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Novel adenosine and cAMP signalling pathways in migrating glial cells

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ABSTRACT

This study was aimed at characterizing the effect of purinergic transmission on migration of embryonic ciliary ganglion satellite glial cells. Application of adenosine significantly decreased the rate of migration of glial cells whereas no differences were observed in the presence of ATP. The A_{2B} receptor antagonist reverted this action, but application of an A_{2A} receptor antagonist or a cAMP–protein kinase inhibitor had no effect on the agonist's stimulation. Forskolin, which stimulates adenylate cyclase activity, and the cAMP analogue 8-CPT-2′-O-Me-cAMP, which selectively activates the guanine exchange factor Epac1, mimicked the effect of adenosine.

In addition, intracellular calcium measurements studies revealed that application of either adenosine or ATP induced an increase in $[Ca^{2+}]_i$ and that the adenosine-induced $[Ca^{2+}]_i$ response was due to Ca^{2+} entry and was blocked by an A_{2A} receptor antagonist, SCH 58261, or by high Gd^{3+} concentrations. Furthermore, forskolin, but not 8-CPT-2′-O-Me-cAMP, activated the Ca^{2+} entry which was blocked by Gd^{3+} and was independent of cAMP–protein kinase activity.

These results demonstrate the involvement of purinergic P1 signalling in the regulation of cellular migration, and point to the importance of adenosine as a negative modulator of migration of peripheral developing glial cells and as an activator of Ca^{2+} entry.

1. Introduction

The endogenous purine ribonucleoside adenosine is an important modulator of neuronal activities, including viability, excitability, synaptic transmission, neuron–glia communication and neuroprotection [1–3]. Moreover, alteration of adenosine receptors density and functions has been associated with several neurological diseases [4,5]. The main source of adenosine in the nervous system is ATP which is released by both neurons and glial cells [2,3,6–8], this nucleotide is then dephosphorylated into adenosine by a cascade of ectonucleotidases. Moreover adenosine can also be released from cells by equilibrative transporters [9]. Adenosine binds to purinergic receptors P1, which belong to the superfamily of G protein coupled receptors. There are four types of evolutionary conserved adenosine receptors (ARs; A_1, A_{2A}, A_{2B} and A_3). A_1 and A_3 receptors are coupled to the $G_{i/o}$; A_{2A} receptors are coupled to members of the G_s family, whereas A_{2B} receptors are coupled promiscuously to several G proteins, including G_s, G_q, and G_{12}. Thus, adenosine binding to ARs can simultaneously activate multiple downstream signalling pathways, including cAMP and intracellular Ca^{2+} pathways [2,3].

Migration of neurons and glia is a fundamental event that plays a central role in many physiological and pathophysiological processes during embryonic and adult life, yet no information is available concerning the ability of adenosine to influence this crucial activity. The aim of this study was therefore to investigate the effects of purinergic transmission on the regulation of cell migration. To elucidate this point we employed embryonic chick ciliary glial cells, a highly motile cell population deriving from cranial neural crest cells and that has been used successfully previously as a model to investigate mechanisms and factors affecting migration [10–12]. In this paper we demonstrate that adenosine, but not ATP, slows the rate of migration via the activation of A_{2B} receptors and in a cAMP–protein kinase (PKA)-independent way; in addition we show that this effect is mimicked by forskolin, an adenylate cyclase activator and by the cAMP analogue 8-CPT-2′-O-Me-cAMP that selectively activates the exchange protein directly activated by cAMP Epac1. Finally, performing calcium imaging on these cells, we demonstrate that adenosine triggers Ca^{2+} influx in glial cells via A_{2A} receptor activation, probably through a direct interaction of cAMP with plasma membrane voltage-independent Ca^{2+} channels.

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2. Experimental procedures

2.1. Cell culture

Cell culture protocols have been described in detail previously [13]. Briefly, chick ciliary ganglia were dissected from 7-day-old embryos. Ganglia were collected in phosphate buffered saline (PBS, Invitrogen) and incubated in divalent cation-free PBS containing 0.06% trypsin at 37 °C for 5 min. Trypsin was removed by washing with 1:1 Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Mixture (F-12) (Invitrogen) containing 0.2% bovine serum albumin (BSA). After gentle trituration, cells were then re-suspended in a modified serum free N2 medium [40] composed of a 1:1 mixture of DMEM/F12 supplemented with N-2 supplement (human transferrin 100 μg/ml, insulin recombinant 0.86 μM, progesterone 20 nM, putrescine 100 μM, selenite 30 nM; Invitrogen), BSA (2 mg/ml), sodium pyruvate (1 mM), gentamicin (20 μg/ml), L-glutamine (1 mM), and basic fibroblast growth factor (10 ng/ml, Alomone Labs). Cells were counted and a droplet (150 μl) of cell suspension containing approx. 18,000 cells was plated in the middle of area of a 40 mm glass coverslip coated with poly-D-lysine (100 μg/ml) and laminin (2 μg/cm^2). Cells were incubated for 1–2 h at 37 °C to allow cells to adhere to the substrate and the plastic Petri dish containing the coverslip was covered with N2 medium.

