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# Effects of adenosine $A_1$ and $A_{2A}$ receptor activation on the evoked release of glutamate from rat cerebrocortical synaptosomes

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**1** The effects of adenosine $A_{2A}$ and $A_1$ receptor activation on the release of glutamate were studied in rat cerebral cortex synaptosomes exposed in superfusion to adenosine receptor ligands.

**2** Adenosine (0.1 $\mu$M) produced a significant potentiation of the $Ca^{2+}$-dependent $K^+$(15 mM)-evoked [$^3$H]-D-aspartate overflow (20.4 ± 3.5%), which was blocked by $A_{2A}$ blocker SCH58261 (0.1 $\mu$M). At higher concentrations (10–1000 $\mu$M) adenosine inhibited in a DPCPX-sensitive manner the $Ca^{2+}$-dependent $K^+$-evoked [$^3$H]-D-aspartate overflow. The inhibitory effect of adenosine at 1000 $\mu$M was significantly increased by SCH58261. This inhibition was antagonized by 1 $\mu$M DPCPX. Adenosine did not produce any effect on basal release.

**3** The $A_{2A}$ receptor agonist CGS 21680 was ineffective on basal release, but stimulated the $Ca^{2+}$-dependent $K^+$-evoked overflow of [$^3$H]-D-aspartate ($EC_{50} \simeq 1$ pM). The effect of 0.01 nM CGS 21680 was totally sensitive to the $A_{2A}$ receptor antagonist SCH58261 ($IC_{50} \simeq 5$ nM).

**4** The $A_1$ receptor agonist CCPA inhibited the $Ca^{2+}$-dependent $K^+$-evoked [$^3$H]-D-aspartate overflow ($EC_{50} \simeq 20$ nM). The effect of 100 nM CCPA was abolished by 100 nM of the $A_1$ receptor antagonist DPCPX.

**5** The $K^+$(15 mM)-evoked overflow of endogenous glutamate was enhanced by CGS 21680 (0.01 nM) and inhibited by CCPA (0.1 $\mu$M). The effect of CGS 21680 was abolished by SCH58261 (0.1 $\mu$M) and that of CCPA by DPCPX (0.1 $\mu$M).

**6** It is concluded that adenosine and adenosine receptor agonists modulate glutamate release by activating inhibitory $A_1$ and excitatory $A_{2A}$ receptors present on glutamatergic terminals of the rat cerebral cortex.

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**Abbreviations:** CCPA, 2-chloro-$N^6$-cyclopentyladenosine; CGS 21680, 2-4-(-2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine; DPCPX, 3-cyclopentyl-1,3-dipropylxanthine; SCH58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazole-[4,3-*e*]-1,2,4 triazolo [1,5-*c*]pyrimidine

## Introduction

Adenosine is an ubiquitous neuromodulator that acts at four G-protein-coupled receptors termed $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ (Fredholm *et al*., 2001). One major role of $A_1$ and $A_{2A}$ receptors is to modulate neurotransmitter release (for a review see Sebastião & Ribeiro (1996)). The distribution of these two receptors is discrete: $A_{2A}$ receptors are abundant in the corpus striatum but rather sparse in the cortex and hippocampus, where the density of $A_1$ is higher than that of $A_{2A}$ receptors (Cunha *et al*., 1996; Fredholm *et al*., 1998).

There is evidence that adenosine mediates inhibition of neurotransmitter release through receptors of the $A_1$ type, whereas $A_{2A}$ receptor activation is thought to facilitate release (for a review see Sebastião & Ribeiro (1996)). Important exceptions exist, however. For instance, adenosine $A_{2A}$ receptors when activated enhance the evoked release of GABA from rat hippocampus synaptosomes (Cunha & Ribeiro, 2000) whereas, in synaptosomes of rat striatum, they mediate inhibition of the evoked GABA release (Kirk & Richardson, 1994; Kurokawa *et al*., 1994).

