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Differential adenosine uptake in mixed neuronal/glial or purified glial cultures of avian retinal cells: Modulation by adenosine metabolism and the ERK cascade

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ABSTRACT

Adenosine is an important modulator of neuronal survival and differentiation in the CNS. Our previous work showed that nucleoside transporters (NTs) are present in cultures of chick retinal cells, but little is known about the mechanisms regulating adenosine transport in these cultures. Our aim in the present work was to study the participation of the adenosine metabolism as well as the ERK pathway on adenosine uptake in different types of retinal cultures (mixed and purified glial cultures). Kinetic analysis in both cultures revealed that the uptake reached equilibrium after 30 min and presented two components. Incubation of cultures with S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) or dipyridamole, different inhibitors of equilibrative nucleoside transporters (ENTs), produced a significant and concentration-dependent uptake reduction in both cultures. However, while dipyridamole presented similar maximal inhibitory effects in both cultures (although in different concentrations), the inhibition by NBTI was smaller in glial cultures than in mixed cultures, suggesting the presence of different transporters. Moreover, pre-incubation of [^{3}H]-adenosine with adenosine deaminase (ADA) or adenosine kinase (ADK) inhibition with iodotubercidin promoted significant uptake inhibition in both cultures, indicating that the uptake is predominantly for adenosine and not inosine, and that taken up adenosine is preferentially directed to the synthesis of adenine nucleotides. In both cultures, the MEK inhibitors PD98059 or UO126, but not the inactive analog U0124, induced a significant and concentration-dependent uptake decrease. We have not observed any change in adenosine metabolism induced by MEK inhibitors, suggesting that this pathway is mediating a direct effect on NTs. Our results show the expression of different NTs in retinal cells in culture and that the activity of these transporters can be regulated by the ERK pathway or metabolic enzymes such as ADK which are then potential targets for regulation of Ado levels in normal or pathological conditions.

1. Introduction

Adenosine mediates its effects via specific G-protein-coupled receptors named A1, A2A, A2B and A3 [1,2]. Activation of A1 receptors (A1Rs) has been shown to be neuroprotective during ischemia and epilepsy [3,4], and together with synaptic modifications induced by stress in hippocampus, a down regulation of these receptors was reported [5]. Nevertheless, A1Rs could also exacerbate neurotoxic effects induced by kainate in cultured cortical neurons [6]. Adenosine intra and extracellular levels are regulated by bidirectional NTs and enzymes related to purine metabolism. NTs are classified as concentrative or equilibrative. Three subtypes of concentrative transporters promote nucleoside influx in a sodium-dependent manner [7]. On the other hand, there are four described subtypes of equilibrative transporters, which promote sodium-independent nucleoside transport according to the concentration gradient [7], and ENT1 and ENT2 are the predominant isoforms expressed in the CNS [8,9]. Concerning the enzymes, adenosine kinase (ADK) and ADA are the main players in adenosine inactivation. Few studies have shown that ADK activity can be regulated by different kinases. For example, some reports show the modulation of ADK expression or activity in different cellular models by different kinases and signaling molecules, such as calcineurin [10], ERK 1/2 activated by insulin [11] and nitric oxide [12]. To our knowledge, few studies show measurements of ADK activity in retina [13], but several studies reported the presence of adenosine, its receptors and transporters in retinas from different species [13–17]. In chicken retina, the presence of ENT1 was demonstrated using [^{3}H]-NBTI binding and shown to be present from embryonic day 8 up to post-hatching animals [18]. This work also showed the presence of [^{3}H]-NBTI binding sites mostly in plexiform layers, in a localization similar to that of A1Rs [18]. Co-localization of ENT1 and A1Rs was also observed in other CNS structures [19]. The presence of ENT1 was previously detected in chick retina mixed neuronal/glial cultures [20] as well as in purified chick retina neuronal cultures [16]. Some additional work has already demonstrated that NTs are modulated by protein kinases such as PKC [21], PKA [22]

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and JNK [23]. Our previous study also showed that adenosine uptake is modulated by CAMK II [24]. Here, we have investigated the mechanisms involved in the regulation of adenosine uptake in different types of chick retinal cultures. We found that the uptake kinetic parameters or inhibition profile by NT blockers differ in mixed or glial cultures, but is similarly inhibited by ADK inhibitor or ERK pathway blockers.

