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# Two distinct inwardly rectifying conductances are expressed in long term dibutyryl-cyclic-AMP treated rat cultured cortical astrocytes

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**Abstract** **Long term incubation (1–3 weeks) with 250 μM dibutyryl-cyclic-AMP (dBcAMP) of pure cultured cortical astrocytes from newborn rats leads to the expression of voltage-dependent, inward-rectifying potassium ($K^+$) and chloride ($Cl^-$) currents which are lacking in shortly treated (4–24 h) and in control cultured astrocytes. Both conductances are already activated at the holding potential of −60 mV and are distinguishable for their gating kinetics and pharmacological sensitivity. $K^+$ currents have a fast activation kinetic and show a time- and voltage-dependent inactivation at potentials negative to −120 mV. The conductive property of the $K^+$ currents increases upon elevation of the extracellular $K^+$ concentration ($[K^+]_o$) and they are reversibly blocked by extracellular 0.1 mM barium ions ($Ba^{2+}$). $Cl^-$ currents are activated only at negative membrane potentials; they display a slow activation kinetic, no time-dependent inactivation and are not affected by 0.1 mM $Ba^{2+}$. In individual astrocyte the $K^+$ and $Cl^-$ conductances can be expressed singularly or in combination. The results indicate that the expression of these two conductances is controlled by a cAMP-dependent molecular signalling, presumably by regulating a late gene activation. Thus, the strengthening of this signalling would contribute to promote the maturation of less differentiated astrocytes in culture, implicating the expression of $K^+$ and $Cl^-$ membrane conductances which may operate together in the regulation of $[K^+]_o$ homeostasis via the mechanism of the local accumulation.**

*Key words:* Rat cortical astrocyte; Cyclic-AMP analog; Inward-rectifying current; $K^+$ channel; $Cl^-$ channel; Gene expression regulation

## 1. Introduction

Astrocytes are capable to respond to physiological and pathological stimuli with cell activation and differentiation which are related to changes of their phenotype and, presumably, also of their functions [1]. The intracellular molecular signalling chains controlling such changes which include also the acquisition of functionally relevant ionic conductances are largely unknown. This can be investigated in relatively undifferentiated astrocytes in culture expressing a pattern of voltage-dependent and ligand-gated ion channels [2,3] which can be different from the pattern observed in mature astrocytes studied upon acute dissociation or in brain tissue slices [4,5]. The reason for this discrepancy could depend on the fact that successful culturing of astrocytes requires fetal calf serum which contains trophic factors and/or extracellular signalling molecules different from those controlling the astrocyte properties in the fully developed brain. The expression of voltage-dependent calcium ($Ca^{2+}$) currents and inward-rectifying potassium ($K^+$) currents can be triggered by the co-cultivation of astrocytes with neurons [4,6]. $Ca^{2+}$ currents were also recorded in pure astrocyte cultures after a short time elevation of the intracellular content of cAMP [7,8]. Long-term treatment with dibutyryl-cyclic-AMP (dBcAMP), a permeable analog of cAMP, of astrocyte cultures has been reported to induce morphological and biochemical changes which have also been taken as an indicator for astrocyte differentiation [9–16]. However, little is known whether in astrocytes this goes along with a parallel modification of the electric membrane properties [17].

We investigated this issue in patch-clamp experiments performed on rat cultured cortical astrocytes which had been incubated for 1–3 weeks with 250 μM dBcAMP. The results indicate that the prolonged strengthening of the cAMP signalling causes, in conjunction with morphological/biochemical signs of astrocyte differentiation, the new expression of kinetically and pharmacologically distinct inwardly rectifying $K^+$ and $Cl^-$ conductances which may be implicated in the astrocyte function of extracellular $K^+$ buffering.

## 2. Materials and methods

### 2.1. Cell cultures

Primary cultures of cortical astrocytes from newborn (1–2 days) Sprague–Dawley rats (Charles River, Italia) were obtained according to the slightly modified method of McCarty and deVellis [18]. Briefly, after decapitation and removal of the meningis, the cerebral cortices were collected in Falcon tubes in DMEM medium (Gibco-BRL). Following the mechanical dissociation and the passage through cell strainers (Falcon) the cells were plated in 25 $cm^2$ culture flasks in DMEM medium supplemented with 15% FCS (Gibco-BRL), 100U/ml penicillin (Gibco-BRL), 100 μg/ml streptomycin (Gibco Brl), 1 mM glutamine (Gibco Brl) and mantained in an incubator with humidified atmosphere at 37 C° containing 5% $CO_2$. The culture medium was changed every 3 days; after the first week, the flasks were gently shaken for 5 min before changing the medium, in order to detach the O-2A precursor cells which seeded on top of the layer of protoplasmic astrocytes. This allowed to obtain astrocyte cultures with a purity of about 95%. After 2 weeks the cultures had reached confluency. From this stage the cultivation was continued for 1–3 weeks adding 250 μM dBcAMP (Sigma) to the culture flasks. Three days before electrophysiological recordings astrocytes were plated in Petri dishes at a concentration of $10^4 \times$ dish (cf. Fig. 1C and D) and mantained in DMEM supplemented medium additioned with 250 μM dBcAMP.