It is generally believed that excessive release of the excitatory amino acids glutamate/aspartate is involved in a number of neuropathological conditions. There is evidence that adenosine can play protective roles against excitotoxicity and that extracellular levels of adenosine are elevated following brain oxidative stress and metabolic dysfunction that occur after interruption of the cerebral flow (Rudolphi *et al*., 1992; von Lubitz, 1999 and references therein; Moreau & Huber, 1999 and references therein). A reasonable hypothesis implies that endogenous adenosine inhibits glutamate release by activating $A_1$ receptors. On the other hand, activation of $A_{2A}$ receptors by the compound CGS 21680 was reported to enhance excitatory transmitter release from ischaemic rat cerebral cortex (Simpson *et al*., 1992) suggesting that, under some conditions, endogenous adenosine can activate excitatory $A_{2A}$ receptors. Accordingly, $A_{2A}$ receptor antagonists

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limited cerebral damage caused by global ischaemia (Gao & Phillis, 1994; von Lubitz, 1999) and reduced cortical infarct volume following focal ischaemia (Monopoli *et al*., 1998). Furthermore, brain injury induced by transient focal ischaemia was attenuated in $A_{2A}$ receptor-deficient mice (Chen *et al*., 1999). These results appear compatible with the hypothesis that release-inhibiting $A_1$ receptors and release-enhancing $A_{2A}$ receptors exist on glutamatergic terminals of the cerebral cortex. However, as discussed in the review by Sebastião & Ribeiro (1996), whether elevation and decrease of glutamate release can be produced by adenosine acting directly on glutamatergic nerve terminals or indirectly through more or less complex circuits, has not been established.

In order to ascertain if cerebrocortex glutamatergic nerve endings possess release-regulating adenosine heteroreceptors, we studied the effects of some adenosine receptor ligands on glutamate release using thin layers of synaptosomes in superfusion, a preparation that permits one to establish the localization of a given receptor on a particular family of nerve endings (see Raiteri & Raiteri, 2000). Modulation of glutamate release by selective $A_1$ and $A_{2A}$ receptor agonists as well as by adenosine was investigated.

## Methods

### *Animals*

Adult male Sprague–Dawley rats (200–250 g) were used. Animals were housed at constant temperature ($22 \pm 1°C$) and relative humidity (50%) under a regular light-dark schedule (light 0700–1900 h). Food and water were freely available.

### *Preparation of synaptosomes*

Crude synaptosomes were prepared from rat cerebral cortex as previously described (Raiteri *et al*., 1984). Briefly, rats were killed by decapitation and the cerebral cortex was rapidly dissected. The tissue was homogenized in 40 volumes of 0.32 M sucrose buffered at pH 7.4 with phosphate. The homogenate was centrifuged (5 min, $1000 \times g$) to remove nuclei and debris; synaptosomes were then isolated from the supernatant by centrifugation at $12{,}000 \times g$ for 20 min. The synaptosomal pellet was finally resuspended in a physiological medium having the following composition (mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, $NaH_2PO_4$ 1.0, $NaHCO_3$ 22 and glucose 10 (gassed with 95% $O_2$–5% $CO_2$ at 37°C), pH 7.2–7.4.

### *Release experiments*

Synaptosomes were incubated 15 min at 37°C with [$^3$H]-D-aspartate ([$^3$H]-D-ASP; spec. act. 16.3 Ci mmol$^{-1}$; final concentration 0.08 $\mu$M) in an atmosphere of 95% $O_2$ and 5% $CO_2$. [$^3$H]-D-aspartate is almost universally used (the ratio of papers is about 100 : 1) instead of [$^3$H]-glutamate because it is non-metabolizable; but it is a substrate for the glutamate transporters (Drejer *et al*., 1985). In experiments of endogenous glutamate release, preincubation was performed in the same conditions and no labelling substance was added. Thereafter synaptosomes were distributed in parallel superfusion chambers. (Raiteri *et al*., 1974; Raiteri & Raiteri, 2000). Superfusion was started at a rate of 0.6 ml min$^{-1}$ with aerated standard medium for 46 min in order to remove excess of tritium and/or to equilibrate the system. When $Ca^{2+}$-independent release was to be studied, $Ca^{2+}$-deprived medium (containing $MgCl_2$ 8.8 mM and EGTA 0.1 mM) was used throughout the superfusion. In $K^+$-evoked release experiments, after 46 min of superfusion the following consecutive samples were collected: basal release ($B_1$; 3 min); $K^+$ evoked release (S; 6 min); after-depolarization basal release ($B_2$; 3 min). Synaptosomes were exposed to the depolarizing stimulus (15 mM KCl containing medium) for 90 s, starting at the end of the first fraction collected ($B_1$). Agonists were added concomitantly with the stimulus, whereas antagonists were added 8 min in advance. Antagonists, when used alone to see their effects on release, were added at the end of fraction $B_1$.

