifying chloride current revealed by whole-cell and perforated-patch recordings in cultured rat cortical astrocytes

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Abstract The osmosensitivity of the inwardly rectifying Cl^- current (I_{Clh}), expressed by primary cultured rat neocortical astrocytes long-term treated with dibutyryl cyclic AMP, was investigated in the whole-cell and perforated-patch modes. In whole-cell experiments, whereas hypotonic extracellular solution ($\Delta = 100$ mOsmol) did not cause any change in I_{Clh}, hypertonicity produced a slowly developing, ~40% reversible decrease in current magnitude. By contrast, in perforated-patch experiments, exposure to a less hypertonic saline ($\Delta = 50$ mOsmol) depressed the current to ~50%, and hypotonicity induced a ~50% slow increase in I_{Clh}. These differences in osmosensitivity between the two experimental modes suggest that the osmoregulation of I_{Clh} may be mediated by complex intracellular mechanism(s), which appear(s) to be partly compromised by the dialysis of the astrocytic cytoplasm.

Key words: Astroglia; ClC-2 channel; Perforated patch; Osmoregulation; Regulatory volume decrease

1. Introduction

Astrocytic swelling is an important response to neuronal activity and occurs during normal and pathological states affecting the central nervous system, including ischemia, trauma, hypoxia, and status epilepticus (for a review, see [17]). Osmotic swelling in astrocytes qualitatively affects astrocyte properties themselves. Primary cultured astrocytes exposed to a bath hypotonic medium undergo a rapid initial cell volume increase, which is followed by restoration of cell volume towards normal values in the continued presence of the hypotonic saline, a process termed regulatory volume decrease (RVD) [16]. RVD is achieved primarily by the electrogenic efflux of cellular potassium and chloride (and osmotically driven water) through distinct conductance paths [1,21,22,24]. The molecular basis for regulatory volume decrease is still unknown. Although several Cl^- conductances have been demonstrated to be osmosensitive in various cell types, it remains to be clearly established which of these conductances really contribute to RVD. A further complication lies in the fact that different volume-sensitive Cl^- channels can be expressed in the same cell type [2,24]. To date, two different Cl^- conductances have been reported to respond to osmotic stress in cortical astrocytes. Lascola and Kraig [19] analyzed an outwardly rectifying Cl^- current activated by modification of the actin cytoskeleton and osmotic stress in neocortical astrocytes. Jalonen [13] reported an increase in the occurrence of a large-conductance anion channel in excised patches exposed to hypo-osmotic solution.

Recently, a hyperpolarization-activated, inwardly rectifying Cl^- current (I_{Clh}) has been characterized in primary cultured rat neocortical astrocytes long-term treated with dibutyryl cyclic AMP (dbcAMP) [7]. The treatment with dbcAMP transforms the astrocytes from a flat polygonal to a process-bearing morphological phenotype, and promotes the expression of the I_{Clh} conductance [6]. The properties of I_{Clh} resemble those of the heterologously expressed ClC-2 channel, which has been postulated to be involved in RVD [10,14,27]. Several ClC-2 and ClC-2-like channels regulated by cell swelling have been identified in various non-neuronal cell types, including human intestinal T84 cells [2,9], rat osteoblastic cells [3], mouse mandibular duct cells [18], ascidian embryos [26], and rodent cardiac myocytes [5]. Interestingly, in T84 cells, where a swelling-activated outwardly rectifying Cl^- current is also present, the ClC-2 conductance is not involved in RVD [2]. Furthermore, whereas it has been generally ascertained that hypotonic cell swelling positively regulates the hyperpolarization-activated Cl^- currents and that hypertonicity decreases it, in osteoblasts and mandibular duct cells it was shown that hypotonic and hypertonic challenges produced effects on Cl^- current amplitudes that were just reversed [3,18]. Because of the reported differences in osmosensitivity of the hyperpolarization-activated, inwardly rectifying Cl^- currents, and considering the importance of further clarifying the role of I_{Clh} in astroglial cells, we investigated the responses of this current to changes in osmolarity using the whole-cell patch-clamp and perforated-patch recording techniques. We found that the astrocytic Cl^- inward rectifier is osmoregulated, but that differences in osmosensitivity are evidenced by using the two recording modes.