### 2.2. Fluorescence immunocytochemistry

The fluorescence method was used for the immunological identification of the glial cells. Astrocyte cultures were stained with an antibody for the glial fibrillary acidic protein (GFAP) constituting the intermediate filament specific of the astrocytes, and/or an antibody for

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the surface antigen A2B5 specific of the fibrous (type 2) astrocytes [19]. Cortical protoplasmic astrocytes are GFAP$^+$ A2B5$^-$ [20]. For the GFAP staining the plated astrocytes were fixed in 2% paraformaldehyde for 30 min at 4C°; after several rinses with 0.1 M PBS, they were mantained for 30 min in 0.1 M PBS + 3% BSA to decrease unspecific binding. Then they were incubated overnight at 4C° with a mouse monoclonal primary antibody for GFAP (Sigma) diluted 1:2000 in 0.1 M PBS + 3% BSA + 0.1 M Triton X-100. The labeling with the secondary antibody was performed at room temperature incubating the astrocytes for 1 hour with a biotinylated goat anti mouse policlonal antibody (Sigma) diluted 1:200 in 0.1 M PBS +3% BSA + 0.1% Triton X-100 and then, after several rinses in 0.1 M PBS, for 1 hour in the dark with streptavidin conjugated with the fluorochrome fluorescinethiocyanate (Sigma) diluted 1:400 in 0.1 M PBS +3% BSA + 0.1% Triton X-100. The A2B5 staining was performed in plated astrocytes or in astrocytes fixed in absolute methanol for 10 min at −20 C°. After several washes in 0.1 M PBS they were incubated (1) 30 min with 0.1 M PBS + 3% BSA; (2) 1 hour at room temperature with a mouse monoclonal anti A2B5 Ig M antibody (Boehringer Mannheim) diluited 1:200 in 0.1 M PBS +3% BSA; (3) 1 hour at 37 C° in the dark with a secondary policlonal anti mouse antibody conjugated with rhodamine (Boehringer Mannheim) diluted 1:10 in 0.1 M PBS +3% BSA. In the double labeling experiments the astrocytes were at first stained with the surface antibody and then with the anti GFAP. An epifluorescence microscope (Zeiss MC 63) was used for the visual inspection. Control experiments, performed by incubating the astrocytes without the primary antibody, were negative confirming the specificity of the binding reaction. Since more than 90% of the cultured cells were GFAP$^+$ A2B5$^-$, the cultures were considered to be constituted by protoplasmic (type 1-like) cortical astrocytes. This result was confirmed by electrophysiological experiments revealing that both control ($n$ = 12) and dBcAMP treated ($n$ = 16) astrocytes responded to the application of 100 $\mu$M glutamate by displaying always inward currents with kinetics and pharmacological properties typical of the electrogenic glutamate uptake which is a distinctive feature of protoplasmic astrocytes [21].

*2.3. Electrophysiological recordings*

Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique [22]. The Petri dish containing the astrocytes was mounted on an inverted microscope stage equipped with phase contrast optics (Zeiss ID 03). The patch pipets were pulled (Sutter Instruments P-87) from borosilicate glass capillaries (Clark Electromedical GT 150 T) and fire polished (Narishige MF-83) to have a resistance of 2–4 M$\Omega$ when filled with the standard internal solution which contained (mM): 140 KCl, 2 $MgCl_2$, 5 TES, 5 EGTA, 5 glucose, buffered with KOH at pH 7.3. The standard external solution contained (mM): 140 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 TES, 5 glucose, buffered with NaOH at pH 7.3. All components (Sigma) were of the highest purity grade. When different saline solutions were used, the ions were substituted in equimolar fashion. The solutions and the drugs were delivered by using a gravity driven, multibarrelled microperfusion system with a flow rate of 20–50 $\mu$l/min and positioned at a distance of 100 $\mu$m from the recorded cell. The voltage stimulation and the current recordings were obtained with a patch clamp amplifier (Jens Meyer, Munchen) using a DMA interface (Labamaster TL-1, Axon Instruments) and a microcomputer with P-Clamp (5.5.1) software (Axon Instruments). The currents were low-pass filtered at 3 kHz (−3dB) and acquired at different sampling rates according to the stimulation protocols. Voltage stimulations were not corrected for the junction potential of about −1 mV at the pipet tip. Pipet access resistance (5–8 $\Omega$M) was compensated to 50–70% with the analog circuit of the amplifier. Capacitive transients were nulled as well with an analog circuit and the zeroing values were used to estimate the cell capacitance. The passive leakage component was not subtracted from the current traces. An agar bridge electrode, filled with 150 mM NaCl was used as reference. When the $Cl^-$ content in the bath was changed, a 1 M KCl reference electrode was used in order to minimize the effects of diffusion potentials. The current–voltage relationship plots ($I$–$V$) were constructed by measuring the current values in relation to the zero current level. All the experiments were performed at room temperature (20–25 C°). Statistics for each experibmental condition were performed processing a number of cells ($n$) never <4. Significance tests were performed using Student's $t$-test for paired or unpaired data. A $P$ value < 0.05 was taken as level of significance.

