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Adenosine A_{2A} Receptors Regulate the Extracellular Accumulation of Excitatory Amino Acids upon Metabolic Dysfunction in Chick Cultured Retinal Cells

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The role of endogenous extracellular adenosine as a tonic modulator of the extracellular accumulation of excitatory amino acids (glutamate and aspartate) caused by metabolic inhibition was investigated in cultured retinal cells. The selective adenosine A_{2A} receptor antagonist, 4-[2-[7-amino-2-(2-furyl)(1,2,4)-triazin-5-ylamino]-ethyl]phenol (ZM241385) (50 nM), increased the release of glutamate (three- to four-fold) and of aspartate (nearly two-fold) upon iodoacetic acid-induced glycolysis inhibition, in the presence or in the absence of Ca^{2+}. Blockade of tonic activation of A_{2A} receptors by ZM241385 also increased (nearly two-fold) the ischemia-induced release of glutamate and aspartate. Furthermore, another selective A_{2A} receptor antagonist, 5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261), also increased the release of aspartate and glutamate by about two-fold in cells submitted to glycolysis inhibition. In contrast, the selective adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (100 nM), did not significantly modify the extracellular accumulation of either glutamate or aspartate caused by inducers of chemical ischemia or glycolytic inhibitors. Inhibition of glycolysis also increased (about three-fold) the extracellular accumulation of GABA, which was virtually unchanged by ZM241385. Furthermore, the $GABA_A$ receptor antagonist, bicuculline (10 μM), only increased (nearly two-fold) the iodoacetic acid-induced Ca^{2+}-dependent release of glutamate, whereas the $GABA_B$ receptor antagonist, 3-aminopropyl(diethoxymethyl) phosphinic acid, CGP35348 (100 μM), was devoid of effects on the extracellular accumulation of glutamate and aspartate. These results show that endogenous extracellular adenosine, which rises under conditions of inhibited glycolysis, tonically inhibits the extracellular accumulation of excitatory amino acid through the activation of A_{2A}, but not A_1, adenosine receptors, and this effect is independent of $GABA_A$ and $GABA_B$ functions in the cultured retinal cells.

Key words: aspartate; A_1 adenosine receptors; A_{2A} adenosine receptors; adenosine; glutamate; metabolic stress; retinal cells.

1. Introduction

Extracellular adenosine modulates the function of neuronal cells by acting on G protein-coupled adenosine receptors, named A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm et al., 1994). Adenosine decreases neuronal excitability through activation of A_1 receptors and may also enhance neuronal excitability through activation of high affinity A_{2A} receptors (Cunha et al., 1994). The role of low affinity facilitatory A_{2B} receptors. for which there is a lack of selective pharmacological tools, and the role of the low affinity A_3 receptors are not yet clearly understood. The levels of extracellular adenosine increase upon electrical stimulation of brain preparations (e.g. Cunha et al., 1996) and increase even further during graded hypoxia (Fowler, 1993a), glycolysis inhibition (Rego, Santos and Oliveira, 1997) or ischemia (Phillis, Walter and Simpson, 1991; Pedata et al., 1993). Extracellular adenosine fulfills two parallel roles to modulate cellular excitability (Cunha, 1997): (i) as a homeostatic modulator, a function common to all types of eucaryotic cells, and (ii) as a neuromodulator at the synaptic level, a role which can be part of the homeostatic role of adenosine, but which can also occur independently of changes in neuronal metabolic status (Mitchell, Lupica and Dunwiddie, 1993). It is now established that tonic neuromodulation by endogenous adenosine is a balance between inhibitory A_1 and facilitatory A_{2A} adenosine receptors (Brown et al., 1990; Cunha et al., 1994; Correia-de-Sá, Timóteo and Ribeiro, 1996). In contrast, whereas the homeostatic role of tonic activation of inhibitory A_1 receptors as a neuroprotective mechanism is well understood (e.g. Rudolphi et al., 1992), the homeostatic role of facilitatory A_{2A} receptors is not well established.

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In the retina, ischemia challenges raise the extracellular concentration of adenosine (e.g. Rego et al., 1997; Roth et al., 1997) which is a major neuroprotector of ischemic damage in this tissue (Larsen and Osborne, 1996; Li et al., 1999). It has been recently described that A_1 and A_{2A} receptors appear to mediate different effects in the ischemic retina, with both A_1 and A_{2A} receptors potentiating recovery from short term (5 min) ischemia, whereas A_{2A} receptors enable recovery from long lasting (30–60 min) ischemia, a situation where A_1 receptors are devoid of effects (Li et al., 1999). These strikingly opposite effects of A_{2A} receptor activation according to the duration of the ischemic period may be due to different effects of A_{2A} receptors on the control of retinal blood flow (Gidday et al., 1996; Ostwald et al., 1997), and to eventual opposite effects on glutamate release, the major deleterious mediator of retinal ischemic damage (Yoon and Marmor, 1989).

Retinal cells in culture, where both A_1 and A_{2A} receptors are present (Agostinho et al., 2000), release adenosine and excitatory amino acids in response to chemical ischemia or glycolysis inhibition (Rego et al., 1997) and allow us to distinguish the modulation of glutamate release from the modulation of blood flow (Gidday et al., 1996; Ostwald et al., 1997) or neovascularization (Lutty et al., 1998). Thus, in this study, we used retinal cells in culture to directly investigate the relative homeostatic role of endogenous adenosine acting on A_1 and A_{2A} receptors, by testing the effect of selective A_1 and A_{2A} receptor antagonists on the extracellular accumulation of excitatory amino acids under conditions of chemical ischemia and glycolysis inhibition. Since in some neuronal systems adenosine controls the release of both excitatory and inhibitory neurotransmitters (e.g. O'Regan et al., 1992; Mayfield, Suzuki and Zahniser, 1993; Kirk and Richardson, 1995), we also investigated if adenosine receptor activation was tonically modulating the release of GABA in cultured retinal cells.