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Abbreviations: dbcAMP, dibutyryl cyclic AMP; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; $NMDG^+$, *N*-methyl-D-glucamine; RVD, regulatory volume decrease; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid

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2. Materials and methods

2.1. Cell cultures

Primary cultures of rat cortical astrocytes were obtained as previously described [7,20]. Briefly, 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 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 a humidified atmosphere at 37°C and 5% CO_2 for 1–2 weeks. At confluence, the culturing was continued in the presence of dbcAMP (250 μM) for 2–3 further weeks to induce the morphological differentiation of the type 1 cortical astrocytes and the expression of specific ion channels [6].

2.2. Electrophysiological recordings

The electrophysiological studies were performed at room temperature (20–23°C) using the whole-cell configuration of the patch-clamp technique [11]. Patch pipettes were pulled from borosilicate glass capillaries (Clark Electromedical, UK) and heat-polished to obtain a resistance of 2–4 MΩ when filled with the aforesaid solutions. The external standard solution was composed of (mM): 140 *N*-methyl-D-glucamine (NMDG)–Cl, 2 $CaCl_2$, 2 $MgCl_2$, 10 *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid (TES), 5 glucose, adjusted with $NMDG^+$ to pH 7.3. The standard pipette solution contained (mM): 140 NMDG–Cl, 2 $MgCl_2$, 5 TES, 5 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 5 glucose, adjusted with $NMDG^+$ to pH 7.3. Osmolarity was set at 316 ± 3 mOsmol with mannitol. Osmolarity was measured with a vapor pressure osmometer (Wescor 5500, Delcon Instruments s.r.l.). For experiments in which the effects of the various osmotic solutions were studied, the isotonic (316 ± 4 mOsmol) bath solution contained (mM): 100 NMDG–Cl, 2 $CaCl_2$, 2 $MgCl_2$, 10 TES, 5 glucose, 100 mannitol, pH 7.3 with $NMDG^+$. The pipette internal saline was (mM): 100 NMDG–Cl, 2 $MgCl_2$, 10 TES, 5 EGTA, 5 glucose, 100 mannitol, pH 7.3. Hypotonic bath solution (216 ± 5 mOsmol) was made by removing mannitol from the isotonic solution, while maintaining equal values of ionic strength and pH. Hypertonic bath solution (416 ± 3 mOsmol) was made by adding 100 mM mannitol to the isotonic saline. In perforated-patch experiments, the hypotonic and hypertonic bath solutions were 266 ± 3 and 366 ± 4 mOsmol, respectively. The perforated-patch configuration [12] was obtained by supplementing the pipette internal solution with the polyene antibiotic nystatin. Every day of the experiments, nystatin was freshly dissolved in 50 mg/ml dimethyl sulfoxide (DMSO) and diluted in the pipette solution to a final concentration of 150 or 200 μg/ml. To allow the formation of the giga-seal, the pipette tip was filled with nystatin-free solution, whereas the pipette bulk was backfilled with the nystatin-containing solution. In the control experiments, DMSO alone in the pipette did not cause any modification of the capacitative current transients, thus indicating that the solvent played no role in membrane perforation. Brief steps in holding potential were used to monitor the gradual decrease in series resistance that followed seal formation with the nystatin-containing pipette. A stable value of series resistance of 20–30 MΩ was usually achieved ~20 min after seal formation. In some experiments, the rupture of the perforated patch to establish the whole-cell mode was used to assess the recording configuration. For the preparation of the astrocytic cultures, all the materials used were from Gibco BRL (Life Technologies, Italy). The chemicals of the salt solutions for the electrophysiological measurements were obtained from Sigma (Italy). Membrane currents were recorded using an L/M EPC7 amplifier (List Electronic, Darmstadt, Germany), and were low-pass filtered at 0.3–1 kHz before acquisition. Capacitative current transients were optimized electronically. To reduce voltage errors, a >70% analog compensation for the series resistance was used. Both voltage stimulation and data acquisition were obtained using a 12-bit interface (Axon Instruments) and a microcomputer equipped with pClamp (5.5.1) software (Axon Instruments). Voltage stimulation was performed either by applying families of voltage steps from a holding potential of 0 mV or by depolarizing voltage ramps delivered after the activation of the Cl^- conductance with a voltage step to −120 mV for 3 s. Data are expressed as the mean ± S.D., and the statistical analysis was made according to Student's *t*-test, with $P < 0.05$ taken as the level of significance.