In basal release experiments samples were collected in five consecutive fractions of 3 min each ($B_1$–$B_5$). Agonists were added at the end of fraction $B_1$, while antagonists were present in the superfusion medium 8 min before the sample collection started.

In both experimental procedures fraction $B_1$ was not influenced by the presence of agonist drugs, while antagonists could not produce any effect, since the neurotransmitters were immediately removed from the superfusion chambers after being released and did not act on presynaptic receptors.

### *Calculation*

In experiments with synaptosomes prelabelled with [$^3$H]-D-ASP (used as a marker for glutamate release; see, for instance, Drejer *et al*., 1985) the fractions collected and the superfused synaptosomes were counted for radioactivity. The amount of radioactivity released into each fraction was expressed as a percentage of the total synaptosomal tritium present at the start of the respective collection period.

The $K^+$-evoked overflow was estimated subtracting from the $K^+$-evoked release (S) the basal release ($B_1 + B_2$). The $Ca^{2+}$-dependent $K^+$-evoked overflow was calculated, in each experiment, by subtracting the $Ca^{2+}$-independent $K^+$-evoked overflow from the $K^+$-evoked overflow. In our experiments the $Ca^{2+}$-independent $K^+$-evoked release amounted to 40–50% of the $K^+$-evoked overflow. All the drugs studied did not modify the $Ca^{2+}$-independent $K^+$-evoked overflow. Drug effects were evaluated as the per cent ratio of the overflow in presence of the drug vs the overflow in control conditions (run in parallel).

In basal release experiments neurotransmitter efflux was calculated as fractional rate by performing the ratio between the radioactivity present in the second and following fractions and that collected in the first ($B_x/B_1$). Drug effects were evaluated by comparing each ratio in presence of the drug with the corresponding ratio in control conditions (run in parallel) and expressing variations in per cent. Final data are means ± s.e.mean of the given number of experiments ($n$).

Data were analysed by one-way ANOVA followed by Dunnett's multiple comparison test and considered significant for $P < 0.05$ at least.

### *Determination of endogenous glutamate*

Endogenous glutamate was determined by high performance liquid chromatography analysis following precolumn deriva-

tization with *o*-phthalaldehyde and separation on a $C_{18}$ reverse-phase chromatographic column (Chrompack, Middleburg, The Netherlands; $10 \times 4.6$ mm, 3 $\mu$m; 30°C) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm). Buffers and the gradient program were as follows: *solvent A*, 0.1 M sodium acetate (pH 5.8)/methanol 80 : 20; *solvent B*, 0.1 M sodium acetate (pH 5.8)/methanol, 20 : 80; *solvent C*, sodium acetate (pH 6.0)/methanol, 80 : 20; gradient program, 15% B and 85% C for 1 min from the initiation of the program; 50% B and 50% C in 1 min; 30% B and 70% A in 1 min; 100% B in 0.5 min; isocratic flow 2 min; flow rate 0.9 ml $min^{-1}$. Homoserine was used as internal standard.

The amount of endogenous glutamate released was expressed as pmol mg $protein^{-1}$. The KCl-evoked overflow was estimated by subtracting the amount of neurotransmitter in the two 3-min fractions representing the basal release from the neurotransmitter present in the 6-min fraction collected during and after the depolarization pulse. Protein content was determined according to Bradford (1976).

### *Drugs*

[$^3$H]-D-aspartate (specific activity, 16.3 Ci $mmol^{-1}$) was purchased from Amersham Radiochemical Centre (Buckinghamshire, U.K.). Adenosine, 2-chloro-$N^6$-cyclopentyladenosine (CCPA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). CGS 21680 hydrochloride ¼ $H_2O$ was produced by Tocris Cookson Ltd. (Bristol, U.K.). SCH58261 was synthesized at the Schering-Plough Research Institute (Kenilworth, NJ, U.S.A.).

## Results

Adenosine has rarely been used in neurotransmitter release studies. Since the characteristics of our superfusion technique (see Raiteri & Raiteri, 2000) permit the agents added to the medium to hit their targets directly before being metabolized or taken up by transporters, we studied the effects of adenosine on the release of [$^3$H]-D-ASP (used as a tracer for endogenous glutamate/aspartate). Rat cortical synaptosomes were prelabelled with [$^3$H]-D-ASP and then exposed during superfusion to adenosine. The drug added at 0.1, 10 $\mu$M or 1 mM did not produce any effect on the basal release of [$^3$H]-D-ASP (values of $B_2/B_1$ ratio: controls = $0.99 \pm 0.03$, $n = 5$; in presence of 0.1 $\mu$M adenosine = $1.030 \pm 0.04$, $n = 5$; in presence of 10 $\mu$M adenosine = $0.99 \pm 0.05$; $n = 5$; in presence of 1 mM adenosine = $1.05 \pm 0.06$, $n = 5$).