2. Materials and Methods

Materials

D-[^{3}H]Aspartate (27 Ci mol^{-1}) was obtained from Amersham International (Amersham Centre, U.K.). Basal Medium of Eagle (Earle's salts—BME) was purchased from Sigma (U.S.A.), trypsin from GIBCO (U.K.) and fetal calf serum from BioChrom KG (Berlin, Germany). DPCPX, 1,3-dipropyl-8-cyclopentylxanthine was from RBI (MA, U.S.A.). ZM241385, 4-[2-[7-amino-2-(2-furyl)(1,2,4)-triazin-5-ylamino]-ethyl]phenol was a generous gift from Dr Simon Poucher (Zeneca Pharmaceuticals, Cheshire, U.K.) and SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine) was kindly provided by Dr E. Ongini (Schering-Plough, Milan, Italy). (−)Bicuculline methbromide 1(S),9(S), referred to throughout as (−)bicuculline, was from RBI (MA, U.S.A.) and 3-aminopropyl (diethoxymethyl) phosphinic acid (CGP35348) was a generous gift from CibaGeigy (Basel, Switzerland). All other reagents were of analytical grade.

Retinal Cell Cultures

Retinal cells were prepared from 8 day old chick embryos and cultured as previously described (Agostinho et al., 1994; Rego, Santos and Oliveira, 1996; Rego et al., 1997). The cells were plated at a density of $2{\cdot}1 \times 10^6$ cells cm^{-2} on Costar 12 multiwell plates. Cells were cultured for 6 days at 37°C in an atmosphere of 95% air and 5% CO_2, and the culture medium was changed every 2 days. Similar cultures were previously described to be enriched in amacrine neuron-like cells (Huba and Hofmann, 1990; Agostinho, Duarte and Oliveira, 1996). These cultures also contain neurons resembling bipolar cells (Agostinho et al., 1994) and glial cells.

Metabolic Inhibition and Incubation of Retinal Cells

After removal of the culture medium, the retinal cells were allowed to equilibrate in saline solution, containing (in mM): NaCl 140·0, KCl 5·0, $CaCl_2$ 1·5, $MgCl_2$ 1·0, NaH_2PO_4 1·0, glucose 5·6 and Hepes 20·0, at pH 7·4. Metabolic inhibition was induced for 15 min (37°C), in the presence of: (1) glycolysis inhibitors: iodoacetic acid, IAA (0·5 mM), which inhibits the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, or 2-deoxyglucose, DG (2 mM) in glucose-free medium, which substitutes for glucose in the incubation medium and is not metabolized by the cells; or (2) ischemia inducers: in the presence of glycolysis inhibitors (IAA or DG) plus oligomycin, OL (5 μg ml^{-1}) and sodium cyanide, CN (2 mM), to inhibit ATP synthase and cytochrome c oxidase, respectively. Incubation in the presence of OL plus CN induced hypoxia-like situations. Control retinal cells were incubated in the absence of the chemicals. Some experiments were carried out in a saline medium without Ca^{2+} containing 200 μM EGTA. Under conditions of chemical ischemia or glycolysis inhibition, induced for 15 min, the viability of retinal cells was not changed, as shown previously by the analysis of lactate dehydrogenase activity, an index of cellular disruption (Rego et al., 1996).

Extracellular accumulation of the excitatory amino acids, aspartate and glutamate, and GABA was determined by collecting the incubation medium 15 min after initiation of the reaction. The endogenous amino acid content was determined in an alkaline extract, treated with 1 M NaOH. When assessing the influence of A_1 adenosine receptors activation by endogenous adenosine on the extracellular accumulation of aspartate or glutamate, 100 nM DPCPX, a

selective A_1 receptor antagonist (Bruns et al., 1987), was used. When assessing the influence of A_{2A} adenosine receptors activation by endogenous adenosine, we used the compound ZM241385, a non-xanthine adenosine receptor antagonist selective for the A_{2A} receptors (Poucher et al., 1995), at concentrations of 20, 50, 100 and 200 nM. A_{2A} receptor function was also evaluated by using the compound SCH58261 (50 nM). To allow sufficient time for receptor occupancy, DPCPX and ZM241385 were preincubated for 60 min, whereas SCH58261 was preincubated for 45 min, in saline solution containing Ca^{2+}, but in the absence of inducers of chemical ischemia or glycolysis inhibitors, and were present throughout the experiment. When present, 10 μM (−)bicuculline, an antagonist of $GABA_A$ receptors (Piredda, Lim and Gale, 1985), and 100 μM CGP35348, a selective antagonist of metabotropic $GABA_B$ receptors (Olpe et al., 1990), were incubated for 15 min before and during IAA-induced glycolysis inhibition.

HPLC Analysis of the Amino Acids

The recovered samples, both the incubation medium and the alkaline extract, containing the amino acids were centrifuged at 15 800 ***g***, for 2 min, to eliminate cell debris. The amino acids were analysed in a Gilson-ASTED HPLC system as described previously (Rego et al., 1996). The amino acids were detected as fluorescent derivatives after derivatization with *o*-phthaldialdehyde/2-mercaptoethanol, at 340 nm excitation and 410 nm emission. The concentrations of amino acids in the samples were determined by comparison with the peak areas of external standards. The extracellular accumulation of aspartate, glutamate and GABA was expressed as a percentage of total amino acid content, that is, the extracellular plus the intracellular content.

D-[^{3}H]Aspartate Uptake

Cultured retinal cells were incubated with 5·5 nM D-[^{3}H]aspartic acid (1 μCi ml^{-1}) in saline solution for 10 min at 37°C. When the effect of ZM241385 (50 nM) was evaluated, a 45 min preincubation was performed, followed by incubation in the presence or in the absence of IAA (0·5 mM) in medium without Ca^{2+} for 15 min, and incubation with the radiolabeled amino acid. At the end of the experiments the cells were disrupted with 0·2 M HCl and the radioactivity was measured using UNIVERSOL scintillation Cocktail (ICN) and a Packard-2000 Spectrometer provided with d.p.m. correction.