3. Results

3.1. Effect of osmotic stress on astrocytic inwardly rectifying Cl^- current recorded using the tight-seal whole-cell recording configuration

Hyperpolarization-activated, inwardly rectifying Cl^- currents (I_{Clh}) were recorded from dbcAMP-treated, rat neocortical type 1 astrocytes in primary culture by applying the whole-cell configuration of the patch-clamp technique ($n = 62$). By using intra- and extracellular solutions with symmetrical high Cl^- and monovalent cations replaced by $NMDG^+$, voltage steps ranging between +20 and −120 mV from a holding potential (V_h) of 0 mV evoked slowly activating, non-inactivating inward currents of increasing amplitude (Fig. 1A). The current–voltage relationship revealed a pronounced inwardly rectifying profile (Fig. 1B). At the beginning of the experiments, after breaking the patch to establish the whole-cell clamp, I_{Clh} increased and reached the stationary amplitude after 17 ± 3 min ($n = 45$). Fig. 2A shows the time

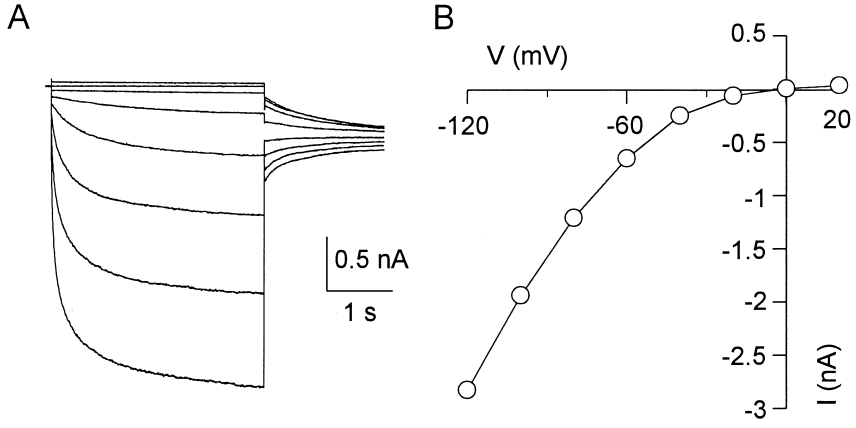

Fig. 1. Whole-cell inwardly rectifying Cl^- current (I_{Clh}) in dbcAMP-treated cultured neocortical astrocytes. A: Representative family of current traces evoked 30 min after accessing the cell. Inward currents were activated from a holding potential (V_h) of 0 mV by 3.5 s voltage jumps from −120 to +20 mV in 20 mV increments and followed by a 2 s step to −50 mV. Pulse interval was 25 s. B: The current–voltage relationship, concerning the current magnitude at the end of the hyperpolarizing voltage pulses, shows a pronounced inwardly rectifying profile. Bath and pipette solutions were symmetrical high 140 mM NMDG–Cl saline.