2. Materials and methods

2.1. Materials

Fertilized White Leghorn eggs were obtained from a local hatchery and incubated at 38 °C in a humidified atmosphere. L-Glutamine, adenosine deaminase (ADA), NBTI, adenosine, 5′-iodotubercidin, 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate (U0126), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), dipyridamole, penicillin G and streptomycin sulfate were obtained from Sigma/RBI Chem. Co. (Missouri, USA). U0124 was obtained from Tocris Bioscience (Ellisville, MO, USA). Minimum essential medium (MEM), heat-inactivated fetal bovine serum (FBS) and trypsin were obtained from GIBCO (New York, USA). [2–^{3}H]-Adenosine (22–28 Ci/mmol) was obtained from GE Healthcare Life Sciences (Buckinghamshire, United Kingdom). All other reagents were of analytical grade.

2.2. Preparation of mixed cultures

Mixed cultures of chick retina cells were prepared as previously described [25]. Briefly, retinas from 8-day-old chick embryos (E8) were dissected and digested with 0.2% trypsin, in calcium and magnesium-free Hank's solution (CMF), for 15 min at 37 °C. Cells were suspended in MEM supplemented with 3% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml) and glutamine (2 mM), and seeded in tissue culture plastic dishes in a density of 2×10^4 cells/mm^2. Cultures were maintained at 37 °C in a humidified incubator with 95% air and 5% CO_2. The medium was changed after 1 day in culture (C1) and experiments were performed at C3–C4.

2.3. Preparation of Purified Muller glia cultures

Purified Muller glia chick retina cultures were prepared as previously described [26], with minor modifications. Cells were