Statistical Analysis

The values are presented as means ± S.E.(M.) of the indicated number of experiments. The significance of the differences between the means was calculated by the unpaired two-tailed Student's *t*-test for the comparison of the means of two Gaussian populations or the one-way ANOVA with the Tukey post-test for multiple comparisons. Values of $P < 0{\cdot}05$ were considered to represent significant differences.

3. Results

Analysis of A_{2A} and A_1 Adenosine Receptor Influence on the Extracellular Accumulation of Aspartate and Glutamate

Figs 1 and 2 show the extracellular accumulation of aspartate and glutamate occurring in the presence or absence of Ca^{2+}, in cells submitted to glycolysis inhibition in the presence of IAA or DG, or subjected to chemical ischemia in the presence of oligomycin and cyanide (hypoxic-like conditions) plus IAA (Isc.IAA) or DG (Isc.DG) for 15 min. In the presence of Ca^{2+}, aspartate release was not significantly changed upon treatment with glycolysis inhibitors or inducers of chemical ischemia (Fig. 1). However, the release of glutamate in the presence of Ca^{2+} increased by about six-fold ($P < 0{\cdot}01$) after treatment with IAA and during chemical ischemia in the presence of IAA (Isc.IAA). In cells submitted to DG or to chemical ischemia in the presence of DG (Isc.DG), glutamate release increased by about nine- ($P < 0{\cdot}001$) or six-fold ($P < 0{\cdot}05$), respectively (Fig. 1).

In the absence of external Ca^{2+}, significant increases in the extracellular accumulation of aspartate after chemical ischemia or glycolysis inhibition were observed: IAA or Isc.IAA increased the release of aspartate by three- ($P < 0{\cdot}01$) or four-fold ($P < 0{\cdot}001$), whereas DG or Isc.DG enhanced extracellular aspartate by about two- ($P < 0{\cdot}05$) or three-fold ($P < 0{\cdot}01$), respectively (Fig. 2). In the absence of Ca^{2+}, extracellular glutamate increased by about eight-fold upon treatment with IAA and about 11-fold after incubation with Isc.IAA. Incubation in the presence of DG or Isc.DG in Ca^{2+}-free medium increased glutamate release by seven- or six-fold, respectively (Fig. 2). Excitatory amino acid release in the absence of Ca^{2+} was higher after chemical ischemia induced in the presence of IAA (Isc.IAA) as observed in Fig. 2.

Analysis of the influence of tonic A_{2A} adenosine receptor activation by endogenous adenosine in regulating the extracellular accumulation of excitatory amino acids was performed by using ZM241385, a highly selective antagonist for the A_{2A} adenosine receptors (Poucher et al., 1995). A_2 adenosine receptors, eliciting an increase in cAMP formation, were shown to be present in the chick embryo retina (Paes de Carvalho and de Mello, 1982), and A_{2A} receptors were identified in the bovine retina (Blazynski and McIntosh, 1993). At a concentration of 50 nM, ZM241385 increased significantly ($P < 0{\cdot}01$) the

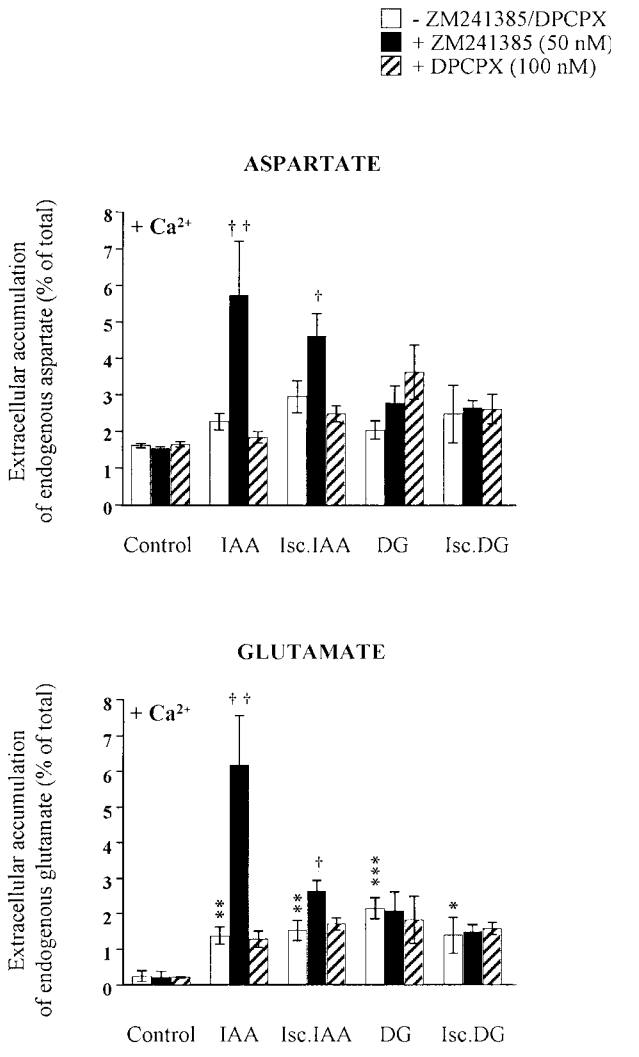

FIG. 1. Influence of ZM241385 or DPCPX on Ca^{2+}-dependent extracellular accumulation of endogenous aspartate and glutamate. Retinal cells were preincubated for 60 min with 50 nM ZM241385 or 100 nM DPCPX. The cells were then incubated with glycolysis inhibitors, in the presence of 0·5 mM IAA or 2 mM DG, or with inducers of chemical ischemia, by exposing the cells to OL (5 μg ml^{-1}) plus CN (2 mM), in the presence of IAA (Isc.IAA) or in the presence of DG (Isc.DG), for 15 min, in saline solution containing 1·5 mM Ca^{2+}. When present, ZM241385 or DPCPX were also incubated with the metabolic inhibitors. Control cells were not exposed to glycolytic inhibitors or inducers of chemical ischemia. Values are the means ± S.E.(M.) of 3–4 experiments run in triplicates. Statistical significance: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ or $***P < 0{\cdot}001$ in cells incubated in the absence of the antagonists, as compared to the control; $\dagger P < 0{\cdot}05$, or $\dagger\dagger P < 0{\cdot}01$, as compared to the same metabolic dysfunction situation, in the absence of the receptor antagonists.