2.2. Calcium imaging

Calcium imaging experiments were performed on cells seeded 1 day prior the experiment. Cells were loaded for 30 min at 37 °C with 0.5 μM Fura-2 AM (Invitrogen) and were transferred in a temperature controlled perfusion chamber (Bioptechs) connected to a peristaltic pump and mounted on an inverted microscope (Eclipse TE 300, Nikon). All experiments were performed at a chamber temperature of 37 °C. Cells were continuously perfused, by means of a gravity microperfusion system, with a control Tyrode solution composed of (in mM): NaCl, 154; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; N-(2-Hydroxyethyl)-piperazine-N′-ethanesulfonic acid (HEPES), 5; glucose, 5.5; pH 7.4 (adjusted with NaOH) and the osmolarity was 335 mOsmol/l. Substances were rapidly and locally applied by switching from this solution to the test solutions. The chamber volume bath was kept at 2 ml and the rate of local microperfusion was 1.2 ml/min.

$[Ca^{2+}]_i$ measurements were performed exciting the dye Fura-2 every 0.3 s alternatively at 340 nm and 380 nm for 100 ms by means of a monochromator (Polychrome IV, T.I.L.L. Photonics GmbH, Germany), and emission was recorded at 510 nm. Images were recorded with a cooled CCD camera (SensiCam, PCO COMPUTER OPTICS GmbH, Germany) and stored on a computer. Dye excitation, image acquisitions and analysis protocols were performed with Axon Imaging Workbench software (Axon Instruments). Fluorescence changes are expressed as the ratio of fluorescence at 340 nm and 380 nm ($R = F_{340}/F_{380}$). In a set of experiments, cells were bathed in a Ca^{2+}-free Tyrode solution containing 0.5 mM EGTA. The final estimated free Ca^{2+} concentration in this solution was about 10 nM as determined using an online web site for calcium/EGTA calculator (http://brneurosci.org/egta.html).

In a set of experiments we tested whether PKA was really inhibited by their specific inhibitor H-89. Since PKA activity controls the regulation of voltage operated calcium channels (VOCCs), 40 mM KCl was used as the depolarization stimulus to activate and open VOCCs and induce a $[Ca^{2+}]_i$ rise in both ciliary ganglion neurons and glial cells. To compare the effects of H-89 on 40 mM KCl-evoked $[Ca^{2+}]_i$, for each cell, changes in fluorescence intensity were expressed as $\Delta R = (R - R_{base})$, where R_{base} is the value before the addition of 40 mM KCl. ΔR traces were then averaged and normalized using the following equation: $\Delta R_N = \Delta R/R_{peak}$, where R_{peak} is the maximum value achieved after 40 mM KCl stimulation.

2.3. Cell migration assay and analysis

Time-lapse microscopy experiments were performed on cells seeded 1 day prior the experiment and bathed in N2 medium. The cells were monitored using a phase-contrast optic in a Nikon Eclipse TE 300 microscope equipped with a heated (37 °C) live-cell closed chamber system (Bioptechs) connected to a peristaltic pump. During experiments cells were bathed in N2 medium, alone or in the presence of the test substances. Images were acquired with a cooled CCD camera (SensiCam, PCO COMPUTER OPTICS GmbH, Germany) using the camera control software. The magnification was 20× (1 pixel = 0.331 μm) and the sampling time interval was 20 s. The duration of all experiments was at least 3 h. At least three experiments were performed for each experimental condition. Cell trajectory was determined as the time sequence of the centroid position of the cell soma using a custom made software written by SITEM, Italy. Instantaneous cell speed (μm/min) was calculated from displacement between two subsequent cell positions.

2.4. Chemicals and reagents

The 8-CPT-2′-O-Me-cAMP was purchased from Biaffin GmbH & Co KG, Kassel (Germany). All other reagents used in this study, if not otherwise specified, were purchased from Sigma.

2.5. Morphological and functional identification of glial cells

At embryonic stage E7, ciliary ganglia contain both neurons and non-neuronal cells. After 24 h of culture in N2 medium, >99% of cells express the placodal and neural crest cell marker HNK-1 (Human Natural Killer-1) ([14]; Gilardino and Torre, unpublished data). In this culture, neurons are round phase-bright cells bearing processes and express neuronal markers such as microtubule associated protein 1b (MAP1b) [15]. Neurons (MAP1b positive cells) are easily distinguishable morphologically from non-neuronal cells which have a flat, polygonal phase-dark appearance. Previous studies indicate that only a small subset (about 5%) of neuronal precursors is still present in the ganglion at stage E7 [15]. The remaining large majority of non-neuronal HNK-1-positive cells are developing satellite glial cells or proliferating precursors committed to the glial cell lineage.

In the present study, therefore, glial cells were at first identified on morphological and functional criteria. As previously reported [11], both ciliary ganglion neurons and glial cells respond to a depolarizing high potassium stimulus with an increase of $[Ca^{2+}]_i$ via VOCC activation. However, in morphologically identified glial cells, the calcium elevation is much lower in amplitude as compared to neurons. For example, the mean peak Fura-2 fluorescence ratio increase induced by the perfusion of a Tyrode solution containing 40 mM KCl was 1.37 ± 0.08 ($n = 11$) in ciliary ganglion neurons and 0.28 ± 0.06 ($n = 14$) in glial cells from the same fields. Thus, the morphological identification of the cells was confirmed by the amplitude of Fura-2 ratio increases in response to a brief (3 s) high potassium stimulation applied at the end of experiments.

2.6. Statistics

Experiments were repeated at least three times on three separate days on three separate primary cultures. Data are presented as the mean values ± SEM. Student's t test or one-way ANOVA followed by a statistical test for multiple comparisons (Origin, Tukey's test) were applied to compare experimental treatments. A probability level of <0.05 was considered statistically significant.