The effect of adenosine was therefore studied in synaptosomes depolarized with 15 mM KCl. When synaptosomes were exposed to a large range of adenosine concentrations (from 0.01 $\mu$M to 1 mM), a significant potentiation of the $Ca^{2+}$-dependent $K^+$-evoked [$^3$H]-D-ASP overflow could be observed only at 0.1 $\mu$M concentration ($20.4 \pm 3.5$%) (Figure 1A). This effect was totally counteracted by the $A_{2A}$ blocker SCH58261 (0.1 $\mu$M; Figure 1B). Adenosine significantly inhibited the $Ca^{2+}$-dependent $K^+$-evoked [$^3$H]-D-ASP overflow when added at concentrations higher than 1 $\mu$M with a maximal inhibitory effect of $37.44 \pm 2.6$% (Figure 1A). The inhibition produced by 1 mM adenosine was significantly increased in presence of $A_{2A}$ antagonist SCH58261 (0.1 $\mu$M), but completely abolished by 1 $\mu$M of the $A_1$ receptor antagonist DPCPX (Figure 1B).

The modest stimulation brought about by 0.1 $\mu$M adenosine acting at $A_{2A}$ receptors might have been due to concomitant activation of inhibitory $A_1$ receptors. However, the presence of 1 $\mu$M DPCPX was unable to potentiate the releasing effect of 0.1 $\mu$M adenosine (Figure 1A). The Figure also shows that the effect of 1 $\mu$M adenosine (slightly inhibitory) was reverted into a stimulatory one by 1 $\mu$M DPCPX. Also the maximal inhibitory effect obtained with 10 $\mu$M adenosine became a stimulatory effect in presence of 1 $\mu$M DPCPX. However, the stimulations of [$^3$H]-D-ASP release observed with 1 or 10 $\mu$M adenosine (having blocked $A_1$ receptors) did not differ significantly from that produced by 0.1 $\mu$M alone. Added at 1 mM with 1 $\mu$M DPCPX, adenosine was unable to cause any effect on [$^3$H]-D-ASP release (Figure 1A). Panel B of Figure 1 shows that 1 $\mu$M DPCPX or 0.1 $\mu$M SCH58261 did not modify, on its own, the $K^+$-evoked release of [$^3$H]-D-ASP, as expected considering the characteristics of the superfusion technique used in this work.

As illustrated in Figure 2A, CGS 21680 increased, in a concentration-dependent manner, the $Ca^{2+}$-dependent $K^+$-evoked overflow of [$^3$H]-D-ASP from cortical synaptosomes. The $EC_{50}$ value amounted to about 1 pM, the maximal effect ($47.69 \pm 6.2$%) being reached at $\sim$ 0.01 nM. The basal release remained unaffected by CGS 21680 (not shown).

The potentiating effect of 0.01 nM CGS 21680 on the $Ca^{2+}$-dependent $K^+$-evoked [$^3$H]-D-ASP overflow was counteracted by the selective $A_{2A}$ receptor antagonist SCH58261 (Zocchi *et al*., 1996) in a concentration-dependent manner (Figure 2B). The calculated $IC_{50}$ value amounted to 5 nM.

The $A_1$ receptor agonist CCPA produced a concentration-dependent inhibition of the $Ca^{2+}$-dependent $K^+$-evoked overflow of [$^3$H]-D-ASP from superfused rat cortical synaptosomes (Figure 3). The calculated $EC_{50}$ of the compound was $\sim$ 20 nM; the maximal effect ($50.18 \pm 4.8$% inhibition) was reached when CCPA was added to the superfusion medium at 100 nM. This effect was completely antagonized by 100 nM DPCPX.

A set of experiments was finally performed to investigate the effects of $A_1$ or $A_{2A}$ receptor ligands on the depolarization-evoked overflow of endogenous glutamate from rat cortical synaptosomes. As illustrated in Figure 4, the $K^+$(15 mM)-evoked overflow of endogenous glutamate was significantly potentiated by 0.01 nM CGS 21680; the potentiation was counteracted by 0.1 $\mu$M SCH58261. Conversely, the $A_1$ receptor agonist CCPA, added at 0.1 $\mu$M, inhibited the depolarization-evoked overflow of endogenous glutamate, an effect antagonized by 0.1 $\mu$M DPCPX.