## 3. Results

*3.1. Long term treatment with dBcAMP of cortical astrocytes induces modifications of their phenotype*

Long term treatment with dBcAMP of astrocyte cultures induced marked changes in cell shape. After 4 weeks in control medium, cultured astrocytes had formed a confluent layer of cublestone cells (Fig. 1A). At the same cultivation stage but following treatment with 250 $\mu$M dBcAMP for the last two weeks, astrocytes had transformed into multipolar processes-bearing cells (Fig. 1B and D). This change of the cell phenotype became evident already 3 hours after starting the dBcAMP treatment, but the maximum morphological changes, together with a strong increase in the expression of GFAP (Fig. 1C), was reached after 7–15 days.

Treatment with 250 $\mu$M sodiumbutyrate of the cultures (1–3 weeks) did not cause any modification of the astrocyte phenotype indicating that the dBcAMP induced morphological changes reflect a specific effect mediated by the sustained elevation of the intracellular cAMP.

*3.2. Long term treatment with dBcAMP of cortical astrocytes induces modifications of their electric membrane properties*

To test whether the prolonged intracellular cAMP elevation also induces changes in the pattern of membrane conductances expressed, voltage-dependent ionic currents were studied comparatively in control and dBcAMP treated astrocytes. Control astrocytes ($n$ = 28) cultured for at least 4 weeks displayed an homogenous pattern of currents. The zero current potential ($V_r$) of the astrocytes, measured with KCl-filled pipets in current clamp 5–10 min after accessing the cells, was −35 ± 8 mV (mean ± S.D.; $n$ = 10). The apparent input resistance ($R_i$) was 0.46 ± 0.14 G$\Omega$ ($n$ = 14) and the cell capacitance ($C_m$) was 65 ± 12 pF ($n$ = 11). Depolarizing steps ($V_m$) above −40 mV from an holding potential ($V_h$) of −60 mV activated time- and voltage-dependent outward currents displaying a slow temporal inactivation (Fig. 2A). In contrast, hyperpolarizing steps up to −140 mV did not evoke any significant time- and voltage-dependent currents as reflected also by the outward rectification of the $I$–$V$ plot (Fig. 2B). At +40 mV, extracellular application of 10 mM tetraethylammonium blocked reversibly the peak currents by 56 ± 6% and the currents at the end of the pulses by 34 ± 9% ($n$ = 7). Outward sustained currents were never observed when the intracellular $K^+$ was substituted with the impermeable larger cation $N$-methyl-D-glucamine ($NMDG^+$) ($n$ = 8, data not shown). The findings suggest that these outward currents were due to the activation of a $K^+$ channel and resemble the outward $K^+$ conductance which has been described in rat and mouse cultured cortical astrocytes [23,24].

Astrocytes treated with 250 $\mu$M dBcAMP for 4–24 hours ($n$ = 32) had a $V_r$ of −36 ± 8 mV ($n$ = 16), a $R_i$ of 0.42 ± 0.12 G$\Omega$ ($n$ = 14) and a $C_m$ of 71 ± 9 pF ($n$ = 13). The outward currents had gating kinetics and a pharmacological sensitivity identical to control cultures. No other time- and voltage-dependent currents were observed, indicating that in this electrophysiological condition, only time- and voltage-dependent outward $K^+$ currents were noticeably expressed. In Fig. 2C and E are shown the patterns of currents in astrocytes treated for 12 days with 250 $\mu$M dBcAMP. Outward $K^+$ currents resembling for time and voltage dependence those described in control

