Journal of Neurochemistry, 2005, **92**, 433–441 doi:10.1111/j.1471-4159.2004.02887.x

Co-localization and functional interaction between adenosine A_{2A} and metabotropic group 5 receptors in glutamatergic nerve terminals of the rat striatum

Ricardo J. Rodrigues, Tiago M. Alfaro, Nelson Rebola, Catarina R. Oliveira and Rodrigo A. Cunha

Centre for Neuroscience of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, Portugal

Abstract

The anti-Parkinsonian effect of glutamate metabotropic group 5 (mGluR5) and adenosine A_{2A} receptor antagonists is believed to result from their ability to postsynaptically control the responsiveness of the indirect pathway that is hyperfunctioning in Parkinson's disease. mGluR5 and A_{2A} antagonists are also neuroprotective in brain injury models involving glutamate excitotoxicity. Thus, we hypothesized that the anti-Parkinsonian and neuroprotective effects of A_{2A} and mGluR5 receptors might be related to their control of striatal glutamate release that actually triggers the indirect pathway. The A_{2A} agonist, CGS21680 (1–30 nM) facilitated glutamate release from striatal nerve terminals up to 57%, an effect prevented by the A_{2A} antagonist, SCH58261 (50 nM). The mGluR5 agonist, CHPG (300–600 μM) also facilitated glutamate release up to 29%, an effect prevented by the mGluR5 antagonist, MPEP (10 μM). Both mGluR5 and A_{2A} receptors were located in the active zone and 57 ± 6% of striatal glutamatergic nerve terminals possessed both A_{2A} and mGluR5 receptors, suggesting a presynaptic functional interaction. Indeed, submaximal concentrations of CGS21680 (1 nM) and CHPG (100 μM) synergistically facilitated glutamate release and the facilitation of glutamate release by 10 nM CGS21680 was prevented by 10 μM MPEP, whereas facilitation by 300 μM CHPG was prevented by 10 nM SCH58261. These results provide the first direct evidence that A_{2A} and mGluR5 receptors are co-located in more than half of the striatal glutamatergic terminals where they facilitate glutamate release in a synergistic manner. This emphasizes the role of the modulation of glutamate release as a likely mechanism of action of these receptors both in striatal neuroprotection and in Parkinson's disease.

Keywords: A_{2A} receptor, adenosine, glutamate, mGluR5, release, striatum.

J. Neurochem. (2005) **92,** 433–441.

The role of the striatum in the control of motor function is essentially viewed as a balanced activation of its two main pathways, the direct and indirect pathways that cause opposite control of thalamic relays, which then impinge on the motor cortex (Obeso *et al*. 2000). These two main striatal pathways are both constituted by medial spiny GABAergic neurons and are both triggered by cortico-thalamic glutamatergic fibres (Calabresi *et al*. 1996). These two pathways are modulated in an opposite manner by important modulators of locomotion, mainly dopamine and also adenosine. Thus, medial spiny neurons of the indirect pathway are endowed with inhibitory dopamine D_2 and facilitatory adenosine A_{2A} receptors, whereas the medial spiny neurons of the direct pathway are endowed with facilitatory D_1 receptors and inhibitory A_1 receptors (Svenningsson *et al*. 1999). Dopamine is considered the chief modulator of the balanced activation of striatal pathways. Thus, in Parkinson's disease, the most common basal ganglia motor dysfunction, there is a severe decrease of striatal dopamine levels that causes a hyperactivation of the indirect pathway (Obeso *et al*. 2000). As facilitatory A_{2A} receptors are selectively located in neurons of the indirect pathway, antagonists of A_{2A} receptors have been developed as anti-parkinsonian drugs (Schwarzschild *et al*. 2002). Furthermore, it is now recognised that the

Received June 3, 2004; revised manuscript received July 28, 2004; accepted August 3, 2004.