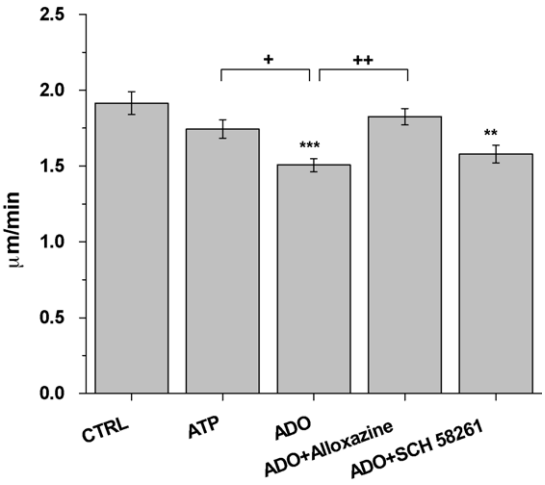

Fig. 1. Adenosine slows the rate of migration through A_{2B} receptors. Bar graph compares the mean modulus of the glial cell velocity in control conditions (CTRL), in the presence of 1 μM ATP, in the presence of 10 μM adenosine (ADO), in the presence of 10 μM adenosine and 10 μM of the A_{2B} receptor antagonist alloxazine (ADO + Alloxazine) and in the presence of 10 μM adenosine and 10 μM of the A_{2A} receptors antagonist SCH 58261 (ADO + SCH 58261). Alloxazine but not SCH 58261 reverts the effect of adenosine on cell migration. $^{+}P < 0.05$; $^{++}P < 0.02$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Effects of ATP and adenosine on cell migration rate

In order to determine whether purinergic messengers control the migratory behaviour of ciliary ganglion (CG) glial cells, we used quantitative time-lapse video microscopy on moving glial cells bathed, for at least 3 h, in N2 medium alone or in N2 medium containing 1 μM ATP or 10 μM adenosine.

When bathed in N2 medium, the speed of migration of glial cells was 1.91 ± 0.07 μm/min ($n = 56$), and no significant difference was observed in the mean speed of cells stimulated with ATP 1 μM (1.73 ± 0.06 μm/min, $n = 38$). On the other hand in the presence of adenosine 10 μM, the mean speed was 1.51 ± 0.04 μm/min ($n = 82$): the nucleoside significantly ($P < 0.001$) decreased the rate of migration to 79% of the control rate (Fig. 1).

The ability of adenosine to slow cells motion was assessed with a different experimental protocol, as follows. In a set of experiments cells were initially bathed in control medium for 3 h and then the whole medium was exchanged to fresh control medium alone or to one containing 10 μM adenosine and maintained for at least 3 h. This protocol allowed us to measure, the percentage of speed change for each cell and that of responsive cells. In control conditions we observed a reduction of the mean speed to 90.7% in 7 out of 10 cells. Adenosine 10 μM significantly ($P < 0.001$) decreased the speed to 73.9% (in 12 out of 13 cells). These data confirm the inhibitory role of adenosine in cell rate migration. No further decrease was observed at higher agonist concentration (data not shown).

Of the four cloned adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3, type 2 receptors are expressed in avian neural crest [16], a transient embryonic cell population that gives rise to satellite glial cells in the ciliary ganglion [17]. Therefore, we tested whether adenosine's effect could be blocked by the selective A_{2A} antagonist SCH 58261 and/or by the A_{2B} receptor antagonist alloxazine.

As shown in Fig. 1, the mean speed of cells obtained in the presence of adenosine and 10 μM SCH 58261 was 1.58 ± 0.06 μm/min ($n = 24$); this value did not differ significantly from the value obtained in the presence of adenosine alone. However, the mean rate of migration of cells incubated with adenosine and 10 μM alloxazine was 1.82 ± 0.05 μm/min ($n = 22$), a value that was significantly different ($P < 0.02$) from the mean speed in the presence of adenosine alone. In conclusion, these results suggest that adenosine inhibits the rate of migration through the activation of A_{2B} receptors.

3.2. Adenosine elicits Ca^{2+} influx in glial cells

In previous studies we have demonstrated that the rate of migration of CG cells is dependent on both resting Ca^{2+} levels and amplitude of spontaneous Ca^{2+} oscillations generated in glial cells [11], and it would therefore be plausible that adenosine affects migration via modifications of calcium signals.

Stimulation of cells bathed in standard Tyrode solution with 10 μM adenosine induced an increase of $[Ca^{2+}]_i$ in $n = 338$ out of 898 glial cells (38%, Fig. 2). Two different patterns of responses were observed: the agonist induced oscillating calcium rises in 53% of cells and promoted a sustained increase in $[Ca^{2+}]_i$ in the remaining 47%. In both cases changes in $[Ca^{2+}]_i$ ceased before or soon after the removal of the agonist from the external medium. The mean amplitude of $[Ca^{2+}]_i$ ΔR (measured as the difference between the maximum value of R during the response to adenosine and the R value before the adenosine perfusion) was 1.13 ± 0.01 ($n = 179$) for sustained responses and 1.14 ± 0.01 ($n = 159$) for oscillating ones, with a mean occurrence of oscillations of 2.0 ± 0.1 events per minute. Moreover, in experiments in which the agonist was applied twice separated by a washout interval of 120 s, most cells (65%, $n = 65$ out of 101) responded to both stimulations. Finally we examined the effect of other three different concentrations of adenosine, namely 0.1 μM, 1 μM and 100 μM: at the lowest concentration no cell responded (0 out of 27), while at the other concentrations percentages were respectively 7% (4 out of 54 cells) and 41% (31 out of 76 cells). These results indicate that adenosine induces a $[Ca^{2+}]_i$ response in glial cells in a concentration-dependent manner with maximal effects at 10 μM. Adenosine was still able to elicit calcium responses also if experiments were performed in N2 medium (data not shown), instead of Tyrode's solution.