## Discussion

The technique here used to monitor transmitter release (a very thin layer of synaptosomes up-down superfused) is best suited to identify and characterize targets that are located on a given family of nerve endings. As described in detail in a recent article (Raiteri & Raiteri, 2000), all the substances released by the preparation are quickly removed by the

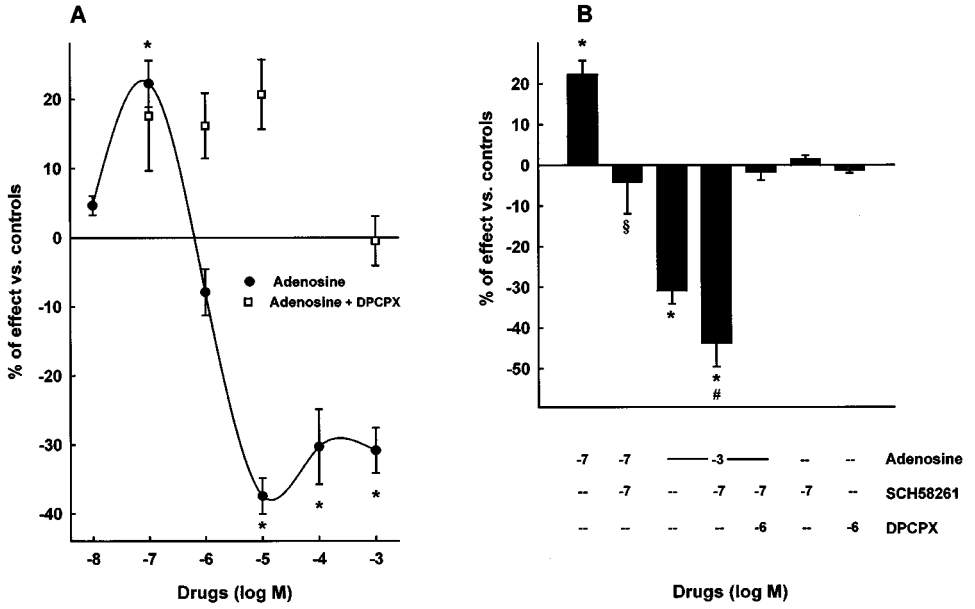


**Figure 1** (A) Effects of adenosine in presence or in absence of $10^{-6}$ DPCPX, on the 15 mM $Ca^{2+}$-dependent KCl-evoked [$^3$H]-D-ASP overflow from rat cortical synaptosomes. Data are means ± s.e.mean of at least six experiments run in triplicate. *$P<0.05$ at least versus controls. (B) Effects of adenosine in presence of the $A_1$ receptor antagonist DPCPX or the $A_{2A}$ receptor antagonist SCH58261 on the 15 mM KCl-evoked [$^3$H]-D-ASP overflow from rat cortical synaptosomes. Data are means ± s.e.mean values of at least five experiments run in triplicate. *$P<0.05$ at least versus controls; $^{\#}P<0.05$ at least versus $10^{-3}$ M adenosine; $^{\S}P<0.05$ at least versus $10^{-7}$ M adenosine.