Address correspondence and reprint requests to Rodrigo A Cunha, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004–504 Coimbra, Portugal. E-mail: racunha@clix.pt

Abbreviations used: CGS 21680, 2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine; CHPG, (RS)-2-chloro-5-hydroxyphenylglycine; DPCPX, 1,3-dipropyl-8-cyclopentyladenosine; mGluR5, glutamate metabotropic group 5; MPEP, 2-methyl-6-phenylethynyl)pyridine; SCH, 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; vGluT, vesicular glutamate transporter.

control of the responsiveness of the medial spiny neurons of the indirect pathway depends not only on D_2 and A_{2A} receptors but also on metabotropic glutamate receptors of the subtype 5 (mGluR5), which form a trimeric complex to control the responsiveness of medial spiny neurons of the indirect pathway (Ferré *et al*. 2003). And, as occurs with antagonists of A_{2A} receptors (Schwarzschild *et al*. 2002), antagonists of mGluR5 receptors also display anti-parkinsonian properties (Ossowska *et al*. 2001; Breysse *et al*. 2002).

Interestingly, antagonists of either A_{2A} (Fredholm *et al*. 2003) or mGluR5 receptors (Bruno *et al*. 2000; Battaglia *et al*. 2002) are also neuroprotective in different models of brain injury that involve glutamate excitotoxicity. However, it is not clear if this neuroprotective effect is associated with the ability of A_{2A} and mGluR5 receptors to control glutamate release, a key event in different neurological disorders (Lipton and Rosenberg 1994). And if these two receptors would control glutamate release, it could be hypothesised that the anti-parkinsonian effects of A_{2A} or mGluR5 receptors antagonists might not only be because of their ability to control the responsiveness of neurons of the indirect pathway but also to their ability to control the release of glutamate that is actually the trigger of striatal pathways (Calabresi *et al*. 1996). One fundamental issue that needs to be demonstrated to entertain this hypothesis is to show if A_{2A} and mGluR5 receptors are indeed located in glutamatergic nerve terminals impinging in the striatum and if they facilitate glutamate release.

As our interest is to focus on the possible presynaptic location of A_{2A} and mGluR5 receptors, we chose the most adequate experimental model to study presynaptic phenomena, i.e. purified nerve terminals or synaptosomes (Cunha 1998), which rule out possible confounding effects intrinsic to experimental manipulation where the striatal circuitry is preserved like electrophysiological recordings in striatal slices or microdialysis in living animals (Corsi *et al*. 2000, 2003). This allowed us to conclude that both A_{2A} and mGluR5 receptors are actually co-located in nearly half of the glutamatergic nerve terminals in the rat striatum where they facilitate the evoked release of glutamate, operating in a synergistic manner.

Materials and methods

Male Wistar rats (6–8 weeks old, 140–160 g, obtained from Harlan Ibérica, Barcelona, Spain) were used throughout this study and were handled according with the EU guidelines for use of experimental animals, the rats being anaesthesized under halothane atmosphere before being killed by decapitation.

[^{3}H]glutamate release studies

The release of [^{3}H]glutamate from rat striatal nerve terminals was performed as previously described (Lopes *et al*. 2002). The nerve terminals were prepared using a combined sucrose/Percoll centrifugation protocol and were re-suspended in oxygenated Krebs solution of the following composition (in mM): NaCl 124, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 25, $MgSO_4$ 2, $CaCl_2$ 2 and glucose 10, which was gassed with a 95% O_2 and 5% CO_2 mixture. The nerve terminals were equilibrated at 37°C for 10 min, loaded with [^{3}H]glutamate (0.2 μM) for 5 min at 37°C, washed, layered over Whatman GF/C filters and superfused (flow rate: 0.8 mL/min) with Krebs solution for 20 min before starting collection of the superfusate. The synaptosomes were stimulated with 20 mM K^+ at 3 and 9 min after starting sample collection (S_1 and S_2), triggering a release of tritium that was found to be mostly [^{3}H]glutamate, released in a Ca^{2+}-dependent manner (Lopes *et al*. 2002). Tested agonists were added 2 min before S_2 onwards, whereas antagonists were added from 10 min before starting sample collection onwards. Radioactivity was expressed in terms of disintegrations per second per milligram of protein (Bq/mg) in each chamber (Lopes *et al*. 2002).