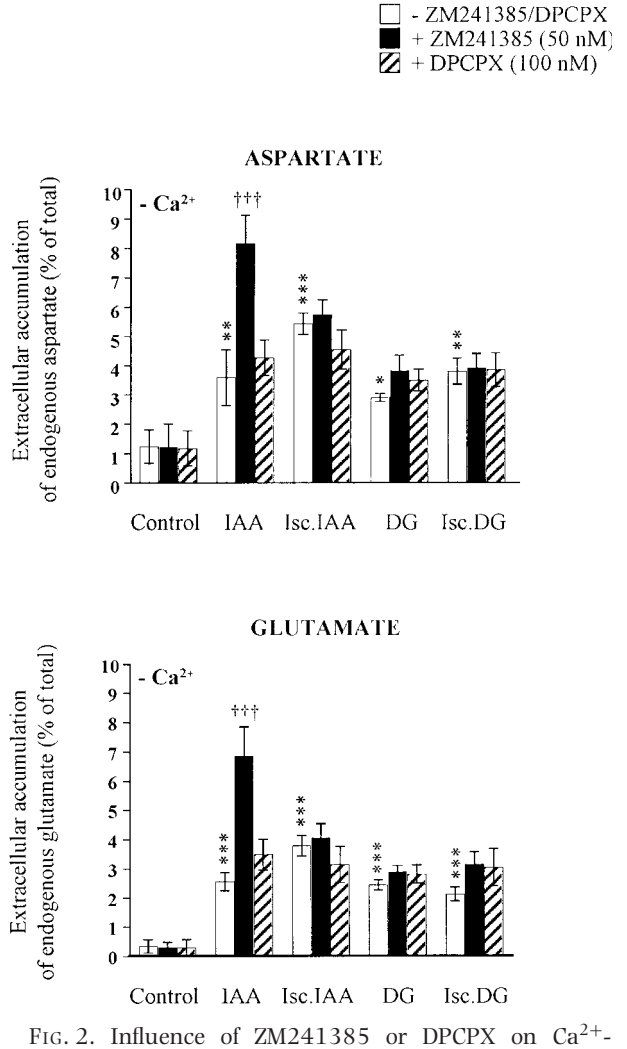

FIG. 2. Influence of ZM241385 or DPCPX on Ca^{2+}-independent extracellular accumulation of endogenous aspartate and glutamate. Retinal cells were preincubated for 60 min with 50 nM ZM241385 or 100 nM DPCPX. Similarly, as shown in Fig. 1, the cells were exposed to glycolysis inhibitors or inducers of chemical ischemia, except that the 15 min incubation was carried out in saline solution without Ca^{2+}, but containing 200 μM EGTA. Values are the means ± S.E.(M.) of four experiments, each one run in triplicates. Statistical significance: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ or $***P < 0{\cdot}001$ in cells incubated in the absence of ZM241385 or DPCPX, as compared with the control; $\dagger\dagger\dagger P < 0{\cdot}001$, as compared with the same metabolic dysfunction condition, in the absence of the receptor antagonists.

release of aspartate (5·53 ± 1·53%, corresponding to 2·1-fold increase) and the release of glutamate (6·18 ± 1·39%, corresponding to 4·5-fold increase) over IAA-induced glycolysis inhibition, in the presence of Ca^{2+} (Fig. 1). In the absence of Ca^{2+}, in cells incubated with IAA, ZM241385 (50 nM) increased significantly ($P < 0{\cdot}001$) the release of aspartate to 8·15 ± 0·96% (2·3-fold increase) and the release of glutamate to 6·86 ± 0·98% (corresponding to 2·7-fold increase), as compared to IAA alone (Fig. 2). After incubation with Isc.IAA, 50 nM ZM241385 also enhanced ($P < 0{\cdot}05$) the release of aspartate by 1·6-fold and the release of glutamate by 1·7-fold in retinal cells incubated in the presence of Ca^{2+}, but no significant changes were observed in the absence of Ca^{2+}. Furthermore, 50 nM ZM241385 did not induce significant changes after treatment of retinal cells with DG alone or with DG plus hypoxia inducers, OL and CN (Isc.DG), as shown in Figs 1 and 2.

To reinforce the role of A_{2A} receptor modulation of glutamate and aspartate release, we also analysed the

effect of another non-xanthine A_{2A} receptor selective antagonist, SCH58261, on the extracellular accumulation of endogenous aspartate and glutamate in retinal cells submitted to glycolysis inhibition in the presence of IAA. Fig. 3 shows that 50 nM SCH58261 significantly ($P < 0{\cdot}05$) increased the extracellular accumulation of aspartate and glutamate in cells incubated in the presence of Ca^{2+} by about two-fold. However, no significant differences were observed in cells incubated with SCH58261 in medium without Ca^{2+} (Fig. 3). These results show that tonic activation of adenosine A_{2A} receptors by endogenous adenosine decreases the release of the amino acids upon IAA-mediated glycolysis inhibition.

Inhibitory A_1 adenosine receptors were also shown to be present in the retina (Paes de Carvalho and de Mello, 1985; Blazynski, 1987; Agostinho et al., 2000). Thus, the influence of A_1 adenosine receptors in mediating the extracellular accumulation of aspartate or glutamate was evaluated by testing the effect of DPCPX, a selective A_1 receptor antagonist (Bruns et al., 1987). DPCPX (100 nM) did not induce significant changes in excitatory amino acid release, either in the presence or absence of Ca^{2+}, after each cell treatment (Figs 1 and 2). At a concentration of 200 nM, DPCPX did not affect the extracellular accumulation of aspartate or glutamate after metabolic inhibition (data not shown).