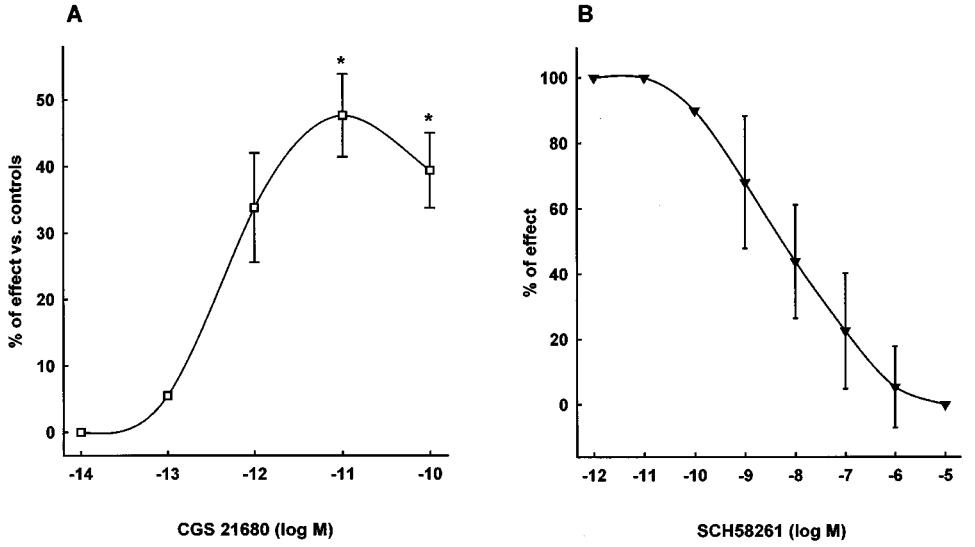


**Figure 2** (A) Effects of CGS 21680 on the $Ca^{2+}$-dependent 15 mM KCl-evoked [$^3$H]-D-aspartate ([$^3$H]-D-ASP) overflow from rat cortical synaptosomes. Data are means ± s.e.mean of at least six experiments run in triplicate. Where s.e.mean is not shown, data are mean values of single experiments run in triplicate. *$P<0.05$ at least versus controls. (B) Effect of the $A_{2A}$ receptor antagonist SCH58261 on the $10^{-11}$ M CGS 21680-evoked potentiation of the [$^3$H]-D-ASP overflow from rat cortical synaptosomes. Data are means ± s.e.mean of six experiments run in triplicate. Where s.e.mean is not shown, data are mean values of single experiments run in triplicate.

superfusion fluid before they can feedback on the releasing terminal or act on neighbouring particles. The targets present on the terminals remain therefore virtually ligand-free but can be activated by agents added to the superfusion medium. These agents reach their targets at the desired concentration which is maintained by the continuous flow in spite of possible enzymatic degradation and uptake. In such a system, indirect effects are prevented or minimized and modulations of release of a given transmitter by the compounds added to the superfusion medium can be assumed to be consequent to direct actions on the nerve terminals releasing that transmitter. This is very important in studies of adenosine as modulator of transmitter release because the contribution of endogenously released adenosine, of adenosine originating from released ATP or of ATP itself are minimal, if any, because of the quick removal of these agents.

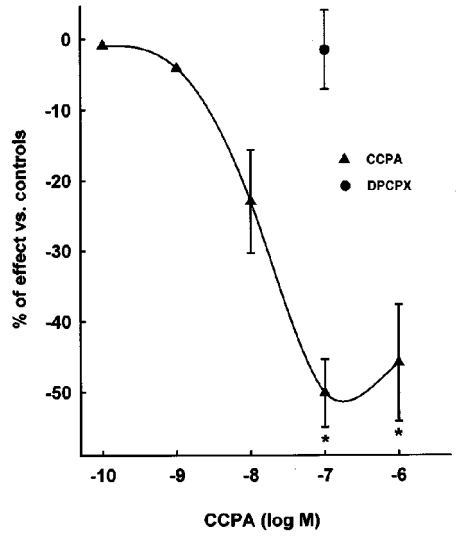


**Figure 3** Effects of the $A_1$ receptor agonist CCPA in presence or in absence of $10^{-6}$ M DPCPX on the $Ca^{2+}$-dependent 15 mM KCl-evoked [$^3$H]-D-ASP overflow from rat cortical synaptosomes. Data are means$\pm$s.e.mean values of at least five experiments run in triplicate. Where s.e.mean is not shown, data are mean values of single experiments run in triplicate. *$P<0.05$ at least versus controls.