To determine the source of the calcium signals, we performed a set of experiments in which cells were bathed in a solution containing 0 mM $CaCl_2$ and 0.5 mM EGTA: in these conditions, adenosine failed to generate intracellular calcium changes in all cells tested ($n = 59$). This result was confirmed by other experiments like the one shown in Fig. 3a. A control response recorded in normal Tyrode solution showed adenosine–calcium signals in 41 out of 66 cells (62%). After washing out the agonist we bathed the cells in Ca^{2+}-free solution and we performed a second application of the agonist that did not produce any elevation of cytosolic calcium. Finally to test whether the lack of response was due of the ability of the calcium-free/EGTA treatment to affect the filling state of the stores, we applied 1 μM ATP; the nucleotide elicited a transient release of Ca^{2+} in 39 out of the 41 cells that had responded to the adenosine administration in the presence of extracellular Ca^{2+} but that had not responded in calcium-free/EGTA. Taken together, these data indicate that the $[Ca^{2+}]_i$ rise induced by adenosine is solely due to Ca^{2+} influx from the extracellular medium. To characterize the channel further, we also performed experiments with Gd^{3+}. Micromolar or submicromolar concentrations of the lanthanide are sufficient to block, among others, VOCCs [18]. Indeed, in these cells 1 μM Gd^{3+} blocked high K^+-induced Ca^{2+} responses (data not shown). To investigate the effect of Gd^{3+}, the cells were subjected to adenosine in the presence of 1 μM Gd^{3+}. Under these conditions, the $[Ca^{2+}]_i$ response was observed in 42% of the cells tested ($n = 87$ out of 205 cells, Fig. 3b). On the contrary the response to adenosine was completely inhibited when the Gd^{3+} concentration was increased to 100 μM ($n = 97$ cells, Fig. 3c). These data suggest that VOCCs are not the major mediators of adenosine-induced Ca^{2+} entry in glial cells.

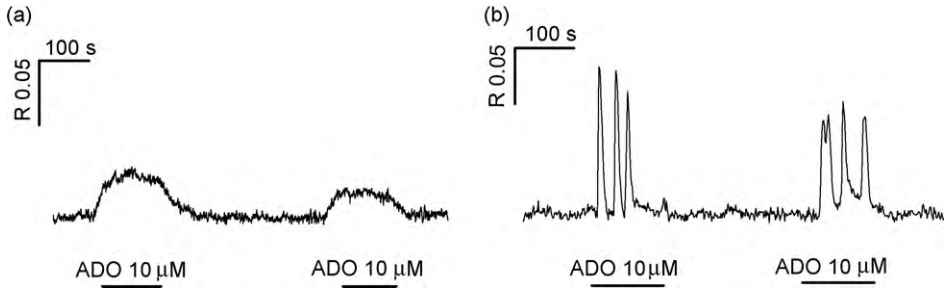

Fig. 2. Calcium signals induced by adenosine. Ciliary ganglion cells were incubated with Fura-2 AM, washed, and continuously perfused with Tyrode saline solution in the presence or absence of 10 μM adenosine. Changes in $[Ca^{2+}]_i$ are expressed as $R = F_{340}/F_{380}$. Bars indicate the duration of agonist application. Adenosine application promotes two different pattern of calcium response in glial cells: (a) sustained and (b) oscillating. As shown in the representative traces, most cells (65%) respond to a second agonist application always with the same kind of response (i.e. oscillating or sustained), usually of less amplitude.

Furthermore, the absence of any measurable Ca^{2+} release would suggest that the store-operated Ca^{2+}channels do not participate either.

Finally, we investigated the effect of A_2 receptor antagonists on Ca^{2+} influx. As stated previously and shown in Fig. 2a, a second application of adenosine to the same cell yields a response in 65% of cells. Therefore, to analyze the effect of the antagonists, we opted to stimulate cells with adenosine twice, first in the absence (control) and then in the presence of SCH 58261 or alloxazine. In these experiments, 78% of cells that responded to the first adenosine stimulation (46%, $n = 21$ out of 46) still responded in the presence of 10 μM of alloxazine (Fig. 4a). On the other hand, no cell responded in the presence of SCH 58261 (10 μM; $n = 68$; Fig. 4b).

We conclude that the A_{2A} purinergic receptor subtype is involved in the generation of adenosine-induced Ca^{2+}entry.

3.3. Forskolin activates a PKA-independent Ca^{2+}influx

In an attempt to better understand the signal pathway which leads to adenosine–Ca^{2+} influx activation, we stimulated the cells with forskolin, an adenylate cyclase (AC) activator. As shown in the Fig. 5a, 1 μM of forskolin triggers an increase in $[Ca^{2+}]_i$ in 36% of cells tested ($n = 12$ out of 33). Of these 12 cells, 2 cells exhibited a sustained response and 10 cells showed spontaneous oscillations after forskolin stimulation. Forskolin-induced $[Ca^{2+}]_i$ responses were still observed in the presence of 1 μM Gd^{3+} (37%, $n = 15$ out 41, Fig. 5b) but were abolished in the presence of 100 μM of Gd^{3+} ($n = 65$, Fig. 5c).