After exposure of retinal cells to CN plus OL to mimic hypoxia conditions, ZM241385 (50 nM) or DPCPX (100 nM) did not significantly affect the extracellular accumulation of aspartate or glutamate (not shown). The extracellular accumulation of the excitatory amino acids observed in control retinal cells was not significantly affected by ZM241385 (50 nM), SCH58261 (50 nM) or DPCPX (100 nM) (Figs 1–3).

ZM241385 Induced a Concentration-dependent Accumulation of Extracellular Aspartate and Glutamate upon Treatment with IAA

Because ZM241385 induced major changes in the release of endogenous aspartate and glutamate after treatment of retinal cells with IAA (0·5 mM), and to a greater extent in the absence of Ca^{2+} (Fig. 2), we examined the effects of several concentrations of this antagonist (20, 50, 100 or 200 nM ZM241385) on the excitatory amino acid release from IAA-treated cells. As shown in Fig. 4, ZM241385 increased the extracellular accumulation of aspartate and glutamate in a concentration-dependent manner. Significant ($P < 0{\cdot}001$) increments were observed after incubation in the presence of 50, 100 or 200 nM ZM241385 in cells incubated with IAA in the absence of Ca^{2+}.

These data suggest that activation of A_{2A} adenosine receptors, particularly during IAA-induced glycolysis inhibition, triggers inhibitory processes that prevent a major increase in excitatory amino acid release from the retinal cells.

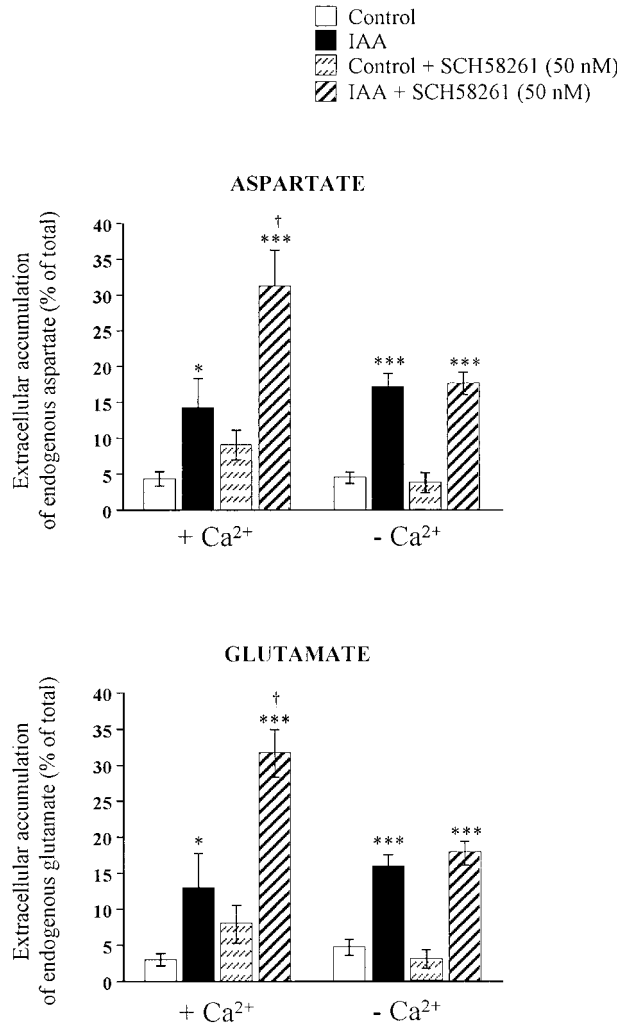

FIG. 3. Influence of SCH58261 on the extracellular accumulation of endogenous aspartate and glutamate. The cells were incubated for 45 min with 50 nM SCH58261 prior to the incubation with IAA (0·5 mM) in the presence ($+Ca^{2+}$) or in the absence of Ca^{2+} ($-Ca^{2+}$) for 15 min. Control cells were not exposed to IAA. The amino acids were analysed by HPLC, as described under Materials and Methods. Data are the means ± S.E.(M.) of triplicates from two independent experiments. Statistical significance: $*P < 0{\cdot}05$ or $***P < 0{\cdot}001$ as compared to the control in the absence of the antagonist; $\dagger P < 0{\cdot}05$ as compared to IAA alone. Levels of significance of IAA + SCH58261-treated cells in the presence of Ca^{2+} as compared to the control in the presence of SCH58261 were $P < 0{\cdot}05$ for aspartate and $P < 0{\cdot}01$ for glutamate.

Effect of ZM241385 on the Uptake of [^{3}H]Aspartate

Since the increased extracellular accumulation of aspartate and glutamate upon adenosine A_{2A} receptor blockade could result from the inhibition of the amino acid uptake or from stimulation of amino acids release, we tested the effect of ZM241385 directly on the uptake of [^{3}H]aspartate in retinal cells submitted to glycolysis inhibition. Table I shows that treatment with 0·5 mM IAA significantly inhibited the uptake of [^{3}H]aspartate by about 33%. Nevertheless, incubation with ZM241385 (50 nM) had no effect on