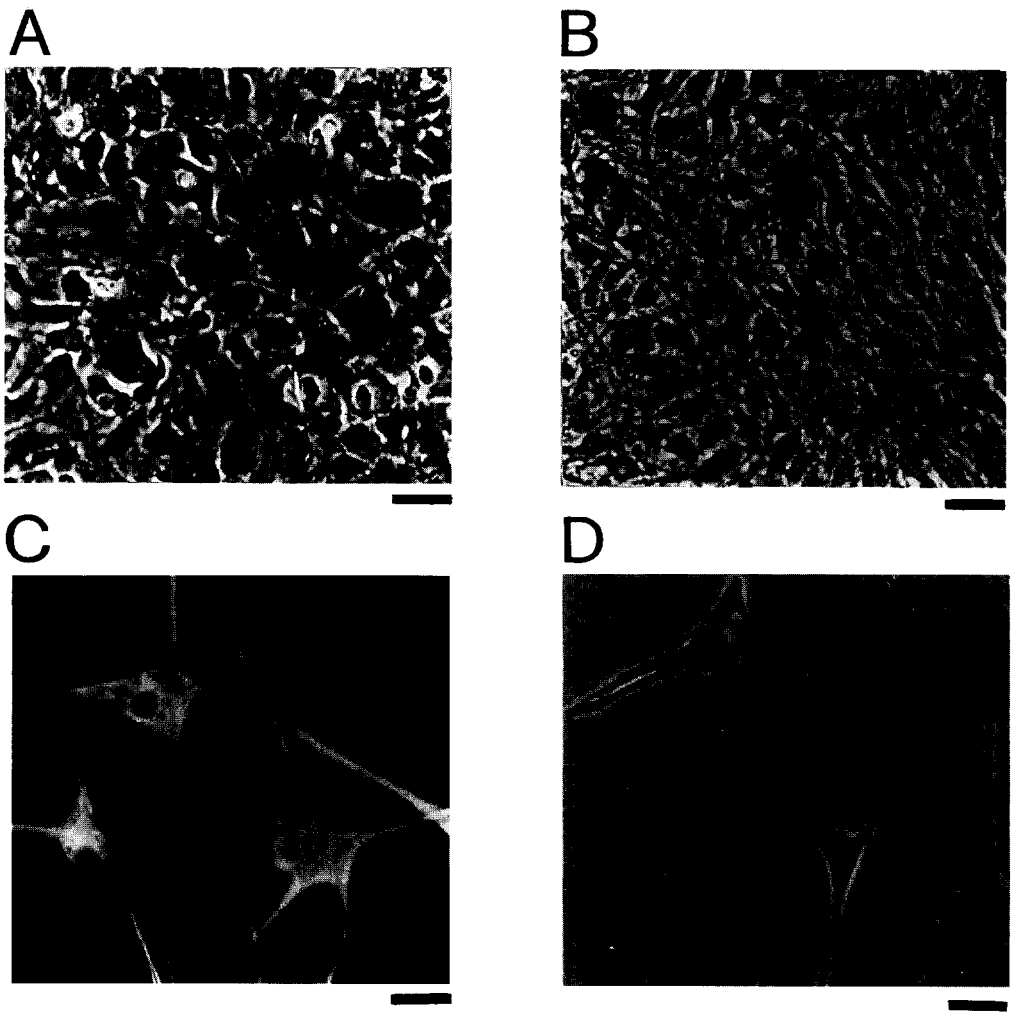


Fig. 1. Morphological and immunological characterization of cultured astrocytes from rat neocortex. (A,B) Phase contrast microphotographs of confluent astrocytes in control condition (A) and after dBcAMP treatment (B) showing the marked change of the cell phenotype induced by dBcAMP. (C) Fluorescence microphotograph of dBcAMP treated astrocytes immunostained for GFAP. (D) Phase contrast microphotograph of typical dBcAMP treated astrocytes from which electrophysiological recordings were obtained. Scale bars: 15 μm (A,B) and 10 μm (C,D).

cultures were still observed, and their density was not significantly different. In contrast to control astrocytes, more than 90% of the recorded astrocytes treated 1–3 weeks with dBcAMP ($n$ = 233) had scattered values of $V_r$ ranging from −85 mV to −18 mV (mean −29 ± 10 mV; $n$ = 76), a lower $R_i$ of 0.12 ± 0.04 ΩG ($n$ = 26) and a $C_m$ of 68 ± 18 pF ($n$ = 85). Moreover, hyperpolarizing steps from a $V_h$ of −60 mV evoked inward currents with different time and voltage dependence and marked differences in reversal potential. The similarity between the reversal potential of the inward-rectifying currents obtained from the $I$–$V$ plots (Fig. 2D and F) and the respective astrocyte zero current potential indicates that in this electrophysiological condition, the two patterns of inward currents partecipate in setting the cell resting membrane potential. In order to determine the ionic selectivity and the pharmacological sensitivity of the two kinds of inward currents only those astrocytes with zero current potentials in a range of 10 mV from the extreme values of the measured potentials (see above) were investigated.

In astrocytes with zero current potentials negative to −75 mV ($n$ = 26), hyperpolarizing steps to −120 mV elicited inward currents which activated within tens of milliseconds and did not inactivate (Fig. 3A). At more negative potentials the currents had a biphasic time course: a rapid rise to the peak followed by a slow decay towards a steady-state level. The rate and the extent of the decay increased with larger hyperpolarizations. The $I$–$V$ plots of the peak and the steady-state currents (Fig. 3B) put in evidence a voltage range of increasing membrane resistance for potentials negative to −140 mV. Small and fast decaying inward tail currents were recorded stepping back to the holding potential. The time and voltage dependence of these currents resemble the inward-rectifying $K^+$ currents described in acutely dissociated Muller glial cells from rabbit retina [25]. The ionic selectivity was investigated by changing the extracellular $K^+$ concentration ($[K^+]_o$) from 4 mM to 12 mM. Elevation of $[K^+]_o$ increased the amplitude of the evoked currents and induced a negative shift of the holding current (Fig. 3C). The extrapolated reversal potential moved of +26 ± 5 mV ($n$ = 4), as expected if $K^+$ ions were the charge carriers. The slope conductance increased with the elevation of $[K^+]_o$ (Fig. 3D) confirming that the currents were generated by ion fluxes through inward-rectifying $K^+$ channels [26]. The inward $K^+$ currents were reversibly blocked by 79 ± 6% with 0.1 mM bar-