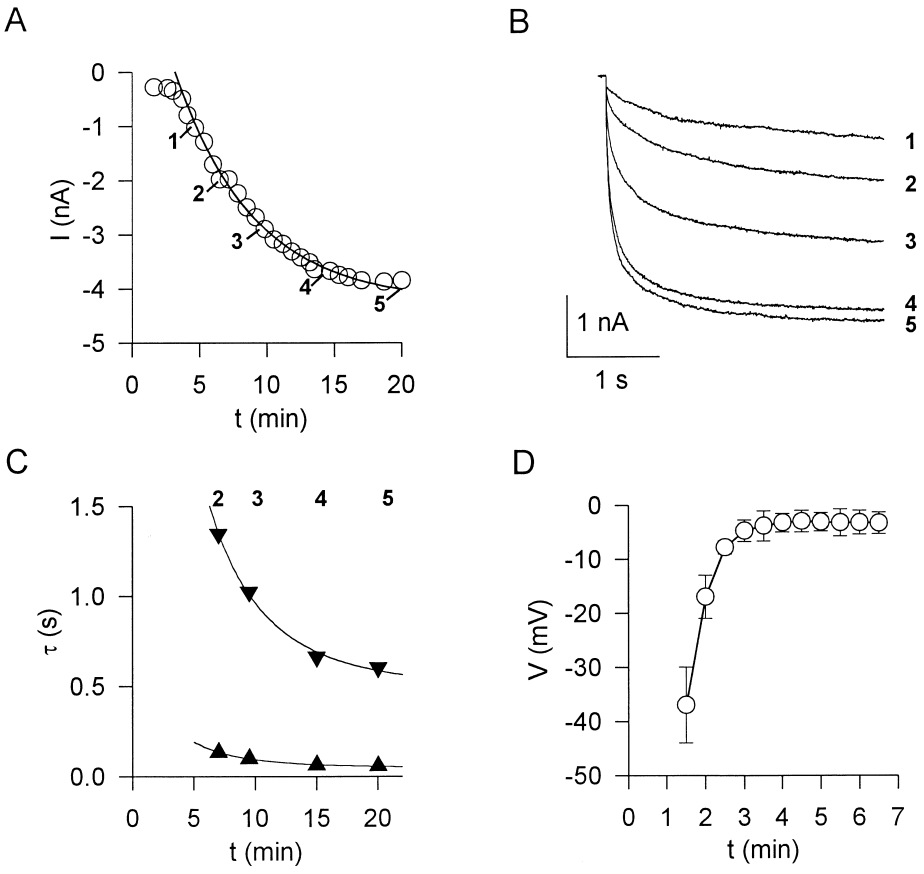

Fig. 2. Characterization of the increase in magnitude of whole-cell I_{Clh} during the recording. A: Representative time course of I_{Clh} currents evoked in response to a repetitive hyperpolarizing voltage step to −120 mV from V_h 0 mV delivered every 30 s. Time zero represents the point at which the astrocyte was accessed to establish the whole-cell configuration. The stationary amplitude was reached ~18 min after establishing the whole-cell clamp. The current increase (run-up) was fitted by an exponential function with a time constant of 6.12 min. B: Current traces at −120 mV recorded at the time points indicated in A. C: Plots versus time of both the slow (▲) and fast (▼) time constants resulting from the fits of the current traces (2–5) in B. Lines are spline curves through the data points. D: Time course of the changes in I_{Clh} reversal potential in symmetrical high Cl^- (bath vs. pipette) showing that the reversal potential reached the stationary level (~0 mV) within 4 min of accessing the cell. Solutions as in Fig. 1.

course of the current development evoked by repetitive hyperpolarizing voltage steps to −120 mV from a V_h of 0 mV. The time course of the current increase (run-up), starting 3 min after accessing the cell, was fitted by a single exponential function with a time constant of 6.12 min (6.06 ± 2.46 min; $n = 7$). The activation kinetics of I_{Clh} was also dependent on the duration of the recordings; the time-dependent components of I_{Clh} at −120 mV, measured at different time points, were best fitted by a double exponential function whose time constants became smaller as the recording duration increased (Fig. 2B,C). Because I_{Clh} gating is dependent on the intracellular Cl^- content [7], we had to rule out that the observed increase in current magnitude was due to the intracellular Cl^- loading during the recording. The time-dependent changes in intracellular Cl^- were examined by measuring the reversal potential of the instantaneous I_{Clh} at different time points by applying a depolarizing ramp from −120 to +60 mV (180 mV/100 ms), after activating the current with a 3 s pulse to −120 mV. In symmetrical high Cl^- (bath vs. pipette), the zero current potential shifted from the initial value of −37 ± 7 mV to a stationary value of −3.3 ± 2.1 mV ($n = 5$) within 4 min of accessing the cell (Fig. 2D). This suggests that the current development during the first 20 min of the recording was not due to the increase in cytoplasmic Cl^- but rather to the washout of some inhibitory factor, which was removed during the intracellular dialysis.