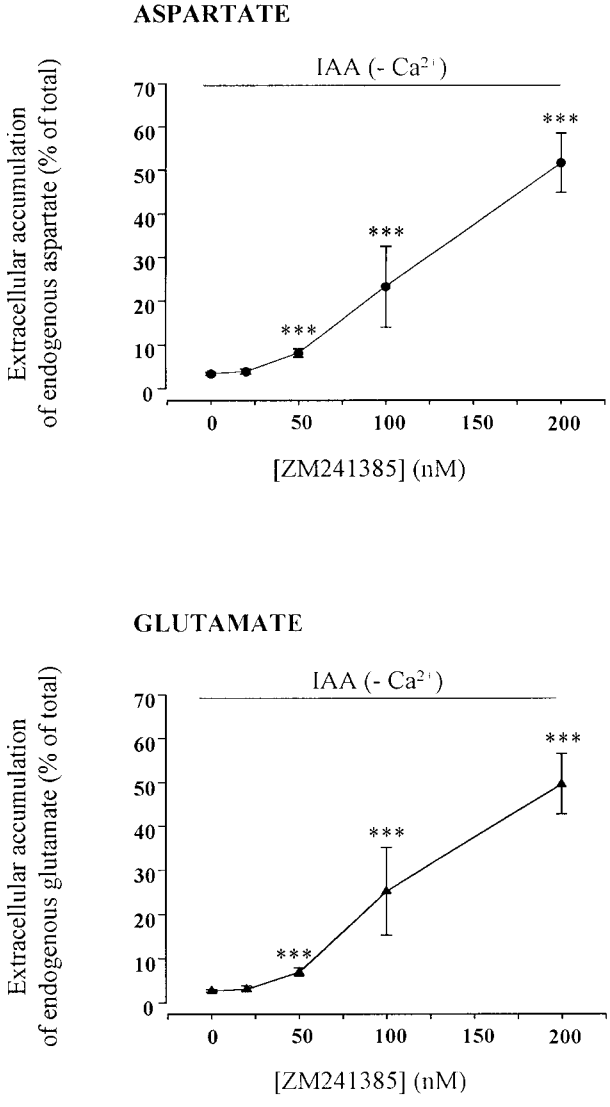

FIG. 4. ZM241385 concentration-dependent increase of extracellular accumulation of aspartate and glutamate upon treatment with IAA. After preincubation for 60 min in the presence of 20, 50, 100 or 200 nM ZM241385, retinal cells were exposed to IAA (0·5 mM) for 15 min in the absence of Ca^{2+}, in saline medium containing 200 μM EGTA. Data are the means ± S.E.(M.) of duplicates or triplicates from three experiments. Levels of significance when compared with IAA-treated cells in the absence of ZM241385 are: ***$P < 0{\cdot}001$.

TABLE I

Influence of ZM241385 on the uptake of [^{3}H]aspartate by cultured retinal cells

	D-[^{3}H]Aspartate uptake (% of control)
Control	99·9 ± 3·7
Control + ZM241385	104·4 ± 2·3
IAA	67·4 ± 4·3***
IAA + ZM241385	66·6 ± 0·6***

The cells were preincubated with 50 nM ZM241385 for 45 min and further treated with IAA (0·5 mM), in the absence of Ca^{2+}, for 15 min. The cells were then incubated for 10 min with D-[^{3}H]aspartate (5·5 nM), and the radioactivity retained by the cells was determined by scintillation counting.

Data are the means of triplicates from two independent experiments.

Levels of significance: *** $P < 0{\cdot}001$ as compared to the control, in the absence or in the presence of the antagonist.

the uptake of [^{3}H]aspartate (Table I), implying that the regulation of amino acid release by A_{2A} adenosine receptors does not involve modulation of excitatory amino acids uptake.

Extracellular Accumulation of Endogenous GABA after Treatment with IAA

Because activation of A_{2A} receptors was reported to influence the release of GABA, an inhibitory neurotransmitter (e.g. O'Regan et al., 1992; Mayfield et al., 1993; Kirk and Richardson, 1995), we examined the eventual A_{2A} receptor-mediated modulation of the extracellular accumulation of endogenous GABA. In the retina, GABA is used by subpopulations of horizontal and amacrine cells (Huba and Hofmann, 1990; reviewed by Lam, 1997) and was shown to colocalize with adenosine (Perez and Bruun, 1987, but see Studholme and Yazulla, 1997).

In this study, the retinal cells were exposed to 0·5 mM IAA-induced glycolysis inhibition, in the absence of external Ca^{2+}, because under these conditions a larger extracellular accumulation of GABA was observed (Rego et al., 1996). Table II

TABLE II

IAA-mediated extracellular accumulation of endogenous GABA in cultured retinal cells

	Control	IAA	IAA + ZM241385
Extracellular GABA (% of total)	2·48 ± 0·18 (4)	8·55 ± 0·80 (5)*	10·25 ± 0·46 (6)*

Retinal cells were incubated with 0·5 mM IAA in the absence of external Ca^{2+}, in medium containing 200 μM EGTA, for 15 min. When present, ZM241385 (50 nM) was incubated for 60 min before and during the incubation with IAA. After the reaction, the incubation medium was recovered and 1 M NAOH was added to the cells, to determine the cellular content of GABA. Extracellular and intracellular GABA was analysed by HPLC, as described under Materials and Methods.

The values are the means ± S.E.(M.) of the number of experiments in brackets.

Statistical significance: *$P < 0{\cdot}05$ as compared to the control, in the absence of IAA. No significant differences were found between cells treated with IAA in the absence or in the presence of ZM241385.

shows that 0·5 mM IAA, in the absence of Ca^{2+}, mediated an increase in extracellular GABA accumulation by 3·4-fold ($P < 0·05$). Furthermore, the accumulation of extracellular GABA that occurred in the presence of IAA plus ZM241385 (50 nM) was not significantly different as compared to IAA alone, indicating that A_{2A} adenosine receptors did not modulate the release of GABA in retinal cells in culture.

Involvement of GABA Receptors in Regulating the Release of Excitatory Amino Acids

We further determined the contribution of GABA receptors on the extracellular accumulation of aspartate or glutamate after incubation in the presence of IAA-induced glycolysis inhibition, shown to significantly increase the release of endogenous GABA (Table II). Thus, the efficiency of two antagonists of GABA receptors, (−)bicuculline, an antagonist of ionotropic $GABA_A$ receptors (Piredda et al., 1985), and CGP35348, an antagonist of presynaptic $GABA_B$ receptors (Olpe et al., 1990), was tested. Presynaptic metabotropic $GABA_B$ receptors and ionotropic $GABA_A$ receptors were shown to be present on amacrine and bipolar retinal cells (Slaughter, 1995).