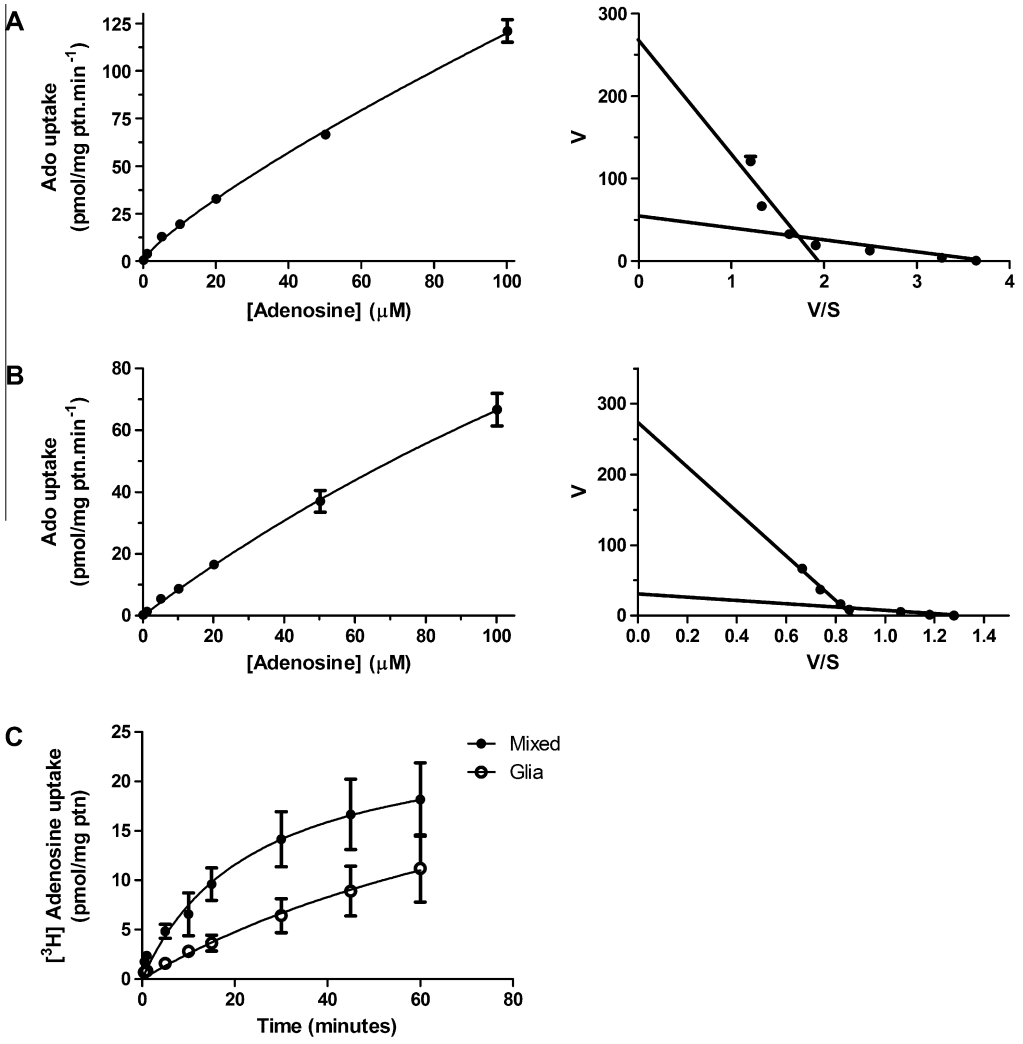

Fig. 1. Kinetic characterization of adenosine uptake in mixed (A and C) and glial cultures (B and C). (A and B) Cultures were washed and incubated with [^{3}H]-adenosine (0.2 μCi/ml) plus different non-labeled adenosine concentrations for 15 min at 37 °C or incubated with [^{3}H]-adenosine (0.2 μCi/ml) for different periods of time (C), rinsed in buffer, and lysed in water to determine intracellular radioactivity. Values represent means ± SEM and the results shown are from three separate experiments performed in triplicate. The points without bars represent the results in which the deviation from the mean was smaller than the symbol size. (A and B, right): Eadie–Hofstee plot of the data.

seeded in tissue culture plastic dishes in a density of 1.8×10^6 cells/ml. Cultures were maintained at 37 °C in a humidified incubator with 95% air and 5% CO_2. The medium was changed every 3 days and experiments were performed at C21.

2.4. [^{3}H]-Adenosine uptake assays

All adenosine uptake assays were conducted at 37 °C in HEPES-buffered salt solution (HBSS) containing 140 mM NaCl, 5 mM KCl, 20 mM HEPES, 4 mM glucose, 1 mM $MgCl_2$ and 2 mM $CaCl_2$, pH 7.4. Before the experiments, the medium was removed and the wells rinsed twice with HBSS solution. Cultured cells were pre-incubated for 10 min in absence or presence of different drugs, [^{3}H]-adenosine (0.2 μCi/ml, ~150 nM) was added and cultures further incubated for 15 min. The dishes were then rinsed twice with HBSS solution and lysed with water for determination of intracellular radioactivity by liquid scintillation.

2.5. Identification of intracellular radioactivity by thin layer chromatography (TLC)

In order to analyze the intracellular radioactivity after [^{3}H]-adenosine uptake, cells were lysed with 5% TCA, removed from the dishes, the material centrifuged at 20,627*g* for 15 min and the supernatant mixed with standards, applied to TLC plates and run in a mixture of butanol, ethyl acetate, methanol and ammonium hydroxide (7:4:3:4) [27]. The spots detected under UV light were scrapped off from the plates and the radioactivity determined by liquid scintillation.

2.6. Statistical analysis

Data are presented as mean ± SEM from *n* experiments. Statistical significance was assessed by one-way ANOVA followed by the Bonferroni test, using GraphPad Prism 5 software. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of NTs involved in adenosine uptake by cultured retinal cells