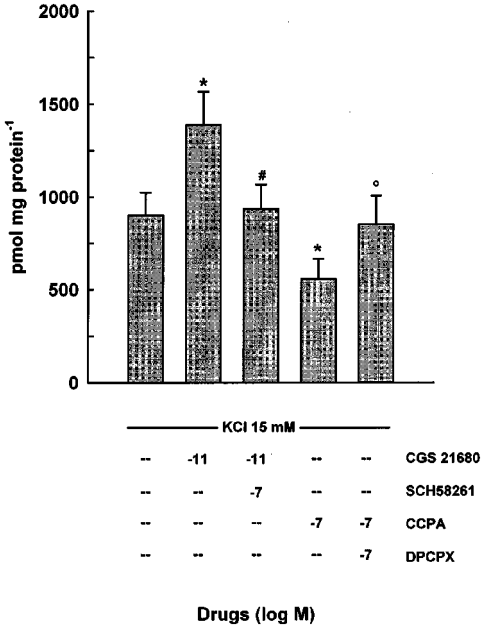


**Figure 4** Effect of CGS 21680, alone or in presence of SCH58261, and of CCPA, alone or in presence of DPCPX, on the 15 mM KCl-evoked glutamate overflow from rat cortical synaptosomes. Data are mean$\pm$s.e.mean of $n$ experiments run in triplicate. *$P<0.05$ at least versus controls; $^{\#}P<0.05$ at least versus $10^{-11}$ M CGS 21680; $^{\circ}P<0.05$ at least versus $10^{-7}$ M CCPA. The basal release of endogenous glutamate, calculated on an average of six experiments, was $300\pm20$ pmoles mg protein$^{-1}$.

In fact, the natural ligand has rarely been employed as an exogenous release modulator in biochemical studies of transmitter release. In a very recent report (Bennett & Boarder, 2000) adenosine (300 $\mu$M) was found to attenuate the depolarization-evoked release of glutamate from rat striatal slices. Previously, adenosine had been reported to inhibit glutamate and aspartate release evoked by electrical stimuli from rat hippocampal slices (Corradetti *et al*., 1984). The veratridine-evoked release of [$^3$H]-acetylcholine from rat cortical synaptosomes was inhibited by exogenous adenosine apparently with no potentiation at low concentrations (Cunha *et al*., 1994).

The findings illustrated in Figure 1 are in keeping with the affinity reported for adenosine on $A_1$ and $A_{2A}$ receptors. The $A_{2A}$ receptor is thought to have high affinity (0.1–1 $\mu$M) for adenosine (Daly *et al*., 1983; Ribeiro, 1999); according to Nishimura *et al*. (1990), adenosine is excitatory on neurotransmission between 0.01 and 1 $\mu$M, but becomes inhibitory between 10 $\mu$M and 1 mM, reflecting a lower affinity for $A_1$ receptors. Based on the characteristics of the superfusion technique employed, our results show that glutamatergic axon terminals in the rat cerebral cortex are endowed with $A_{2A}$ and $A_1$ receptors mediating respectively potentiation and inhibition of the evoked glutamate release.

CGS 21680 is currently used as a selective $A_{2A}$ receptor agonist (see Ongini & Fredholm, 1996). However, in brain regions where the $A_{2A}$ receptors are sparse, such as the cerebral cortex, CGS 21680 binds to additional non-specific undefined sites (Johansson & Fredholm, 1995; Cunha *et al*., 1996). On the other hand, these sites are insensitive to the selective $A_{2A}$ receptor antagonist SCH58261 indicating that they are unrelated to $A_{2A}$ receptors (Lindström *et al*., 1996). As shown in Figure 2B, the elevation of the $K^+$-evoked [$^3$H]-D-ASP overflow produced by the maximally effective concentration (0.01 nM) of CGS 21680 was abolished by SCH58261. The $IC_{50}$ for SCH58261 is 5 nM, which is close to the reported affinity from binding studies ($K_i$ values around 2 nM) and the $IC_{50}$ values obtained in functional tests (see Ongini & Fredholm, 1996). Our results show that the CGS 21680 effect on the release of [$^3$H]-D-ASP or of endogenous glutamate from cortical nerve endings is exclusively mediated by $A_{2A}$ receptors.

Comparing the results obtained with low concentrations of adenosine and those obtained with CGS 21680 it can be seen that the release potentiation produced by the natural modulator acting at $A_{2A}$ receptor ($\sim$ 20%) is significantly lower than that caused by the $A_{2A}$ agonist ($\sim$ 50%). The possibility that 0.1 $\mu$M adenosine concomitantly activates release-inhibiting $A_1$ receptors, thus counteracting the $A_{2A}$-mediated release stimulation, seems unlikely, however, because the effect of adenosine did not increase when the $A_1$ receptors were blocked by DPCPX. No further potentiation of release could be observed when, keeping blocked $A_1$ receptors, the concentrations of adenosine were increased to 1 or 10 $\mu$M, suggesting that, under our experimental conditions, adenosine can produce only a relatively modest potentiation of glutamate release. Why release potentiation by adenosine is lower than that provoked by CGS 21680 is at present unclear.