After treatment with IAA in the presence of external Ca^{2+}, inhibition of $GABA_A$ receptors with 10 μM (−)bicuculline significantly increased the release of glutamate to 3·24 ± 0·31%, when compared to the release occurring after incubation with IAA alone (1·91 ± 0·41%), as shown in Fig. 5. However, (−)bicuculline did not significantly influence the release of glutamate occurring through a Ca^{2+}-independent process, or the release of aspartate, occurring in the presence or in the absence of Ca^{2+} (Fig. 5). Fig. 5 also suggests that extracellular endogenous GABA acting on $GABA_B$ presynaptic metabotropic receptors did not influence the release of excitatory amino acids during incubation with IAA, because CGP35348 did not significantly affect the extracellular accumulation of aspartate or glutamate, occurring in the presence or in the absence of external Ca^{2+}.

4. Discussion

The present results show that, upon inhibition of glycolysis, tonic activation of adenosine A_{2A} receptors by endogenous extracellular adenosine decreases excitatory amino acid release in cultured retinal cells, because blockade of adenosine A_{2A} receptors with two selective A_{2A} antagonists, ZM241385 or SCH58261, greatly enhanced the extracellular accumulation of excitatory amino acids after treatment with IAA, without affecting the uptake of the amino acids. Despite the increase in endogenous extracellular GABA after IAA-mediated glycolysis inhibition, the observation that antagonists of

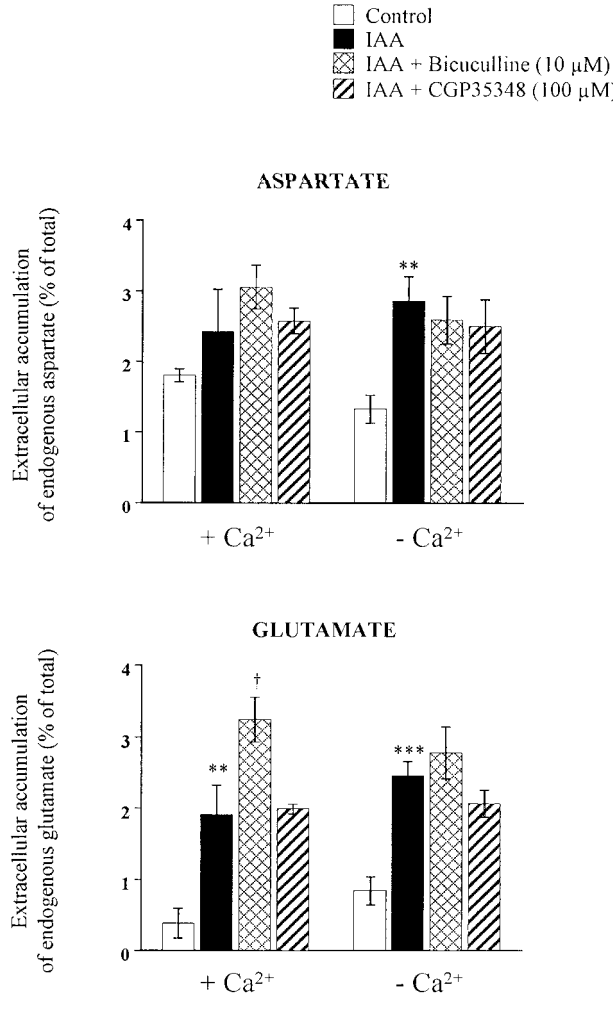

FIG. 5. Effect of (−)bicuculline and CGP35348 on the extracellular accumulation of aspartate and glutamate after treatment with IAA. Retinal cells were preincubated with 10 μM (−)bicuculline and 100 μM CGP35348 for 15 min. The cells were then incubated with 0·5 mM IAA, in the presence (+Ca^{2+}) or in the absence of Ca^{2+} (−Ca^{2+}) for 15 min. When present, (−)bicuculline or CGP35348 were incubated with the glycolytic inhibitor. Control cells were not exposed to IAA. The values of the control in the presence of (−)bicuculline (2·05 ± 0·04% or 1·07 ± 0·09% aspartate and 0·51 ± 0·10% or 1·01 ± 0·10% glutamate, respectively, in the presence or in the absence of Ca^{2+}) or CGP35348 (2·21 ± 0·05% or 1·15 ± 0·03% aspartate and 0·48 ± 0·20% or 0·91 ± 0·07% glutamate, respectively, in the presence or in the absence of Ca^{2+}) are not significantly different from the control values in the absence of these compounds. Data are the means ± S.E.(M.) of 3–5 experiments run in triplicate. Levels of significance from comparison between IAA-treated cells and the control: $^{**}P < 0·01$ or $^{***}P < 0·001$. Statistical significance when compared with IAA conditions, but in the absence of (−)bicuculline are: †$P < 0·05$.

$GABA_A$ or $GABA_B$ receptors did not significantly interfere with the extracellular accumulation of excitatory amino acids, and that ZM241385 did not affect the release of GABA, indicate that the inhibitory process triggered by adenosine A_{2A} receptor activation did not involve modulation of $GABA_A$ and $GABA_B$ function. This confirms a previous study concluding that the adenosine and GABA systems are weakly

inter-related in the retina (Studholme and Yazulla, 1997). It remains to be explored if the role of the GABAergic system in the control of excitatory amino acid release may burst upon blockade of the adenosine system, as has been shown to occur in the modulation of hypoxia-induced depression of synaptic transmission in hippocampal slices (Lucchi et al., 1996).

Metabolic inhibition, including the incubation with IAA, or ischemia leads to an increase in the extracellular levels of adenosine that mainly occurs through the reversal of nucleoside transporters (Rego et al., 1997). Adenosine is generally recognized to play a neuroprotective role in hypoxia and/or in ischemic situations in the central nervous system (for a review see, Rudolphi et al., 1992). This neuroprotective effect is normally associated with activation of inhibitory A_1 adenosine receptors (Rudolphi et al., 1992), whereas the role of A_2 receptor activation is unclear. Thus, upon severe temporary forebrain ischemia in mongolian gerbils, activation of A_{2A} receptors decreases neuronal loss (Sheardown and Knutsen, 1996), and A_2 receptor activation reverts ischemia/reperfusion-induced lung endothelial damage (Khimenko, Moore and Taylor, 1995).