We have characterized the kinetic parameters involved in adenosine uptake in mixed neuronal/glial and purified glial cultures in order to define the time of incubation with [^{3}H]-adenosine, as well as the adenosine concentration to be used in subsequent experiments. Adenosine transport in both cultures was concentration-dependent (Fig. 1A and B) and exhibited at least two uptake components, as demonstrated by Eadie–Hofstee plot, which was curvilinear in both cultures (Fig. 1A and B, right panels). The K_m values observed in mixed cultures were 14.4 ± 2.4 and 130.9 ± 42.0 μM and V_{max} values 51.1 ± 6.5 and 258.6 ± 64.7 pmols/mg protein. minute. In contrast, different values were obtained in glial cultures (K_ms of 30.4 ± 7.2 and 300.8 ± 29.7 μM and V_{max}s of 38.1 ± 7.6 and 263.8 ± 23.0 pmols/mg protein.minute). The time course of adenosine uptake was linear for incubation times ranging from 0.5 to 15 min in both cultures and tended to equilibrium in mixed cultures but not in glial cultures (Fig. 1C). All subsequent experiments were performed using the incubation time of 15 min for both types of culture. We have not observed any change on [^{3}H]-adenosine uptake when using sodium-free HBSS (data not shown), suggesting an absence of sodium-dependent concentrative nucleoside transporters in both cultures. In order to characterize the relative contribution of ENT1 and 2, we decided to use NBTI and dipyridamole, two different inhibitors of ENTs. Our results demonstrated that treatment with each inhibitor led to inhibition of [^{3}H]-adenosine uptake in both cultures in a concentration-dependent fashion (Fig. 2A and B). Interestingly, the maximal inhibition with NBTI was larger in mixed than in glial cultures (Fig. 2A) but the same was not observed with dipyridamole (Fig. 2B), which presented a very similar maximal inhibition but with very distinct concentrations. In mixed cultures, dipyridamole and NBTI presented a very similar inhibition profile, a fact that was not observed in glial cultures (compare Fig. 2A and B).

3.2. ADK is a main driving force for adenosine uptake in retinal cultures

Pre-incubation of [^{3}H]-adenosine with ADA (2 U/ml) for 45 min before addition to cultures strongly reduced the uptake in both mixed and glial cultures (Fig. 3A), demonstrating a low efficiency of cells to take up inosine, the product of adenosine deamination. In order to verify if ADK was an important driving force for adenosine uptake in retinal cultures, we used 5′-iodotubercidin, a known inhibitor of ADK. A significant reduction of uptake was observed in both cultures after treatment with this compound at different concentrations (Fig. 3B).

3.3. Inhibition of adenosine uptake by MEK inhibitors

Few studies have shown that ERK is able to modulate the expression and/or activity of neurotransmitter transporters, as for example glutamate transporters [28], as well as dopamine transporters [29]. PD98059 or UO126, two MEK inhibitors, produced concentration-dependent inhibitory effects on [^{3}H]-adenosine uptake in both mixed and glial cultures (Fig. 4A and B, respectively). We have also

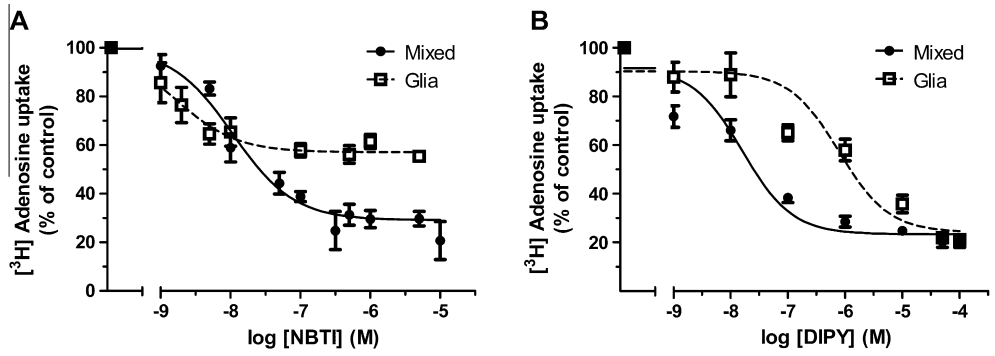

Fig. 2. Effects of NBTI and dipyridamole on [^{3}H]-adenosine uptake in mixed and glial cultures. (A and B) Cells were pre-incubated in absence or presence of different concentrations of NBTI or dipyridamole for 10 min and subsequently incubated for 15 min at 37 °C after addition of [^{3}H]-adenosine. The values are the mean ± SEM from at least three separate experiments performed in triplicate and error bars are not shown where the SEM values were smaller than the size of the symbol.