Western blot analysis in subsynaptic fractions

The relative mGluR5 and A_{2A} receptor immunoreactivity was evaluated by western blot analysis, as previously described (Rebola *et al*. 2003a) using antibodies against either adenosine A_{2A} receptor (1 : 500 dilution) or mGluR5 receptors (1 : 3000 dilution). This analysis was carried out in the purified presynaptic and postsynaptic components of the active zone as well as in the non-active zone fraction of rat striatal nerve terminals, separated upon solubilization of the synaptosomal fraction as initially described by Phillips *et al*. (2001). We have previously confirmed that this subsynaptic fractionation method allows an over 90% effective separation of active zone (syntaxin and SNAP25), postsynaptic density (PSD95 and NMDA receptor subunit 1) and extra-synaptic (synaptophysin) markers and can be used to access the subsynaptic distribution of metabotropic receptors (Rebola *et al*. 2003b).

Immunocytochemical analysis in nerve terminals

For immunochemical analysis, striatal synaptosomes were obtained through a discontinuous Percoll gradient, as previously described (Diáz-Hernández *et al*. 2002), with minor modifications. Striatal tissue was homogenized in a medium containing 0.25 M sucrose and 5 mM TES (pH 7.4). The homogenate was spun for 3 min 2000 *g* at 4°C and the supernatant spun again at 9500 *g* for 13 min. Then, the pellets were re-suspended in 8 mL of 0.25 M sucrose and 5 mM TES (pH 7.4) and 2 mL were placed onto 3 mL of Percoll discontinuous gradients containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol and 3, 10, or 23% Percoll, pH 7.4. The gradients were centrifuged at 25 000 *g* for 11 min at 4°C. Synaptosomes were collected between the 10 and 23% Percoll bands and diluted in 15 mL of HEPES buffered medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). After centrifugation at 22 000 *g* for 11 min at 4°C, the synaptosomal pellet was removed. This procedure for preparation of the synaptosomes (in the absence of calcium) is crucial to allow reducing the amount of postsynaptic density material. In fact, immunocytochemical analysis of the synaptosomes obtained with this discontinuous Percoll gradient showed that less than 3% of the synaptophysin-positive elements were labelled by an anti-PSD-95 antibody (data not shown).

These striatal synaptosomes were placed onto cover-slips previously coated with poly L-lisine, fixed with 4% paraformaldehyde for

15 min and washed twice with phosphate-buffered saline (PBS) medium (140 mM NaCl, 3 mM KCl, 20 mM NaH_2PO_4, 15 mM KH_2PO_4, pH 7.4). The synaptosomes were permeabilized in PBS with 0.2% Triton X-100 for 10 min and then blocked for 1 h in PBS with 3% bovine serum albumin (BSA) and 5% normal rat serum. The synaptosomes were then washed twice with PBS and incubated with goat anti-A_{2A} receptor (1 : 500), guinea pig anti-vGluT1 (1 : 5000) and guinea pig anti-vGluT2 (1 : 5000), together with rabbit anti-mGluR5 (1 : 1000) or mouse anti-synaptophysin (1 : 200) for 1 h at 23–25°C. It should be emphasized that anti-vGluT1 and anti-vGluT2 were applied together to identify the population of rat striatal glutamatergic nerve terminals. The synaptosomes were then washed three times with PBS with 3% BSA and incubated for 1 h at room temperature with AlexaFluor-488 (green) labelled donkey anti-goat IgG antibody, AlexaFluor-598 (red) labelled goat anti-guinea pig IgG and AlexaFluor-350 (blue) labelled goat anti-rabbit or goat anti-mouse IgG antibodies (1 : 200 for all). To avoid recognition of the goat anti-guinea pig, goat anti-rabbit and goat anti-mouse antibodies by the donkey anti-goat antibody, first we applied the donkey anti-goat and, after washing with PBS, we applied the other secondary antibodies. After washing and mounting onto slides with Prolong Antifade, the preparations were visualized in a Zeiss Axiovert 200 inverted fluorescence microscope equipped with a cooled CCD camera and analysed with MetaFluor 4.0 software. Each cover-slip was analyzed by counting three different fields and in each field a total amount of 100 individualized elements.