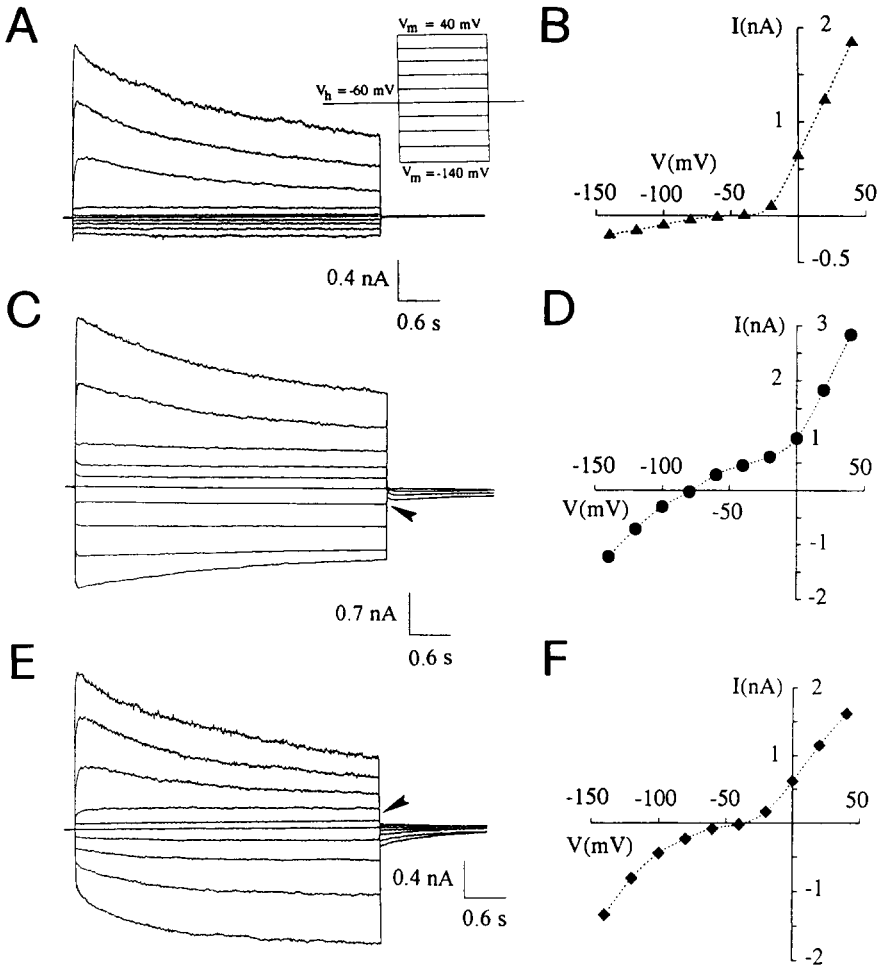


Fig. 2. Whole-cell membrane currents recorded from cortical astrocytes in control condition and after long-term treatment with dBcAMP. (A) Family of currents elicited in a control astrocyte from a $V_h$ of −60 mV by voltage steps ($V_m$) from +40 mV to −140 mV with −20 mV increments and delivered every 20 s (inset). (B). The *I–V* plot obtained from the peak currents shows an outward rectifying profile due to the activation of time- and voltage-dependent outward currents at potentials positive to −40 mV. (C–E) Different families of currents from two long term dBcAMP treated astrocytes activated by using the same stimulation protocol shown in A. (C) In addition to outward currents, time- and voltage-dependent inward currents having a fast activation kinetic and a slow temporal decay for potentials negative to −120 mV were elicited in an astrocyte with a zero current potential of −81 mV. (D) The *I–V* plot of the peak currents denotes, at negative membrane potentials, an inwardly rectifying behaviour. (E) Astrocyte with a zero current potential of −28 mV displaying a different pattern of inward currents characterized by a slower activation time course and no inactivation. (F) The *I–V* plot depicts a marked inward rectification at potentials negative to −40 mV. The arrows in C and E indicate the zero current levels.

ium ions ($Ba^{2+}$) ($n$ = 5) which at submillimolar concentration is a rather potent and selective blocker of inward-rectifying $K^+$ currents [26,27]. $Ba^{2+}$ blocked both the holding and the evoked currents (Fig. 3E). The inhibition of the inward $K^+$ currents was time- and voltage-independent and was accompained by a significant change in the zero current potential from −79 ± 4 mV to −45 ± 6 mV ($n$ = 4). The *I–V* plot (Fig. 3F) confirms that the $Ba^{2+}$ sensitive currents were carried by $K^+$ ions as it is shown by the voltage intercept (−82 mV) of the control with the residual currents.

A different pattern of inward currents was observed in those astrocytes with zero current potentials positive to −25 mV ($n$ = 96). Hyperpolarizing steps from a $V_h$ of −60 mV elicited time- and voltage-dependent currents which activated slowly and did not inactivate even for long voltage steps (Fig. 4A). In addition, slow outward deactivating currents were observed stepping back to the holding potential. Sustained inward currents were elicited already after accessing the cells but 5–10 min were required to stabilize their amplitude and time course. These inward currents were not affected by elevation of $[K^+]_o$ to 12 mM ($n$ = 24) or by the application of 0.1 mM $Ba^{2+}$ ($n$ = 32) (Fig. 4B and C) and they also did not change when the extracellular $Na^+$ content ($[Na^+]_o$) was decreased to 40 mM ($n$ = 10) by substitution with the larger cation $NMDG^+$ (Fig. 4D). Therefore, this conductance is kinetically and pharmacologically different from the previously determined time- and voltage-dependent inwardly rectifying $K^+$ conductance and overall it is not permeable to monovalent cations. Accordingly, slow activating sustained inward currents were still elicited by hyperpolarizing steps using intra and extracellular solutions $K^+$ and $Na^+$-free replaced with $NMDG^+$ ($n$ = 37, Fig. 4E). The corresponding reversal potential obtained from the *I–V* plot (Fig. 4F) of the steady-state currents activated by voltage steps ranging from +90 mV to −150 mV was at −3 mV (mean −2 ± 4 mV, $n$ = 8). The tail current analysis indicated that deactivating currents recorded by using extracellular solutions with different