Next, in order to investigate the involvement of PKA in forskolin-activated $[Ca^{2+}]_i$ responses we tested the effect of the PKA inhibitor H-89. Cells were pre-incubated for 30 min with 1 μM H-89. In the presence of H-89 both forskolin and adenosine still generated $[Ca^{2+}]_i$ responses (68%, $n = 36$ out of 53, 29 sustained and 7 oscillating responses and 41%, $n = 22$ out of 53, respectively; see Fig. 5d for a cell in which both agents elicited responses). Finally calcium increases were never observed in response to forskolin application in calcium-free solution ($n = 32$, Fig. 5e). To test the validity of H-89 in our system, we opted to analyze the effect of this compound on VOCC activity, as it is well known that inhibition of PKA produces a reduction of the plateau phase of 40 mM KCl-induced calcium influx [19]. As expected, H-89, also at 0.1 μM, had a significant effect (Fig. 1g), supporting the validity of our conclusions.

The guanine nucleotide exchange factor Epac is a direct target of cAMP [20]. The most ubiquitously expressed variant of Epac is Epac1, which couples cAMP production to the small GTPase protein Rap [21]. In order to explore the possibility that Epac1 is involved in mediating the effect of adenosine and forskolin on Ca^{2+} influx, we verified the effect of the cell permeant cAMP analogue 8-CPT-2′-O-Me-cAMP, a selective activator of Epac1. However, no $[Ca^{2+}]_i$ responses were observed after application of 10 μM or 50 μM

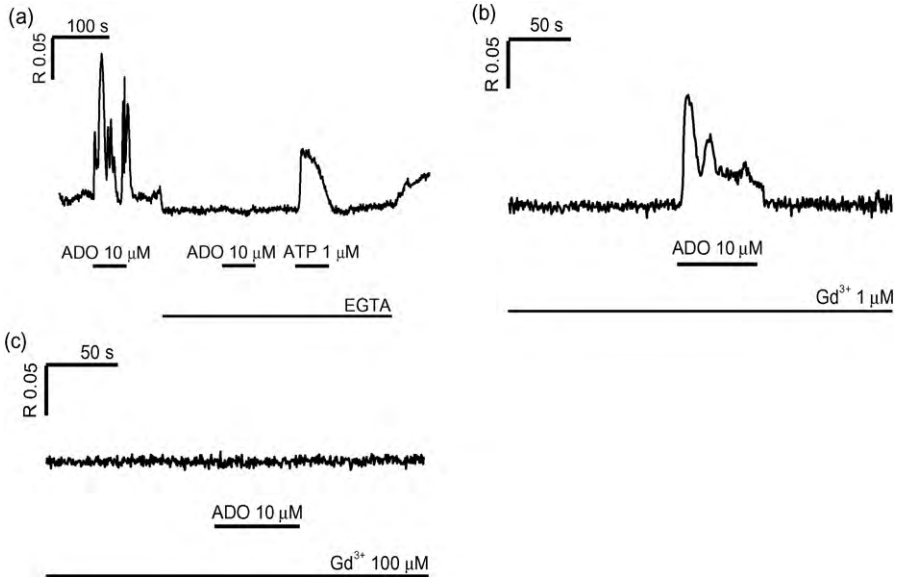

Fig. 3. Adenosine induces Ca^{2+} entry but not Ca^{2+} release. (a) When cells were bathed in a nominally Ca^{2+}-free Tyrode solution containing 0.5 mM EGTA, 10 μM adenosine, unlike 1 μM ATP, fails to evoke a $[Ca^{2+}]_i$ response. (b) Lack of effect of Gd^{3+} (1 μM) on adenosine-induced $[Ca^{2+}]_i$ responses. (c) Gd^{3+} 100 μM completely inhibits the $[Ca^{2+}]_i$ response activated by adenosine.

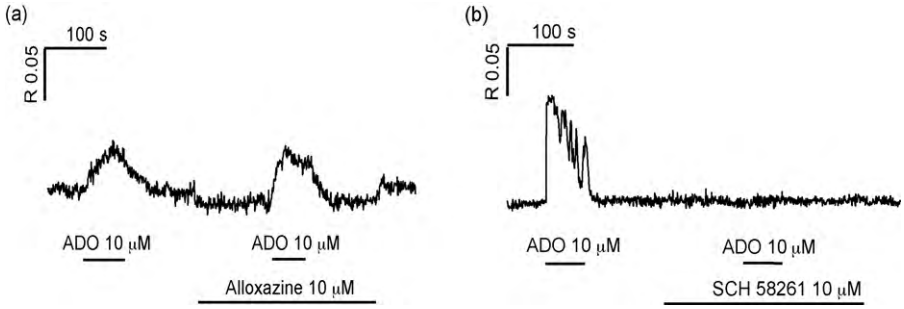

Fig. 4. The A_{2A} purinergic receptor subtype is involved in the generation of adenosine-mediated calcium entry. Representative traces showing the effects of A_2R antagonists on adenosine-induced $[Ca^{2+}]_i$ response. (a) The $A_{2B}R$ antagonist alloxazine fails to inhibit $[Ca^{2+}]_i$ rises elicited by adenosine. (b) Adenosine-induced $[Ca^{2+}]_i$ response is completely blocked by the $A_{2A}R$ antagonist SCH 58261.