Statistics

The values are mean ± SEM of n experiments. To test the significance of the effect of a drug versus control, a paired Student's t-test was used. When making comparisons from different set of experiments with control, one-way variance analysis (ANOVA) was used, followed by Dunnett's test. $p \leq 0.05$ was considered to represent a significant difference.

Reagents

2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) and 1,3-dipropyl-8-cyclopentyladenosine (DPCPX) were from Research Biochemicals (Reagente 5, Oporto, Portugal), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) was a kind gift of Scott Weiss (Vernalis, UK) (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) and 2-methyl-6-phenylethylnyl)pyridine (MPEP) were from Tocris (Avonmouth, UK), [^{3}H]glutamate (specific activity 45 Ci/mmol) was from Amersham (Buckinghamshire, UK), goat purified IgG anti-adenosine A_{2A} receptor antibody (200 μg/mL) was from Santa Cruz Biotechnology-Europe (Freelab, Lisbon, Portugal), rabbit purified IgG anti-mGluR5 receptor antibody (100 μg in 232 μL of 0.2 M Tris/glycine buffer, pH 7.5, with 0.15 M NaCl) was from Upstate (Reagente 5, Oporto, Portugal), guinea pig anti-vesicular glutamate transporter type 1 and guinea pig anti-vesicular glutamate transporter type 2 were from Chemicon (Hofheim, Germany), mouse anti-synaptophysin (100 μg in 200 μL water) was from Sigma (Sintra, Portugal) and mouse anti-PSD-95 was from Upstate Biotechnology (Lake Placid, NY, USA). All other reagents were of the highest purity available.

CGS 21680 and SCH 58261 were made up as 5-mM stock solutions in dimethylsulfoxide, DPCPX was made up as a 5-mM stock in 99% dimethylsulfoxide and 1% NaOH 1 M and MPEP was made up as a 5-M stock in 100 mM NaOH. All drug stock solutions were diluted directly into the superfusion solution to the appropriate final concentration and the pH corrected to pH 7.4 when required. Dimethylsulfoxide, in the maximal concentration used, was devoid of effects on [^{3}H]glutamate release.

Results

Modulation of glutamate release by A_{2A} and mGluR5 receptors

The evoked release of tritium, triggered by 20 mM K^+ for 30 s, from superfused nerve terminals previously labelled with [^{3}H]glutamate was essentially constituted by [^{3}H]glutamate that is released in a Ca^{2+}-dependent manner (Lopes *et al*. 2002), suggesting that it may represent a vesicular release of glutamate.

The presence of the selective A_{2A} receptor agonist, CGS 21680 (1–30 nM), facilitated in a concentration-dependent manner the evoked release of glutamate from striatal nerve terminals (Fig. 1). In fact, 1 nM CGS 21680 facilitated the evoked release of glutamate by 20.8 ± 8.5% ($n = 6$), 10 nM CGS 2160 caused a 57.1 ± 9.8% ($n = 5$) facilitation and 30 nM CGS 21680 caused a 50.4 ± 9.0% ($n = 6$) facilitation. This facilitatory effect of 10 nM CGS 21680 was prevented by the selective A_{2A} receptor antagonist, SCH 58261 (50 nM, $n = 5$), but not significantly ($p > 0.05$) modified by the selective A_1 receptor antagonist, DPCPX (50 nM, $n = 4$). This indicates that the facilitatory effect of CGS 21680 on the evoked release of glutamate from striatal nerve terminals is mediated by A_{2A} receptors and is independent of A_1 receptor modulation, in contrast to what was reported in glutamatergic nerve terminals of the rat hippocampus (Lopes *et al*. 2002). It should be noted that neither SCH 58261 nor DPCPX caused any effect per se on the evoked release of glutamate, in accordance with the inexistence of tonic effects of endogenously released modulators in superfused synaptosomes because any released substance is effectively washed out by superfusion (Raiteri and Raiteri 2000).