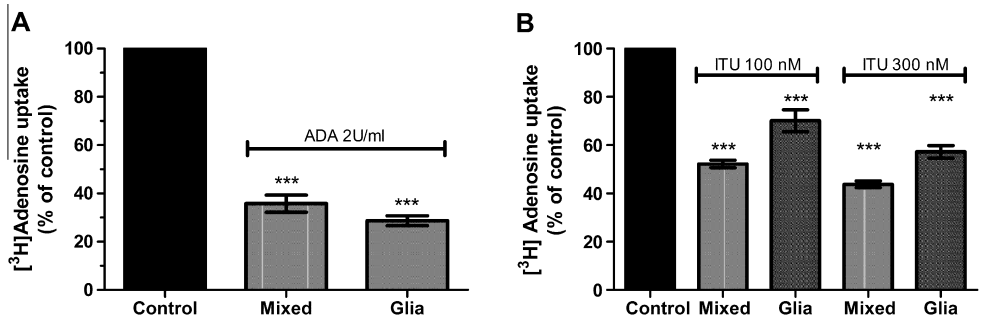

Fig. 3. Effect of [^{3}H]-adenosine pre-incubation with ADA and ADK inhibition on [^{3}H]-adenosine uptake in mixed and glial cultures. (A) [^{3}H]-adenosine (0.2 μCi/ml) was pre-incubated with ADA (2 U/ml) for 45 min at 37 °C and subsequently added to the cultures which were further incubated for 15 min at 37 °C. (B) Cells were pre-incubated in absence or presence of different ITU concentrations (100 or 300 nM) for 10 min and then subsequently incubated for 15 min at 37 °C after addition of [^{3}H]-adenosine. Values are the mean ± SEM from at least three separate experiments performed in triplicate. $^{***}p < 0.001$ (compared to control).