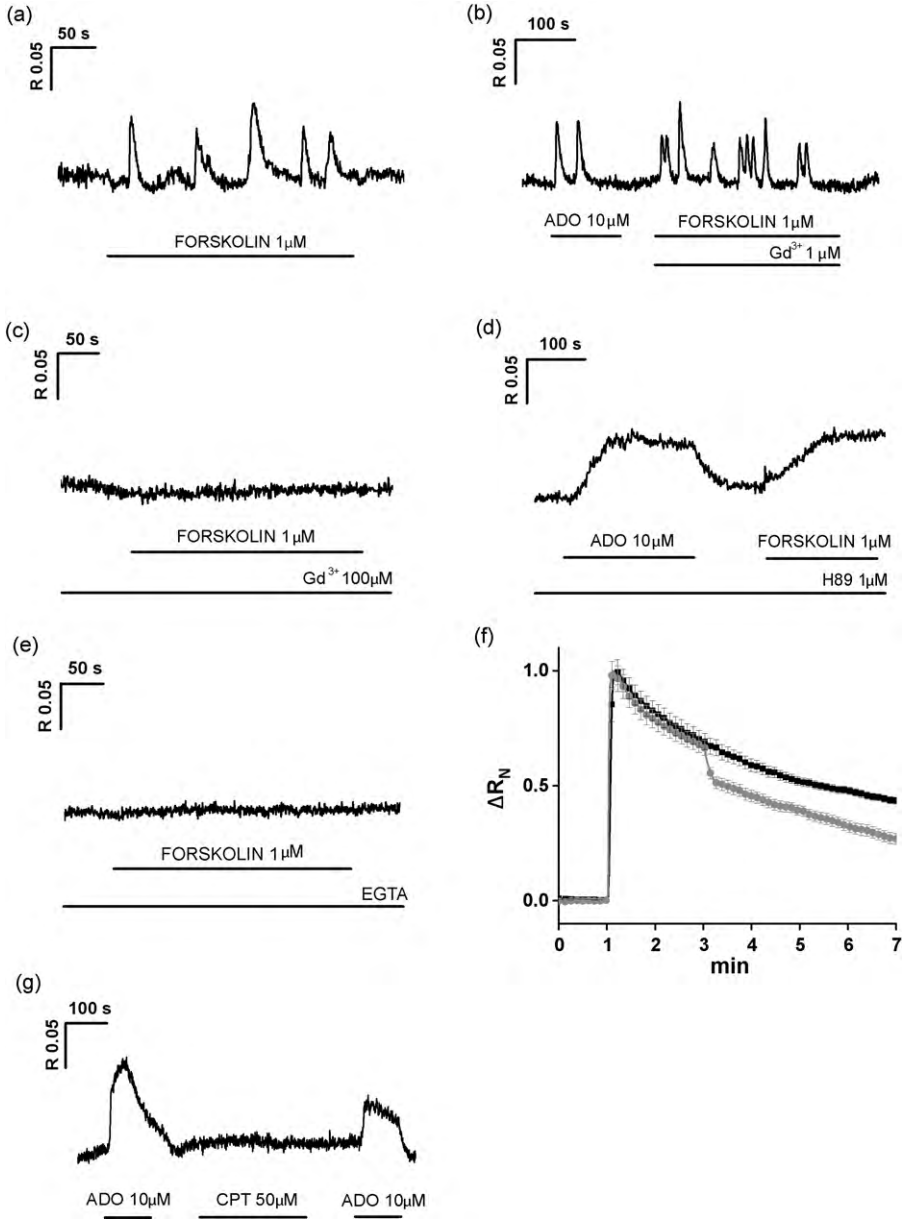

Fig. 5. Forskolin activates Ca^{2+} entry via a PKA- and Epac1-independent mechanism. $[Ca^{2+}]_i$ responses by application of forskolin (a) are still observed in the presence of 1 μM of Gd^{3+} in the external solution (b). Forskolin fails to activate $[Ca^{2+}]_i$ in the presence of 100 μM Gd^{3+} (c) or nominally Ca^{2+}-free Tyrode solution containing 0.5 mM EGTA (e). $[Ca^{2+}]_i$ responses were still elicited by adenosine and forskolin (d) in cells incubated with the PKA inhibitor H-89. (f) Effect of 1 μM H-89 on 40 mM KCl-induced Ca^{2+} influx in ciliary ganglion neurons. To facilitate the comparison, averaged traces were normalized to 1 and superimposed. Black: mean and SEM of normalized $[Ca^{2+}]_i$ response ΔR_N obtained from $n = 11$ ciliary ganglion neurons in response to the 40 mM KCl depolarization stimulus applied at min 1. Gray: Effects of H-89 applied 2 min after depolarization induced by 40 mM KCl ($n = 17$). A ΔR_N reduction of 40.0% was observable at time 7 min. Similar results were obtained in glial cells (48.4%, $n = 5$) and at other H-89 concentration tested (0.1 μM: 62.7% for glial cells ($n = 7$) and 33.1% for neurons ($n = 12$); 10 μM: 53.1% for glial cells ($n = 9$) and 40.0% for neurons ($n = 6$)). (g) 8-CPT-2′-O-Me-cAMP, a selective activator of Epac1, fails to activate calcium signals in glial cells.

8-CPT-2′-O-Me-cAMP in all cells tested ($n = 23$ and $n = 23$, respectively; Fig. 5 f).

Taken together, our data are consistent with a direct action of cAMP on membrane voltage-independent calcium channels.