The selective mGluR5 receptor agonist, CHPG (300–600 μM), also facilitated the evoked release of glutamate from striatal nerve terminals (Fig. 2). In fact, 300 μM CHPG facilitated the evoked release of glutamate by 25.7 ± 6.4% ($n = 6$) and 600 μM CHPG caused a 28.8 ± 5.8% ($n = 6$) facilitation, whereas lower concentrations of CHPG (10–100 μM) failed to significantly ($p > 0.05$) modify the evoked release of glutamate. The facilitatory effect of 300 μM CHPG was prevented by the selective mGluR5 receptor antagonist, MPEP (10 μM, $n = 4$), further confirming that the activation of mGluR5 receptors facilitates the evoked release of glutamate from striatal nerve terminals.

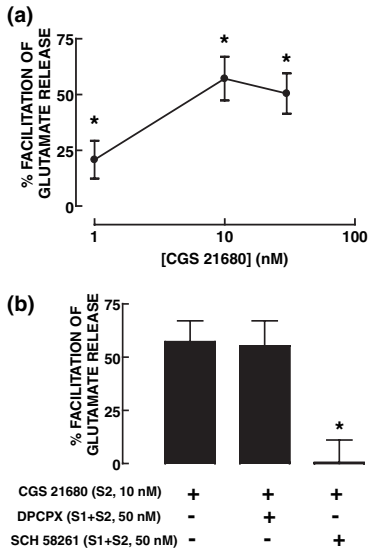

Fig. 1 Concentration-dependent facilitation by the adenosine A_{2A} receptor agonist, CGS 21680, of the evoked release of [^{3}H]glutamate from rat striatal nerve terminals (a) and antagonism of this effect by a selective A_{2A} receptor antagonist, SCH 58261, but not by an A_1 selective antagonist, DPCPX (b). Neither SCH 58261 (50 nM) nor DPCPX (50 nM) modified the evoked release of glutamate by themselves (not shown). *$p < 0.05$ compared with 0% in (a) and with the effect of CGS 21680 (30 nM, first bar from the left) in (b). The results are mean ± SEM of four to six experiments.

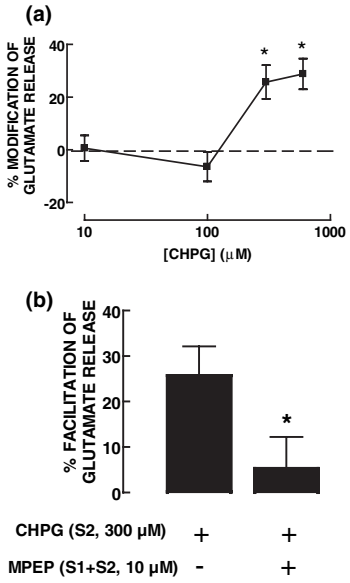

Fig. 2 Concentration-dependent facilitation by the metabotropic glutamate receptor subtype 5 (mGluR5) agonist, CHPG, of the evoked release of [^{3}H]glutamate from rat striatal nerve terminals (a) and antagonism of this effect by a selective mGluR5 antagonist, MPEP (b), which was devoid of effects by itself (not shown). *$p < 0.05$ compared with 0% in (a) and with the effect of CHPG (300 μM, first bar from the left) in (b). The results are mean ± SEM of four to six experiments.

Identification of A_{2A} and mGluR5 receptors in glutamatergic nerve terminals

The ability of both A_{2A} and mGluR5 agonists to facilitate the evoked release of glutamate allows us to predict that these receptors should be located in the active zone of nerve terminals. To confirm this, we used a recent method of subsynaptic fractionation that allows separating the presynaptic active zone and postsynaptic density from other presynaptic proteins not located in synapses (Phillips *et al.* 2001). We have previously validated this technique that allows an over 90% efficiency of separation of these fractions and we have confirmed its usefulness to evaluate the subsynaptic distribution of adenosine receptors (Rebola *et al.* 2003b). As illustrated in Fig. 3, we found that A_{2A} receptors could be identified by western blot analysis in the presynaptic active zone fraction, albeit this receptor is most densely located in the postsynaptic density fraction, as also concluded in electron microscopy studies carried out by others (Hettinger *et al.* 2001). We also found that mGluR5 receptors were located in the active zone (Fig. 3), although mGluR5 immunoreactivity was greater in the postsynaptic density fraction, as also concluded in electron microscopy studies carried out by others (Paquet and Smith 2003).