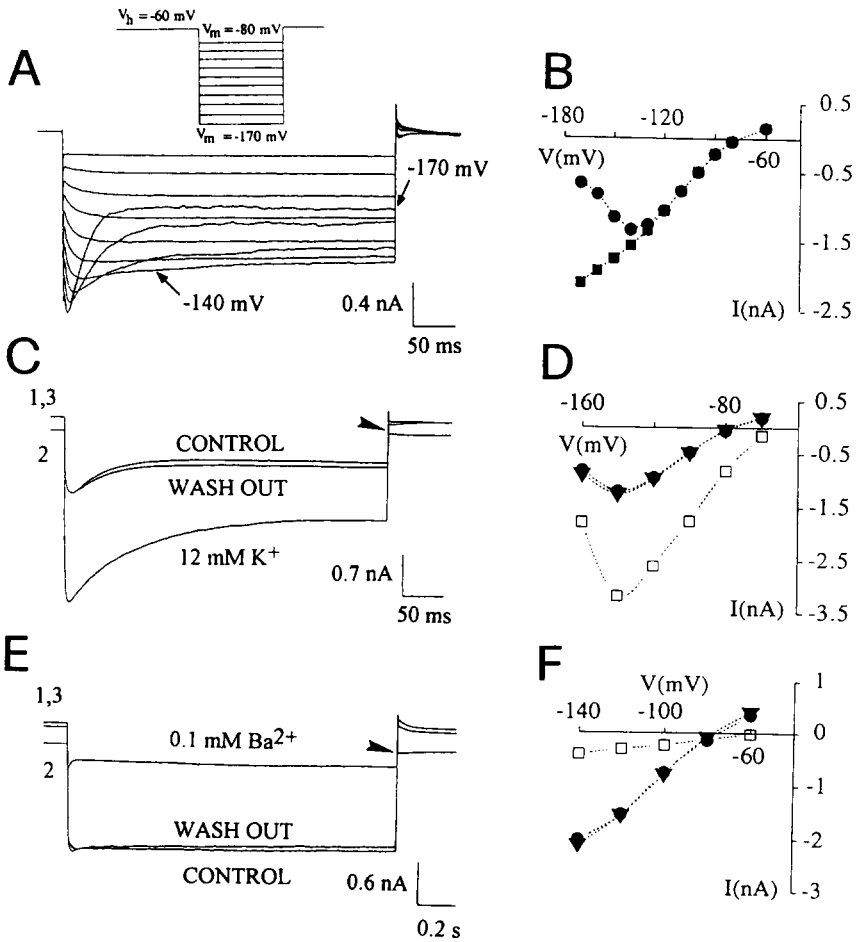


Fig. 3. Fast activating, slow inactivating inward currents identify a $K^+$ conductance in long term dBcAMP treated astrocytes. (A) Family of currents elicited from a $V_h$ of −60 mV by voltage steps ($V_m$) from −80 to −170 mV with −10 mV increments and delivered every 20 s (inset) in an astrocyte with a zero current potential of −80 mV. (B) The *I–V* plots of the peak (■) and of the steady-state currents (●) show a voltage range of increasing membrane resistance for $V_m$ negative to −140 mV. (C) In an astrocyte with a zero current potential of −82 mV, the elevation of $[K^+]_o$ from 4 mM (1) to 12 mM (2) shifted the holding current at −60 mV and increased the current amplitude elicited by a voltage step to −160 mV. The currents came back to the initial values after washing and returning to 4 mM $[K^+]_o$ (3). (D) The *I–V* plots of the steady-state currents activated at different membrane potentials in 4 mM $[K^+]_o$ (●), after elevation to 12 mM $[K^+]_o$ (□) and returning to 4 mM $[K^+]_o$ (▼) show a shift of the extrapolated reversal potential and an increase in slope conductance. (E) In an astrocyte with a zero current potential of −79 mV, the holding current at −60 mV (1) was inhibited (2) reversibly (3) by extracellular application of 0.1 mM $Ba^{2+}$ which also blocked the current elicited by a voltage step to −120 mV. (F) The *I–V* plots of the steady-state currents at different membrane potentials in control condition (●), after application of 0.1 mM $Ba^{2+}$ (□) and after wash-out (▼) confirm that the blocked currents were carried by $K^+$ ions. The arrows in C and E indicate the zero current levels.

$Cl^-$ concentrations changed polarity according to the equilibrium potential for the relative $Cl^-$ gradient (data not shown). Taken together the data indicate that this inwardly rectifying conductance is selectively permeable to $Cl^-$ ions over cations.