3.4. Forskolin and 8-CPT-2′-O-Me-cAMP mimic the effect of adenosine on migration rate

Because A_{2B} receptors are coupled to AC in virtually all cell types, we tested the effect of forskolin on cell migration. Forskolin (1 μM) significantly decreased the mean speed to 64% ($n = 31$), suggesting an important role of cAMP as a negative regulator of glial migration speed. Therefore, we further investigated the involvement of PKA in the adenosine-activated signal transduction pathway that regulates cell migration. In another set of experiments, the cells were pre-incubated for 30 min with H-89 and then exposed to 10 μM adenosine in the presence of H-89 (0.1 μM or 1 μM). As shown in Fig. 6a, we found that under these conditions adenosine still slows the migration rate of cells, as the mean values (1.67 ± 0.04 μm/min, $n = 41$, and 1.55 ± 0.03 μm/min, $n = 48$, respectively) did not differ significantly from the value obtained with the nucleoside alone.

The lack of effect of PKA inhibition therefore suggests the involvement of an alternative pathway. For this reason, we tested the hypothesis that Epac1 signalling regulates cell migration. As shown in Fig. 6a, 10 μM and 100 μM 8-CPT-2′-O-Me-cAMP significantly decreased the speed to 87% ($P < 0.01$, 1.61 ± 0.06 μm/min, $n = 30$) and to 78% ($P < 0.001$, 1.44 ± 0.04 μm/min, $n = 38$, respectively). We conclude that Epac1 activation induces a dose-dependent negative modulation of the mean rate.

Finally, inspection of time-lapse experiments revealed that adenosine and 8-CPT-2′-O-Me-cAMP induce subtle but detectable morphological changes. Migrating satellite glial cells move by protrusion of a broad leading lamellipodium which is actively ruffling. In the presence of adenosine and more markedly of the Epac activator, the ruffling appears to increase (see supplementary material) and the lamellipodium edge results better defined, as indicated by the arrows in the representative images of Fig. 6b_2 and 6b_3. This latter phenomenon can be due to an apparent increase of the thickness of the lamellipodium edge, which may be dependent on the detachment of the membrane edge that refolds or retracts briefly before adhering again to the substrate.

In conclusion, these results are consistent with the involvement of the cAMP/Epac pathway in the adenosine-induced regulation of CG glial cell migration.

4. Discussion

In the present study we provide for the first time evidences that cultured CG satellite glial cells express functional purinergic receptors for adenosine and ATP. Our results identify the nucleoside as a novel extracellular modulator of the migratory activity in the nervous system. We show that adenosine decreases the rate of migration through A_{2B}Rs, which are positively coupled via G_s proteins to adenylate cyclase, via a PKA-independent pathway. In addition, we demonstrate that the adenylate cyclase activator forskolin and the Epac1 activator 8-CPT-2′-O-Me-cAMP were both able to slow the cell speed of migration, suggesting that the inhibitory effect of adenosine on glial cell migration is associated with the AC/cAMP/Epac1 pathway.

Changes of cAMP intracellular concentration have been identified as key events in the modulation of neuronal cell migration by neuropeptides and ethanol [22–24]. In postnatal cerebellar granule cells increases in cAMP levels induced by pituitary adenylate cyclase-activating polypeptide (PACAP) are inhibitory signals of migration that are mediated by an enhancement of PKA activation