Because this fractionation procedure does not allow separating nerve terminals releasing different types of neurotransmitters, we undertook a complementary immunocytochemistry approach aimed at defining whether A_{2A} and mGluR5 receptors immunoreactivity could be detected in glutamatergic nerve terminals, identified with the simultaneous use of antibodies against vesicular glutamate transporters type 1 and 2 (vGluT1 and vGluT2). As illustrated in Fig. 4(a), 45.5 ± 4.6% of the synaptophysin immunoreactive elements were also endowed with A_{2A} receptor immunoreactivity and 52.1 ± 1.4% of the synaptophysin immunoreactive elements were also endowed with mGluR5 receptor immunoreactivity ($n = 3$). In the general population of striatal nerve terminals, identified as synaptophysin-immunoreactive elements, we found a general colocalization of A_{2A} and mGluR5 immunoreactivity (Fig. 4). In fact, 83.8 ± 8.9% ($n = 3$) of A_{2A} receptor-immunopositive nerve terminals were also labelled with the mGluR5 receptor antibody. Likewise, 90.0 ± 2.0% ($n = 5$) of the mGluR5 receptor-immunopositive nerve terminals were also labelled with the A_{2A} receptor antibody. However, glutamatergic nerve terminals (vGluT1- and vGluT2-immunoreactive) only represented 20.2 ± 1.6% ($n = 3$) of striatal nerve terminals, identified as synaptophysin-positive elements. And, as illustrated in Fig. 4, triple labelling studies with antibodies against A_{2A} and mGluR5 receptors and against vGluT1 and vGluT2 indicated that 57.4 ± 1.1% ($n = 4$) of the vGluT1 or -2 positive nerve terminals are equipped with both A_{2A} and mGluR5 receptors and few (< 6.8%) of glutamatergic nerve terminals are equipped with only A_{2A}

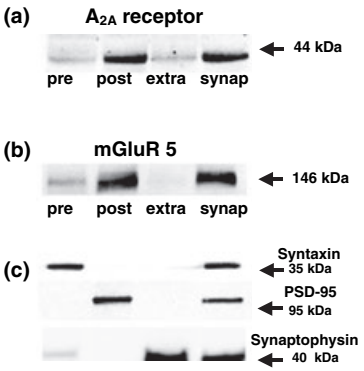

Fig. 3 Subsynaptic distribution of adenosine A_{2A} receptors (a) and metabotropic glutamate receptor subtype 5 (mGluR5) (b). The figures correspond to a western blot comparing the A_{2A} receptor (a) and mGluR5 (b) immunoreactivity, corresponding to the 44-kDa (a) and 146-kDa (b) bands, in a fraction enriched in the presynaptic active zone (pre), in the postsynaptic density (post), in nerve terminals outside the active zone (extra) and in the initial synaptosomal fraction (synap) from where fractionation began. These fractions were over 90% pure, as illustrated by the ability to recover the immunoreactivity for syntaxin in the presynaptic active zone fraction, PSD95 in the postsynaptic density fraction and synaptophysin (a protein located in synaptic vesicles) in the extra-synaptic fraction (c). Forty micrograms of protein of each fraction was applied to the sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) gel. Each blot is representative of four blots from different animals with similar results. Note that, although both mGluR5 and A_{2A} receptors are most abundant in the postsynaptic density fraction, they are also located in the active zone fraction.