In contrast, activation of A_2 receptors exacerbates neuronal injury upon deprivation of oxygen and glucose in guinea pig superior collicular slices (Fujiwara et al., 1994) or upon carotid artery occlusion in gerbils (Gao and Phillis, 1994; Phillis, 1995). In the retina, it was previously observed that A_{2A} receptor activation by endogenous adenosine may have either neuroprotective or deleterious effects upon ischemia (Li et al., 1999). The present observation that blockade of A_{2A} receptors facilitates the release of excitatory amino acids upon IAA-induced glycolysis inhibition suggests that A_{2A} receptors may exert their neuroprotective role in retinal cells through inhibition of glutamate release in the early phase of the ischemic period (see Li et al., 1999). However, in ischemia-like conditions, in the presence of IAA, which are associated with a large decrement of intracellular ATP levels (Rego et al., 1997), we observed a lower effect of ZM241385 as compared to IAA alone, suggesting that a decrease in the extracellular accumulation of the excitatory amino acids mediated by activation of A_{2A} receptors in cultured retinal cells is highly energy dependent.

In most central nervous system preparations, A_{2A} receptors fulfill a facilitatory role (Sebastião and Ribeiro, 1996). Thus, activation of A_{2A} receptors enhances Ca^{2+} uptake (Gonçalves, Cunha and Ribeiro, 1997), neurotransmitter release (e.g. Cunha et al., 1994) or excitatory transmission (Cunha, Constantino and Ribeiro, 1997b). Interestingly, A_{2A} receptors can couple to Gs as well as to Gi/Go proteins (Cunha, Constantino and Ribeiro, 1999), suggesting that, according to the level of A_{2A} receptor expression or activation, different transducing systems may be activated, as has been observed for other Gs-coupled receptors (for a review see, Gudermann, Schöneberg and Schultz, 1997). This helps understanding the post-synaptic inhibitory effects of A_2 receptor activation on the conductance of NMDA receptor channels (Nörenberg et al., 1998), as well as the A_{2A} receptor-mediated inhibition of the release of neurotransmitters, such as ATP release in the medial habenula (Robertson and Edwards, 1998) or GABA release in the striatum (Kirk and Richardson, 1995). In the present study, we excluded the involvement of $GABA_A$ and $GABA_B$ function as a possible mediator of A_{2A} inhibition of IAA-induced excitatory amino acid release, although IAA-induces extracellular GABA accumulation and inhibition of $GABA_A$ receptors enhanced IAA-mediated accumulation of extracellular excitatory amino acids. However, the possible involvement of other neuroactive substances in the inhibitory effect of A_{2A} receptors on IAA-induced accumulation of extracellular excitatory amino acids can not be excluded. Also, the observation that the blockade of A_{2A} receptors enhanced the IAA-induced extracellular accumulation of excitatory amino acids, both in the presence and in the absence of extracellular Ca^{2+}, is suggestive of a possible metabolic role of A_{2A} receptor activation, involving the A_{2A} receptor-mediated control of glycogenolysis in cerebral cortical preparations (Magistretti, Hof and Martin, 1986) and in the retina (Osborne, 1989) or of a direct antioxidant effect of adenosine (Ramkumar et al., 1995; Yokoi et al., 1995; Yavuz et al., 1997).

Another interesting observation in the present work was the absence of involvement of adenosine A_1 receptors in the control of the extracellular accumulation of excitatory amino acids upon metabolic dysfunction of retinal cells. This contrasts with the presynaptic inhibitory role of A_1 receptor activation in hypoxia (Lucchi et al., 1996) or hypoglycemia conditions (Fowler, 1993b) in different models, and with the known deleterious role of acutely applied adenosine A_1 receptor antagonists in ischemic injury models (for a review, see Rudolphi et al., 1992). Previous studies in retinal preparations have demonstrated that adenosine can tonically activate inhibitory A_1 receptors (Larsen and Osborne, 1996) and that activation of A_1 receptors inhibits NMDA currents (Costenla et al., 1999), a central event in ischemic neuronal damage in the retina (Yoon and Marmor, 1989). However, our results are in agreement with the observation that the neuroprotective effect of A_1 receptor activation in the retina is restricted to short periods of ischemia (Li et al., 1999). This reduced contribution of A_1 receptors may have resulted from the generation of arachidonic acid (Rego et al., 1996) or reactive oxygen species after metabolic inhibition in the presence of IAA (Rego, Santos and Oliveira, 1999) that could inhibit A_1 receptor function in retinal cells, similarly as found in brain membranes (Oliveira et al., 1995; Cunha, Constantino and Ribeiro, 1997a). Alternatively, it is

possible that the increase in importance of A_{2A} activation may cause a desensitization of A_1 receptors (Dixon, Widdowson and Richardson, 1997; Lopes, Cunha and Ribeiro, 1999a) and, since the role of endogenous adenosine is a balance between inhibitory A_1 and facilitatory A_{2A} responses (Brown et al., 1990; Cunha et al., 1994), and the A_1/A_{2A} balance changes according to stimulus paradigms (Correia-de-Sá et al., 1996) as well as with aging (Cunha et al., 1995; Lopes, Cunha and Ribeiro, 1999b), it is possible that A_{2A} responses preponderate under the conditions used in the present work. Thus, the A_1/A_2 balance in control or IAA-treated retinal cells needs to be explored to understand why endogenous adenosine activates A_{2A}, but not A_1 receptors, to control the IAA-induced extracellular accumulation of excitatory amino acids.

In conclusion, the present finding that inhibition of A_{2A} adenosine receptors increases the extracellular accumulation of excitatory amino acids caused by metabolic dysfunction in cultured retinal cells, may provide a rationale for the reported A_{2A} receptor-mediated neuroprotective effects of endogenous adenosine in the retina subject to short-term hypoxia. This also stresses the possible neuroprotective role of A_{2A} receptors, which has traditionally been associated with A_1 receptor activation.

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