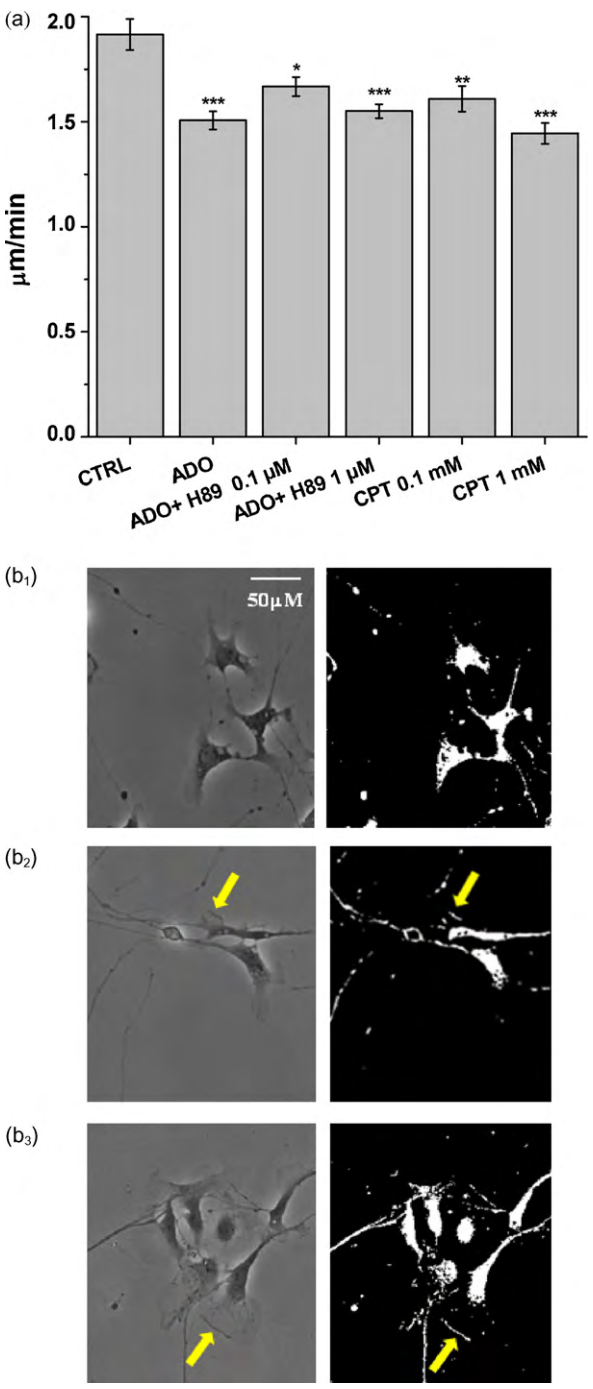

Fig. 6. Effect of the PKA inhibitor H-89 or of the Epac1 activator 8-CPT-2′-O-Me-cAMP on cell migration. (a) Bar graph compares the mean modulus of the glial cell velocity in control conditions (CTRL), in the presence of 10 μM adenosine (ADO) alone, in the presence of 10 μM adenosine (ADO) and 0.1 μM H-89 (ADO + H89 0.1 μM), in the presence of 10 μM adenosine and 1 μM of H-89 (ADO + H89 1 μM) inhibitor H-89, and in the presence of two different concentration (CPT 0.1 mM and CPT 1 mM) of 8-CPT-2′-O-Me-cAMP. Adenosine still slows the rate of migration of cells treated with the PKA inhibitor. The Epac1 activator mimics the effects of adenosine in a dose-dependent manner. $^*P < 0.02$, $^{**}P < 0.01$, $^{***}P < 0.001$. The control and adenosine bars are the same as Fig. 1. (b) Adenosine and 8-CPT-2′-O-Me-cAMP affect the morphology and the dynamics of the lamellipodium. (b_1) Representative image of cell cultures bathed in control conditions. In the presence of 10 μM adenosine (b_2) or 1 mM 8-CPT-2′-O-Me-cAMP (b_3) ruffling increases (see supplementary material) and the lamellipodium edge appears better defined, as indicated by the arrows. To make the effect more clear, the images were also converted and showed as binary images.

[25]. Another neuropeptide, somatostatin, also affects migration of granule cells by inhibiting the cAMP signalling pathway [26] and reduces the effect of PACAP [23]; moreover, alteration of the cAMP/PKA system affects motility and chemotaxis of various other cells types [27,28]. More recently, the small GTPase protein Rap via the cAMP/Epac1 pathway has been found to play a crucial role as a negative regulator of migration in epithelial cells and in ovarian tumor cells [29,30]. In epithelial cells Rap activation inhibits migration by modulating the focal adhesions and lamellipodial activity. We suppose that the nucleoside acts on glial cell by influencing the cell motility machinery with the same or a similar mechanism that modulate the membrane adhesion properties, as suggested by the changes in lamellipodia dynamic which we have observed in ciliary ganglion migrating cells in the presence of adenosine or 8-CPT-2′-O-Me-cAMP.

The ability of adenosine to generate Ca^{2+} signals has already been shown in different types of glial cells, via either A_{2A} or A_{2B} which induce Ca^{2+} release from intracellular stores [31,32]. Herein, we demonstrate the ability of adenosine to induce, via A_{2A}Rs, changes in $[Ca^{2+}]_i$ through the sole activation of Ca^{2+} influx. Moreover, we demonstrate that the adenosine-induced Ca^{2+}influx is still evoked in the presence of 1 μM Gd^{3+} and, taken together, these results indicate the existence of a novel pathway for A_{2A}R that is independent from both Ca^{2+} store depletion and VOCC activation. Because Ca^{2+} and cAMP signalling pathways can interact with each other [33,34], we tried to determine the mechanisms that lead to the Ca^{2+} channel opening in these cells and in particular we have investigated the role of cAMP. Data presented in this study demonstrate that forskolin activates a Ca^{2+} influx which shares all the properties of the Ca^{2+}-signals generated by adenosine. However, neither PKA nor Epac seem to be involved in their activation; therefore, the simplest explanation for the activation of the Ca^{2+} influx pathways is via activation of cyclic-nucleotide gated channels. Cyclic-nucleotide gated channels are widely expressed not only in sensory organs, but also in several other tissues, including the chick embryo nervous system [35,36].

When Ca^{2+} influx was blocked by the specific A_{2A} antagonist, no changes in the modulatory action of adenosine were observed, suggesting that the activation of Ca^{2+} pathway is not the mechanism responsible for the inhibitory response on migration caused by adenosine.

Adenosine and adenosine receptors have been implicated in a number of neurological disorders, including traumatic brain injury [37] and neurodegenerative disorders [38]. Furthermore, these effects by adenosine appear to be attributable to receptors localized on either glia or microglia. Of particular interest is the finding that ATP/ADP release from injured tissue induces brain microglial chemotaxis via G_i-coupled $P2Y_{12}$ receptors. On the other hand, in activated microglia, adenosine derived from ATP breakdown promotes microglia process retraction via G_s-coupled adenosine A_{2A} receptor activation and cAMP–PKA dependent pathways [38,39]. It would therefore be exciting to speculate that the effects played by adenosine on motility and calcium signalling described in this manuscript might play a role in certain neuropathologies and that A_{2B} receptors could be considered, alongside A_{2A}, as new therapeutic targets in these disorders.

In conclusion, this study provides evidence that adenosine regulates migration in developing glial cells via the A_{2B}R/cAMP/Epac1 pathway and reveals that adenosine affects Ca^{2+} homeostasis through a novel mechanism which appears to be dependent on cAMP but independent of PKA and Epac1.

Conflict of interest

Authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ceca.2010.07.004.

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