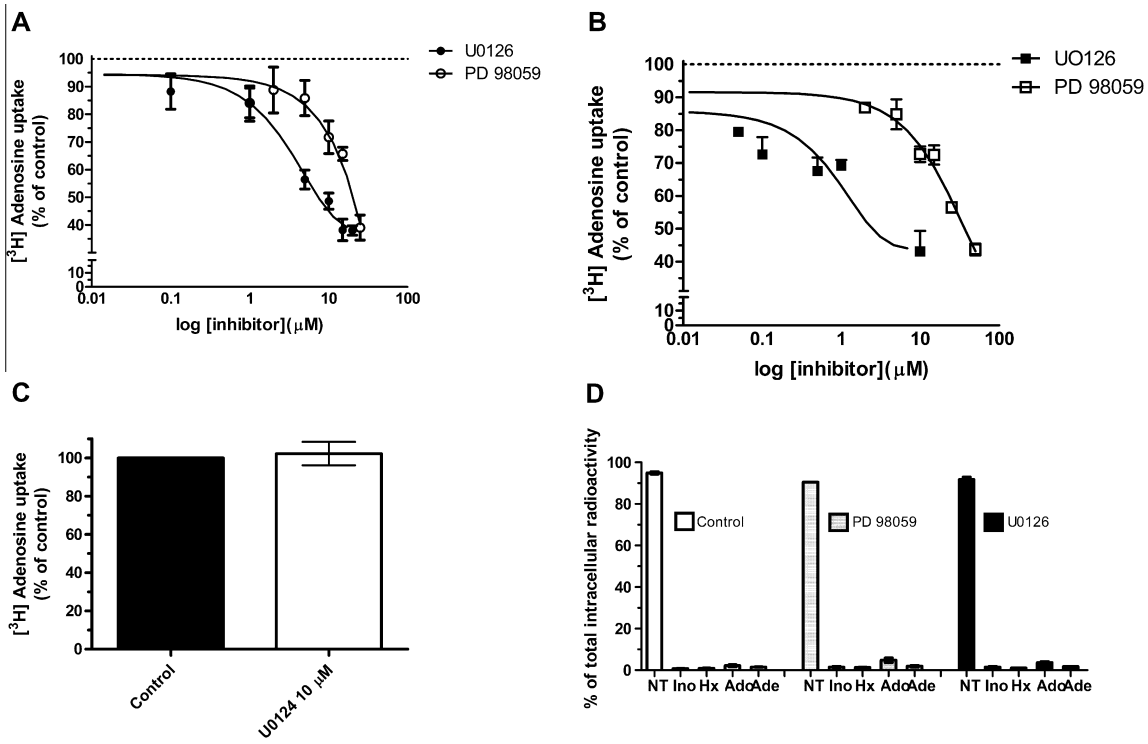

Fig. 4. Effect of MEK inhibitors PD98059 or U0126 on [^{3}H]-adenosine uptake and metabolism by cultured retinal cells. (A and B, mixed and glial cultures, respectively) Cultures were washed and pre-incubated for 10 min with different concentrations of PD98059 or U0126 and subsequently incubated for 15 min at 37 °C after addition of [^{3}H]-adenosine and cell lysis for determination of intracellular radioactivity. (C) Cultures were washed and pre-incubated in absence or presence of U0124 (10 μM) for 10 min and subsequently incubated for 15 min at 37 °C after addition of [^{3}H]-adenosine. Cells were then lysed and the intracellular radioactivity determined. Values represent the mean ± SEM from at least three separate experiments performed in triplicate. (D) Distribution of intracellular radioactivity after [^{3}H]-adenosine uptake in control and PD98059 (25 μM) or U0126 (10 μM)-treated mixed cultures. After incubation with [^{3}H]-adenosine, intracellular radioactivity was extracted and the samples applied with standards to TLC plates. The results are expressed in percent of total radioactivity and represent the mean ± SEM from three separate experiments.

observed a similar effect in purified neuronal cultures (data not shown). Previous studies indicate that some kinase inhibitors could have non-specific effects on NTs in a way independent of kinase inhibition [30,31]. We then used U0124, an inactive analog of U0126 which does not inhibit MEK activity, and found that this compound produced no change on uptake in mixed cultures (Fig. 4C). To verify whether the observed decrease on uptake promoted by different MEK inhibitors was due to inhibition of adenosine metabolism, we performed experiments measuring adenosine metabolites using TLC. As can be observed in Fig. 4D, we have not observed any detectable change in the metabolite profile of mixed cultures after treatment with PD98059 or UO126.

4. Discussion

4.1. ENTs are the main mediators of [^{3}H]-adenosine uptake in retinal cultures

Adenosine markers such as different receptors and transporter systems are known to be expressed in the retina *in vivo* and in cultured retinal cells [16–18,24,32]. However, very little is known about the characteristics of [^{3}H]-adenosine uptake and the mechanisms involved in the regulation of intracellular or extracellular nucleoside levels. Our previous work has already demonstrated that uptake sites labeled with [^{3}H]-NBTI and detected using

binding and auto-radiographic methods are present in developing chicken retinas from E8 up to post-hatching animals [18]. At E8, NBTI binding sites showed a diffuse distribution, but are localized to the plexiform layers of more developed retinas, a similar localization as that found for A1Rs [18]. This co-localization between ENT1 and A1Rs was also observed in different brain structures [19]. In the present study, we have shown that ENTs are the main mediators of [^{3}H]-adenosine uptake in mixed cultures, in a way similar to what was found in cultured chick retinal neurons [16], where [^{3}H]-adenosine uptake was inhibited more than 80% by NBTI. However, glial cultures showed a different profile and a maximum of 50% inhibition of uptake was observed with NBTI. Interestingly, dipyridamole did promote a maximum of 80% inhibition in both cultures, although with different potencies. These results strongly indicate that mixed or glial cultures express uptake sites with different pharmacological properties. One hypothesis is that glial cells grown in purified cultures express ENT2 besides ENT1 transporters with ENT2 being sensitive to dipyridamole but not to NBTI, as shown in other studies [7,33].