To test the osmosensitivities of whole-cell I_{Clh} currents at the stationary level, slow-ramp currents (180 mV/4 s), under control isotonic conditions with symmetrical 100 mM NMDG–Cl saline (316 ± 3 mOsmol), were compared with those obtained after 10 min application of a hypotonic saline (216 ± 5 mOsmol) and upon washout. Under these experimental conditions, there were no differences in current amplitude or in activation kinetics (Fig. 3A, inset), and also the inwardly rectifying shape of the current was unmodified ($P > 0.05$; $n = 18$). However, in four astrocytes that, in control conditions, expressed I_{Clh} as the only time- and voltage-dependent current, application of the hypotonic solution induced the activation of an ohmic Cl^- current that had characteristics similar to those of the osmosensitive Cl^- current previously identified in cultured astrocytes [19]. This current was clearly distinguishable at potentials above 0 mV at which I_{Clh} was negligible, and was abolished upon restoring the isotonic solution (data not shown). When astrocytes were exposed to a hypertonic bath solution (416 ± 3 mOsmol), I_{Clh} was depressed by 40 ± 15% ($P < 0.01$; $n = 11$) to a new steady-state level within 6–8 min (Fig. 3B). The decrease in current magnitude was not paralleled by evident changes in activation kinetics

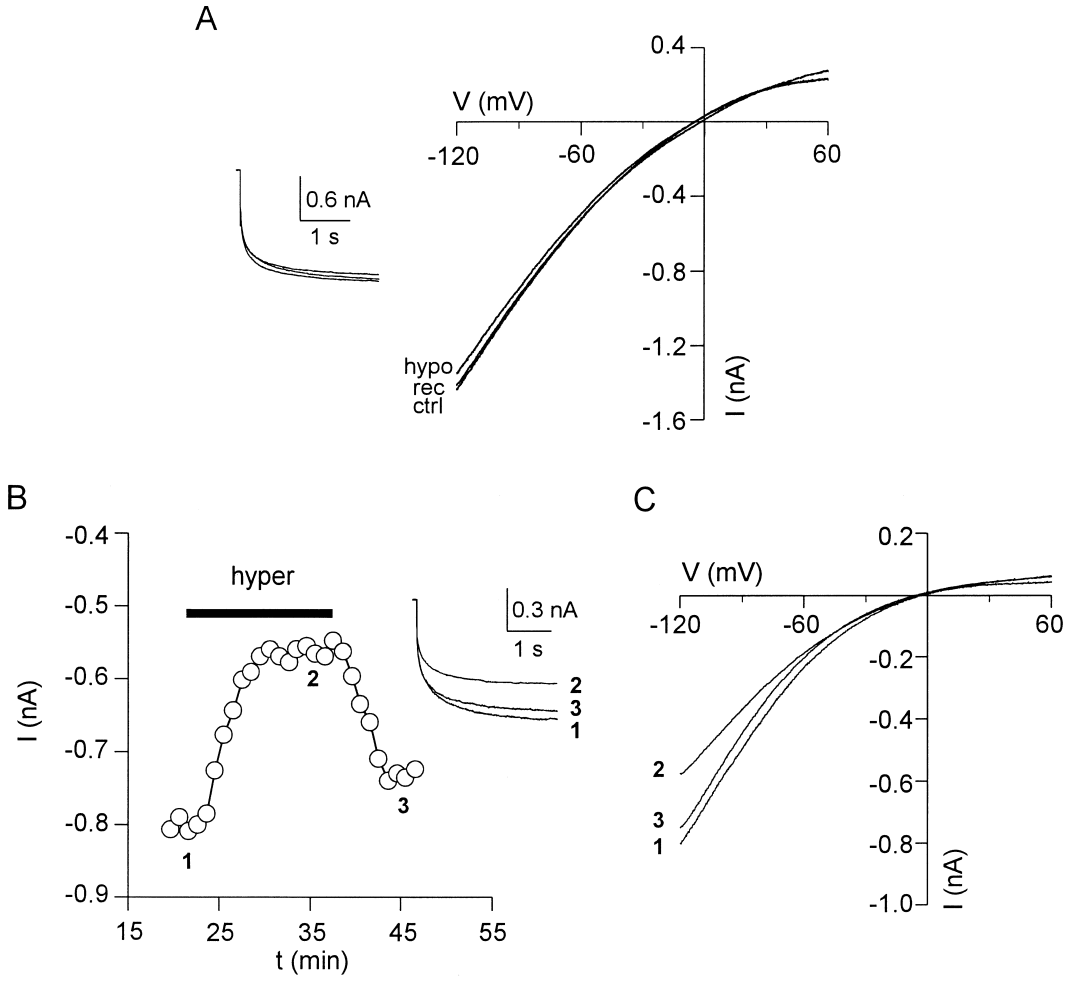

Fig. 3. Effects of changes in extracellular osmolarity on I_{Clh} recorded in the conventional whole-cell mode. A: The Cl^- currents were recorded at the stationary level 23 min after establishing the whole-cell configuration and were elicited in response to a slowly depolarizing (180 mV/4 s) ramp following the activation of I_{Clh} with a 3 s pulse to −120 mV from V_h 0 mV (inset). The amplitude and the profile of the inwardly rectifying current in control condition (ctrl) were not altered after 10 min application of hypotonic solution (hypo) and return to isotonic external solution (rec). B: Amplitudes of representative inward Cl^- currents at −120 mV after the phase of run-up and exposure to an extracellular hypertonic saline (bar) in another astrocyte. Inset: Current traces obtained by repetitive voltage jumps to −120 mV and recorded at the time indicated in the plot. Note that, whereas the onsets of the current changes were rapid, the time courses to reach the maximal effect and recovery were relatively slow. C: I_{Clh} currents activated at the time indicated in the plot. The inwardly rectifying ramp current under isotonic condition (1) decreased in the negative range of membrane potentials following the hypertonic challenge (2) and recovered fully on returning to isotonic saline (3). Bath and pipette solutions were symmetrical 100 mM NMDG–Cl saline.