Adenosine and CGS 21680 were ineffective on the basal release from superfused synaptosomes. In this system the compounds seem to enhance the $Ca^{2+}$-dependent exocytotic component of the release of glutamate evoked by depolarization, but are ineffective on the $Ca^{2+}$-independent component of the $K^+$-evoked overflow. The latter (40–50% of the total overflow) occurs by transporter reversal, being almost totally blocked (85–90%) by the glutamate EAAT1-EAAT3

transporter inhibitor DL-TBOA added at 10 $\mu$M. Similar results had been obtained when the release of [$^3$H]-GABA or [$^3$H]-acetylcholine was studied in superfused hippocampal or striatal synaptosomes. CGS 21680 facilitated the evoked [$^3$H]-GABA or [$^3$H]-acetylcholine release (30 or 15%, respectively) but had no effect on the basal release (Cunha *et al*., 1995; Cunha & Ribeiro, 2000).

The finding that glutamate release is enhanced during depolarization with relatively low [$K^+$] excludes involvement of gliasomes contaminating synaptosomal preparations. In fact, glial preparations release glutamate when exposed to very high concentrations of $K^+$ ($\geqslant$90 mM; Longuemare & Swanson, 1997).

Of interest, the $A_{2A}$ receptors present on glutamatergic terminals exhibit an apparent affinity for CGS 21680 ($EC_{50}$ $\simeq$ 1 pM) about three orders of magnitude higher than that of the $A_{2A}$ receptors present on hippocampal GABAergic terminals ($EC_{50}$ $\simeq$ 1 nM; Cunha & Ribeiro, 2000). On the other hand, CGS 21680 inhibited the $K^+$(15 mM)-evoked release of [$^3$H]-GABA from superfused striatal synaptosomes with an $EC_{50}$ value of 1 pM (Kirk & Richardson, 1994).

The transducing mechanisms by which adenosine and CGS 21680 enhance the evoked release of glutamate remain to be investigated. As discussed recently (Moreau & Huber, 1999; Cunha & Ribeiro, 2000), the facilitation of the $K^+$-evoked release of GABA from hippocampal synaptosomes appears to involve activation of adenylate cyclase and protein kinase A, protein kinase C and P-type $Ca^{2+}$ channels.

The $Ca^{2+}$-dependent $K^+$-evoked [$^3$H]-D-ASP overflow was inhibited by adenosine and by the $A_1$ receptor agonist CCPA with similar maximal effects. Both the inhibitions produced by adenosine and by CCPA were counteracted by the selective $A_1$ receptor antagonist DPCPX. Similar results were obtained by monitoring the $K^+$-evoked release of endogenous glutamate.

Activation of $A_1$ receptors is thought to inhibit the release of various transmitters. In particular, the $A_1$ agonist $N^6$-cyclohexyladenosine depressed the $K^+$- or the 4-aminopyridine-evoked release of endogenous glutamate from guinea-pig cerebral cortex or rat striatal synaptosomes and inhibition of voltage-sensitive $Ca^{2+}$ channels was associated with the $A_1$ receptor-mediated reduction of release (Barrie & Nicholls, 1993; Ambrósio *et al*., 1996). The results obtained by concomitantly adding adenosine at high concentrations and the $A_{2A}$ receptor antagonist indicate that the potentiating effect of adenosine is still present and do not desensitize. This data is in agreement with previous findings showing that $A_{2A}$ receptors remain unaltered upon repeated administration of receptor agonists (Casati *et al*., 1994; Adami *et al*., 1995).

The present demonstration that adenosine receptors of the $A_{2A}$ receptor type able to enhance glutamate release exist on glutamatergic axon terminals in the rat cerebral cortex may help to explain, at least in part, the reduction of the cortical infarct volume observed in rats administered the $A_{2A}$ antagonist SCH58261 after focal cerebral ischaemia (Monopoli *et al*., 1998) and, more generally, the neuroprotective effects of adenosine $A_{2A}$ receptor antagonists. It is worth recalling that extracellular $K^+$ concentrations increase to depolarizing levels during ischaemia and this would facilitate activation of $A_{2A}$ receptors on glutamate terminals. The $A_{2A}$ agonist CGS 21680 was reported to enhance the ischaemia-evoked release of aspartate/glutamate from the rat cerebral cortex *in vivo*, while the basal release was not affected (O'Regan *et al*., 1992). Finally, the above-mentioned higher apparent affinity of CGS 21680 for the $A_{2A}$ receptors present on glutamatergic terminals versus those present on GABAergic terminals, implying a preferential release of glutamate versus that of GABA, may deserve consideration.

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