A large population of astrocytes ($n$ = 68) exhibited inward currents with time- and voltage-dependent kinetics which denoted the concomitant expression of both $K^+$ and $Cl^-$ conductances. In these astrocytes the zero current potential ranged from −70 to −30 mV indicating that the two different components could be expressed in a different ratio. Inward currents developed with a fast kinetic within tens of milliseconds and did not inactivate. However, for large hyperpolarizations inward currents could have a complex time course consisting of a fast activation followed by an inactivating component and a further rising phase which had a time constant of seconds (Fig. 5A). The result was that no voltage range of increasing membrane resistance was observed (Fig. 5B). The finding that the sensibility to the elevation of $[K^+]_o$ and to the application of 0.1 mM $Ba^{2+}$ varied sensibly among the different astrocytes of this population confirms that the two conductances could be expressed differently in individual astrocyte.

No differences were found in the expression of voltage-dependent membrane currents when astrocytes treated with 250 M sodiumbutyrate were compared to control cultures. This suggests that the induction of the inward-rectifying $K^+$ and $Cl^-$ currents is a dBcAMP related event and is the consequence of the specific prolonged elevation of the intracellular cAMP level.

## 4. Discussion

This study indicates that rat cultured cortical astrocytes, after long-term incubation with 250 μM dBcAMP, express two voltage-dependent, inwardly rectifying $K^+$ and $Cl^-$ conductances which are lacking in control cultures and differ from

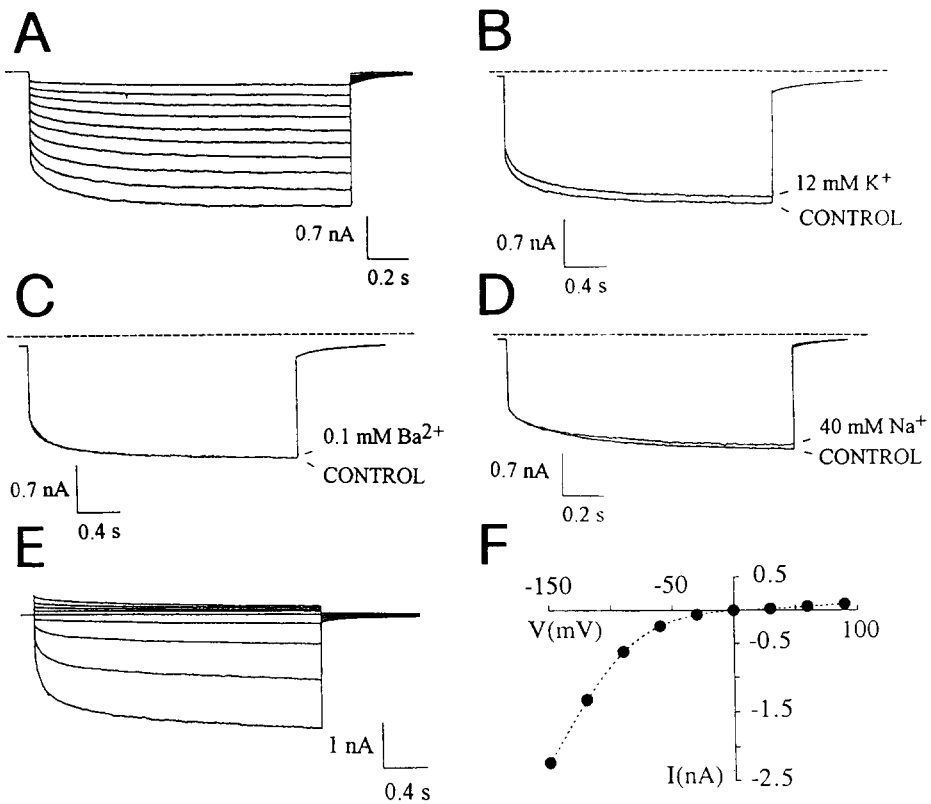


Fig. 4. Slow activating, not inactivating inward currents identify a $Cl^-$ conductance in long term dBcAMP treated astrocytes. (A) Family of currents activated from a $V_h$ of −60 mV by using the same stimulation protocol shown in Fig. 3A and recorded in an astrocyte with a zero current potential of −19 mV. (B,C) Elevation of $[K^+]_o$ to 12 mM or application of 0.1 mM $Ba^{2+}$ in two astrocytes with zero current potentials of −23 mV (B) and −18 mV (C) did not affect either the shape or the current amplitude elicited at −120 mV. (D) Lowering $[Na^+]_o$ from 140 mM to 40 mM did not change the current trace at −120 mV in an astrocyte with a zero current potential of −20 mV. (E). Family of currents evoked from a $V_h$ of −30 mV by voltage steps from +90 mV to −150 mV with −30 mV increments and recorded in an astrocyte using intra- and extracellular $K^+$ and $Na^+$-free solutions. (F). The I-V plot of the steady-state currents denotes a marked inward rectification in the negative range of membrane potentials. The dashed lines in B, C and D indicate the zero current levels.