4.2. Control of adenosine metabolism by different protein kinases

Adenosine uptake systems present in mixed or glial cultures are more efficient in taking up adenosine than inosine, since we had a large decrease of uptake in both cultures when [^{3}H]-adenosine was pre-incubated with ADA. This result is in accordance with data showing that ENT1 presents low affinity for inosine (K_m 170 μM as compared to 40 μM for adenosine), while ENT2 has an affinity almost fourfold higher for inosine than ENT1 (K_m 50 μM) [33,34]. Accordingly, the study by Zamzow et al. [35] indicates that inosine is released through ENT2.

Besides NTs, ADK and ADA activities are also important in the regulation of adenosine levels. Our previous work has already demonstrated that, in mixed and purified neuronal cultures, most of the taken up [^{3}H]-adenosine is converted to adenine nucleotides [16,24], as also observed in mouse primary cultured cortical astrocytes [36]. In purified glial cultures, similarly to what was observed in other retinal cultures, we have observed an intense conversion of taken up [^{3}H]-adenosine to nucleotides (data not shown). These data suggest that chick retinal cultures have a strong ADK activity. On the other hand, some groups have already shown, using different models [36,37], that most taken up adenosine is converted to inosine/hypoxanthine, indicating a larger ADA activity with respect to ADK. However, ADA activity does not seem to have a significant role in our cultures, because incubation with EHNA, an ADA inhibitor, did not induce any change in [^{3}H]-adenosine uptake (data not shown). On the other hand, we have observed a significant reduction of [^{3}H]-adenosine uptake in mixed and purified glial cultures treated with 5′-iodotubercidin, an ADK inhibitor.

4.3. Control of NTs by different protein kinases

NTs may have their activity regulated by activation/inhibition of protein kinases, despite the cellular mechanisms involved still remain not understood. Different kinases were previously reported to modulate adenosine uptake [21,22,24,38–41]. In this study, PD98059 and U0126, two different MEK inhibitors, caused a strong and significant decrease of [^{3}H]-adenosine uptake in both mixed and glial cultures, and a similar effect was observed in purified retinal neuronal cultures (data not shown). Mouse ADK sequence is known to have potential phosphorylation sites for several kinases, including ERK [42]. However, this same work showed that ERK was not able to phosphorylate recombinant mouse ADK *in vitro*. On the other hand, insulin has been shown to modulate ADK expression in rat lymphocytes by a mechanism mediated by MAPK cascade [11]. Our data indicate that the effect of MEK inhibitors on [^{3}H]-adenosine uptake by retinal cultures was not due to inhibition of ADK activity, since we have not detected any significant change in the amount of nucleotides formed after adenosine uptake. This raises the possibility that ERK is directly modulating transporter activity and not adenosine metabolism. To our knowledge, this is the first report demonstrating a role for the MAP kinase pathway in the control of ENT activity. One report shows that all-trans-retinoic acid-induced human CNT3 trafficking to the plasma membrane is mediated by a TGF-β1 and ERK 1/2-dependent mechanism [43].

Huang et al. [30,31] performed a screening work to identify possible effects of different kinase inhibitors on nucleoside uptake by human erythroleukemia K562 cell line, which is a cell line expressing NBTI-sensitive ENTs, and observed that inhibitors of receptor tyrosine kinases, PKC, cyclin-dependent kinases and p38 MAPK can affect nucleoside transport in a kinase-independent fashion. It is interesting to stand out that the authors did not observe any effect on uptake of the same MEK inhibitors used in the present work, PD98059 or U0126. Moreover, U0124, an inactive analog of U0126, had no effect on [^{3}H]-adenosine uptake in our cultures, strongly indicating that the effects of different MEK inhibitors on [^{3}H]-adenosine uptake are due to inhibition of the kinase itself, and not to eventual unspecific effects directly on NTs.

In conclusion, this study showed that both adenosine metabolic enzymes as well as ERKs are regulators of [^{3}H]-adenosine uptake probably through regulation of ENTs, but the mechanisms involved remains to be clarified. There is a great interest in studying the mechanisms involved in the control of adenosine levels as adenosine might be a neuroprotective agent in different pathological conditions.

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