(Fig. 3B, inset) and was fully reversible with a time course comparable to that of the current reduction. Moreover, the inwardly rectifying profile of the ramp-evoked currents was not significantly altered by the hyperosmotic change (Fig. 3C).

3.2. *Effect of osmotic stress on astrocytic Cl^- inwardly rectifying current recorded using nystatin-perforated patches*

We next investigated whether the partial osmosensitivity observed in conventional whole-cell experiments could be dependent on the recording configuration by using the nystatin-perforated-patch method. Under this experimental condition and in symmetrical high Cl^-, smaller I_{Clh} currents than those obtained in the whole-cell mode could be recorded ($n = 12$). Both the voltage dependence and the activation kinetics of the hyperpolarization-activated, inwardly rectifying Cl^- currents were similar to those related to whole-cell recordings (data not shown). However, in contrast to whole-cell experiments, nystatin-perforated astrocytes responded with an increase in current amplitude upon the application of a hypotonic external solution (Fig. 4A, inset). In control isotonic saline (100 mM NMDG–Cl), the current evoked by a test pulse to −120 mV increased because of the progress in perforation (decrease in access resistance) and the intracellular dialysis with Cl^- ions, and reached the steady-state level within an additional 12 min after an apparently stable perforation (Fig. 4A). At the steady-state level, when the bath osmolarity was reduced (266 ± 3 mOsmol; $\Delta = 50$ mOsmol), the current amplitudes increased by 50 ± 20% ($P < 0.01$; $n = 5$), but with a relatively slow time course. The I_{Clh} increases were promptly reversible upon returning to the isotonic saline. The analysis of the ramp-evoked currents reveals that the hypotonic challenge did not affect the inwardly rectifying behavior of the Cl^- current, thus suggesting that the hypo-osmotic challenge regulated I_{Clh} specifically (Fig. 4B). When astrocytes were exposed to a hypertonic bath solution (366 ± 3 mOsmol, $\Delta = 50$ mOs-