each other for gating kinetics and pharmacological sensitivity. The $K^+$ conductance has properties comparable to the inwardly rectifying $K^+$ conductance observed in glial cells (cf. for a review [2]) and other cell types [26], such as time- and voltage-dependent inactivation for large hyperpolarizations, high pharmacological sensibility to submillimolar concentrations of extracellular $Ba^{2+}$ and $[K^+]_o$-dependent gatings. In rat astrocytes this kind of $K^+$ conductance has been observed only in astrocytes from slices [28], upon acute dissociation [29] or by means of the tissue print technique [4]. The inwardly rectifying $Cl^-$ conductance was never demonstrated in rat cortical astrocytes in culture. However, a passive $Cl^-$ flux via a conductive pathway activated in the same range of membrane potentials of the present inward $Cl^-$ currents, has been indirectly demonstrated in astrocytes of olfactory cortex slices from guinea pig [30]. The here described $Cl^-$ conductance shows several similarities with the $Cl^-$ currents activated by hyperpolarizations in vertebrate and invertebrate neurons [31,32] and in *Xenopus* oocytes [33,34] and may also account for the single $Cl^-$ channel currents observed in mouse cultured astrocytes activated upon hyperpolarizations [24]. The fact that the two $K^+$ and $Cl^-$ inwardly rectifying conductances are exhibited only in astrocytes of the mature brain, whereas they are lacking in conventionally cultured rat astrocytes obtained from the immature brain of newborn animals, indicates that the expression of $K^+$ and $Cl^-$ inward currents is coupled to the maturation state of the astrocytes. The finding that 1–3 weeks of dBcAMP treatment induces the expression of these conductances suggests that the responsible mechanism of this functional differentiation includes a sustained strengthening of the cAMP-controlled intracellular signalling. The possibility that the two respective channel proteins are pre-existing in the membrane but not functionally active, and then switched to the functional state by a cAMP-dependent protein kinase phosphorylation seems unlikely since incubation of the astrocytes with dBcAMP for several hours, sufficient to produce a direct protein phosphorylation, did not induce the expression of these inward currents. In contrast, the requirement of a long lasting elevation of intracellular cAMP suggests that the expression of the observed inwardly rectifying conductances relies on a time-dependent new synthesis of channel proteins which could be induced by a cAMP-regulated gene activation.

Although dBcAMP treated astrocytes have been proposed to reflect reactive astrocytes [35], there are evidences indicating that in vitro dBcAMP acts as a signal that induces a physiological astrocyte differentiation with changes in both morphology and function [36,37]. In this respect it is worth to note that also in vivo rat astrocytes require several weeks after birth to reach maturity [38]. This occurs under the influence of noradrenergic fibers coming from the *locus coeruleus* [39] known to stimulate,

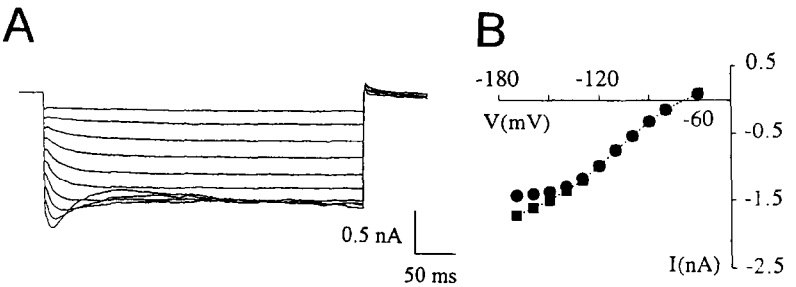


Fig. 5. Inwardly rectifying $K^+$ and $Cl^-$ conductances can be co-expressed in long term dBcAMP treated astrocytes. (A) Family of currents elicited from a $V_h$ of −60 mV by voltage steps from −80 mV to −170 mV with −10 mV increments in an astrocyte with a zero current potential of −68 mV. Note that, for larger hyperpolarizations, the initial inactivation is followed by a further current rising phase. (B) The *I–V* plots of the peak currents (■) and of the currents at the end of the pulses (●) show that the voltage range of increasing membrane resistance has disappeared even at very hyperpolarized potentials.

via β-adrenergic receptors, the intracellular cAMP formation [40,41]. Accordingly, our data can be explained as the result of a cAMP induced physiological differentiation of immature astrocytes.

Interestingly, a peculiarity of the newly expressed inwardly rectifying $K^+$ and $Cl^-$ conductances is that they are activated in the same range of negative membrane potentials. This finding has a relevant functional significance because in vivo, when $[K^+]_o$ raises as a consequence of increased neuronal activity, the concomitant passive influxes of $K^+$ and $Cl^-$ enable the astrocytes to take up the excess of $K^+$ from the extracellular cleft via the mechanism of the local accumulation [42], thus contributing to the astrocyte mediated control of $[K^+]_o$ homeostasis. This mechanism in fact, to be operative, requires the activation of a $Cl^-$ conductance counteracting the restrictive membrane depolarization due to the $K^+$ influx via the inwardly rectifying $K^+$ conductance. Therefore the data, providing evidences that dBcAMP promotes the expression of $K^+$ and $Cl^-$ inwardly rectifying conductances in astrocytes, indicate that a prolonged strengthening of the cAMP-dependent molecular signalling would be necessary for the induction of functionally relevant membrane conductances which may act in the regulation of the $[K^+]_o$ homeostasis.

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