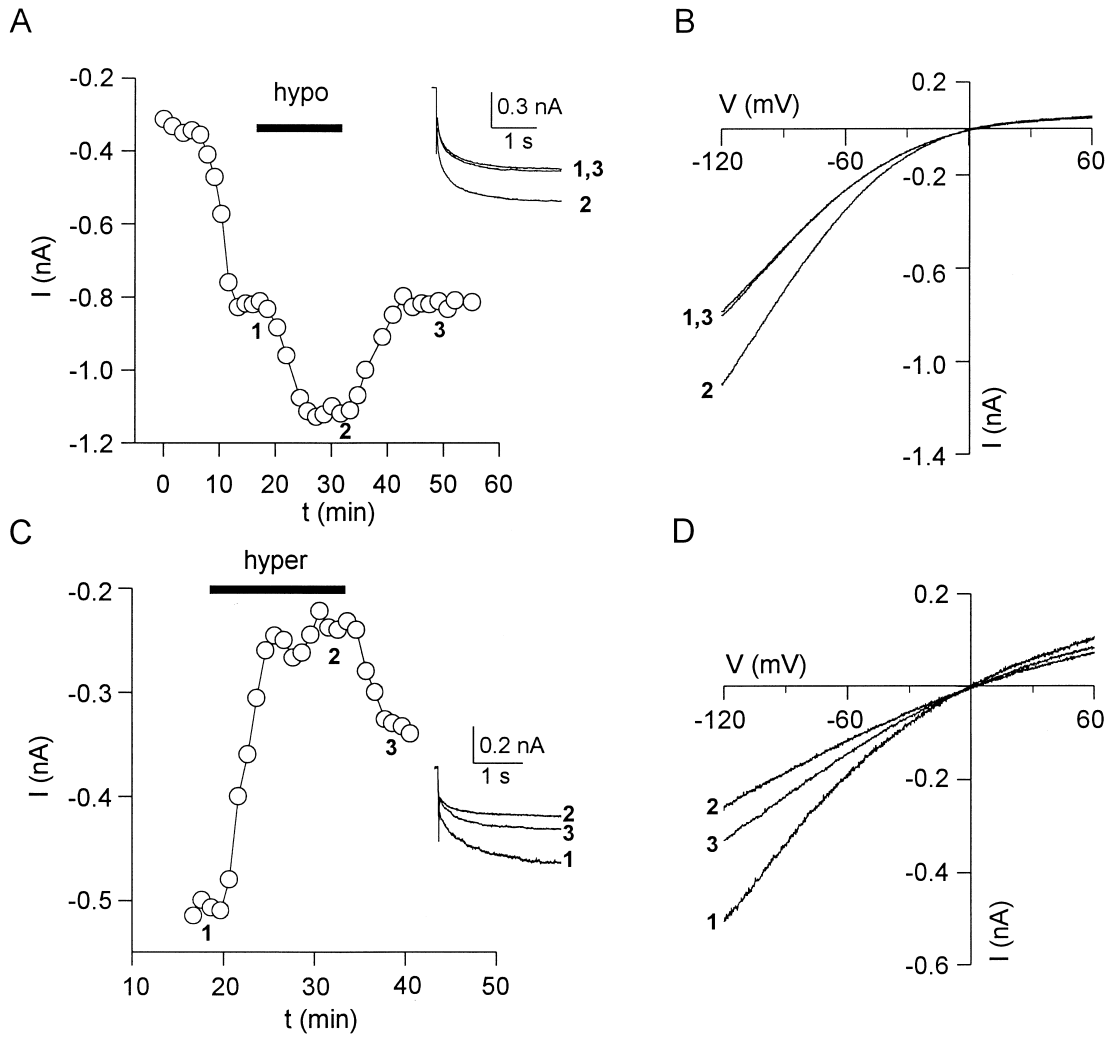

Fig. 4. Effect of changes in I_{Clh} recorded using the nystatin-perforated mode. A: Representative amplitudes of I_{Clh} at −120 mV plotted versus recording time. Current amplitudes increased during the first minutes due to the progress in perforation and intracellular Cl^- loading. At the steady-state level, application of a hypotonic solution (bar) induced a slowly developing current augmentation, which was promptly reversible. Inset: Voltage jump currents at the time indicated in the plot. B: The slow-ramp currents activated at the time points of A show that hypotonicity selectively up-regulated I_{Clh}. C: In another astrocyte, hypertonic solution (bar), applied after inward currents reached the steady-state amplitudes, slowly reduced the voltage step-induced currents to −120 mV. Inset: Current traces recorded at the time indicated in the plot. D: Hypertonicity regulated the slow-ramp inward currents at negative membrane potentials. The effect was only partially reversible even after long exposure to isotonic saline. Solutions as in Fig. 3.

mol), I_{Clh} at −120 mV was reduced by 53 ± 16% ($P < 0.01$; $n = 6$; Fig. 4C) with a time course that was similar to the one observed in whole-cell recordings. The fact that the current decrease was clearly visible at more negative potentials, whereas it was not significant ($P > 0.05$; $n = 5$) in the positive range of membrane voltages, confirms that hypertonicity regulated the hyperpolarization-activated Cl^- current (Fig. 4D).

4. Discussion

The data reported in this study indicate that osmotic stress regulates the I_{Clh} current expressed by primary cultured rat neocortical type 1 astrocytes, which were morphologically differentiated after a long-term treatment with dbcAMP [7]. The results show that a reduction in external osmolarity caused a sustained, reversible increase in current amplitude. This effect, however, occurred only when I_{Clh} was recorded by means of nystatin-perforated patches ($\Delta = 50$ mOsmol) and not when the conventional whole-cell recording configuration ($\Delta = 100$ mOsmol) was adopted. Conversely, an increase in external osmolarity produced a depression of I_{Clh} in both conventional whole-cell and perforated-patch conditions. We also found that, in whole-cell recordings, I_{Clh} activated by a given hyperpolarizing step increased during the first 15–20 min of accessing the cell (run-up), and that this phenomenon was accompanied by an acceleration of the activation kinetics. The removal of some inhibitory intracellular molecule, which regulates the channel activity, may account for this process because the current increases could not be fully explained only by the rise in cytoplasmic Cl^- content. By contrast, under nystatin-perforated-patch conditions, I_{Clh} reached the steady-state level much faster, consistently with the time taken by the progress in perforation (decrease in access resistance) and the elevation of intracellular Cl^-. These different behaviors under isotonic conditions may also partially explain the variable

sensitivities to hypotonic challenges, as the run-up of I_{Clh} may occlude the hypotonic sensitivity in whole-cell recordings. Furthermore, the observation that hypertonicity decreased the whole-cell inward currents in cells that were not affected by hypotonic saline suggests that two molecularly distinct mechanisms may underlie the two processes.

We previously showed [7] that I_{Clh} exhibits biophysical and pharmacological properties resembling those of the ubiquitously distributed ClC-2 channel that is also activated by cell swelling and might be one of the ionic pathways involved in RVD [10,25,27]. However, the variability of the osmoregulation of the several ClC-2 and ClC-2-like channels identified so far (see Section 1) makes it difficult to find an unequivocal functional significance of this type of current in terms of cell volume regulation. In T84 cells, which display a ClC-2-mediated inward current regulated by cell swelling, the anionic conductance contributing to RVD was an osmosensitive outwardly rectifying Cl^- current [2]. It is worth noting that conventionally cultured, undifferentiated neocortical astrocytes also express a time-independent, ohmic Cl^- conductance that is activated by osmotic stress [19]. However, in dbcAMP-treated astrocytes, this channel is activated very rarely either under isotonic conditions or following osmotic stress, suggesting that the Cl^- conductance relevant to volume regulation may be different, depending on the state of astrocyte differentiation. Recently, the ClC-2 channel has been demonstrated to be abundantly expressed at the endfeet of hippocampal astrocytes contacting the capillary endothelium [23]. Interestingly, astrocytic swelling is best seen at the perivascular endings [15]. We previously showed that I_{Clh} is also affected by other conditions such as the intracellular Cl^- content and pathophysiological changes in intra- and extracellular pHs [7,8]. Those data and the I_{Clh} osmosensitivity described here indicate that this inwardly rectifying Cl^- channel may be involved in giving an integrated cellular response to volume challenges, particularly in pathophysiological situations.

A dependence on the recording configuration concerning the osmosensitivity was observed for a hyperpolarization-activated Cl^- current in rat sympathetic neurons [4]. It was also found that dephosphorylation of the channel is the cause of the hypotonicity-induced increase in the hyperpolarization-activated Cl^- current in human intestinal T84 cells [9]. The differential osmosensitivity of I_{Clh} suggests that its osmoregulation is likely to be mediated by intracellular biochemical mechanisms. However, the transduction mechanisms of the osmolarity signal that regulate I_{Clh} remain to be determined.

On the whole, our data demonstrate that the astroglial inwardly rectifying Cl^- current is affected by changes in extracellular osmolarity. These results add further molecular support to the view that the control of intracellular Cl^- homeostasis plays a crucial role in the regulation of the astrocytic volume.

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