receptors or mGluR5 receptors (Fig. 4). This directly shows that A_{2A} and mGluR5 receptors are located in glutamatergic nerve terminals, but it also indicates that only about half of the glutamatergic nerve terminals are equipped with these two neuromodulatory systems. It should also be noted that presynaptic A_{2A} and mGluR5 receptors are not solely located in glutamatergic nerve terminals in the striatum. In fact, only $27.8 \pm 1.6\%$ ($n = 4$) of the A_{2A} receptor immunoreactivity is located in vGluT1 or vGluT2 immunoreactive nerve terminals. Likewise, only $32.3 \pm 1.5\%$ ($n = 4$) of the mGluR5 receptor immunoreactivity is located in vGluT1 or vGluT2 immunoreactive nerve terminals.

Functional interaction between presynaptic A_{2A} and mGluR5 receptors

As we found a near total co-localization of A_{2A} and mGluR5 receptors in about 50% of the glutamatergic nerve terminals of the rat striatum, and it was previously shown that there was a synergy between postsynaptic A_{2A} and mGluR5 receptors (Ferré *et al*. 2002; Nishi *et al*. 2003), we tested if there was also an interaction between presynaptic A_{2A} and mGluR5 receptors in the control of glutamate release. As illustrated in Fig. 5, we found that there was a cross-inhibition by antagonists of these two modulatory systems. In fact, the facilitation of the evoked release of glutamate by the A_{2A} receptor agonist, CGS 21680 (10 nM) was prevented by the mGluR5 receptor antagonist, MPEP (10 μM, $n = 4$). Likewise, the facilitation of the evoked release of glutamate by the mGluR5 receptor agonist, CHPG (300 μM), was prevented by the A_{2A} receptor antagonist SCH 58261 (50 nM, $n = 4$). This suggests a tight interaction between these two presynaptic receptor systems in glutamatergic nerve terminals.

To investigate if the activation of A_{2A} and mGluR5 receptors caused a synergistic facilitation of the evoked release of glutamate, we tested the effect of co-activation of both A_{2A} and mGluR5 receptors. When using submaximal concentrations of each receptor agonist, we observed that the co-administration of 1 nM CGS 21680, together with 100 μM CHPG, caused a $44.3 \pm 6.8\%$ ($n = 9$) facilitation of the evoked release of glutamate (Fig. 5c). This facilitation is greater ($p < 0.05$) than that obtained only with 1 nM CGS 21680 ($20.8 \pm 8.5\%$, $n = 6$) and represents a synergistic effect as 100 μM CHPG did not significantly ($p > 0.05$) modify the evoked release of glutamate. To further confirm that there was a cross-dependency resulting from the simultaneous activation of these two presynaptic neuromodulatory systems, we also studied the effect of the co-activation of A_{2A} and mGluR5 receptors, but now with maximally effective concentrations of each agonist. Under these conditions, one would expect that physically interacting receptors should produce a less than additive effect, because only one rather than two G proteins can bind the complex at a time (e.g. Breitwieser 2004) levelling the agonist efficacy to that of the most efficacious individual receptor system (Kenakin 2002). In fact, as illustrated in Fig. 5(d), the facilitation of glutamate release on co-activation with maximally effective concentrations of A_{2A} and mGluR5 agonists was partially additive but not synergistic. Thus, the co-administration of 10 nM CGS 21680, together with 300 μM CHPG, caused a $72.7 \pm 5.2\%$ ($n = 6$) facilitation. This facilitation is lower than the sum of the facilitatory effects caused by 10 nM CGS 21680 ($57.1 \pm 9.8\%$) or 300 μM CHPG ($25.7 \pm 6.4\%$) when tested alone.

Discussion

The present results provide direct morphological and functional evidence indicating that adenosine A_{2A} receptors and metabotropic glutamate receptors subtype 5 (mGluR5) are co-located in a subset of glutamatergic nerve terminals of the rat striatum. In fact, we found that there was a co-localization of A_{2A} and mGluR5 receptor immunoreactivity in striatal nerve terminals and, in particular, in nerve terminals endowed with the vesicular glutamate transporter type 1 or type 2 (vGluT1or vGluT2), i.e. glutamatergic nerve

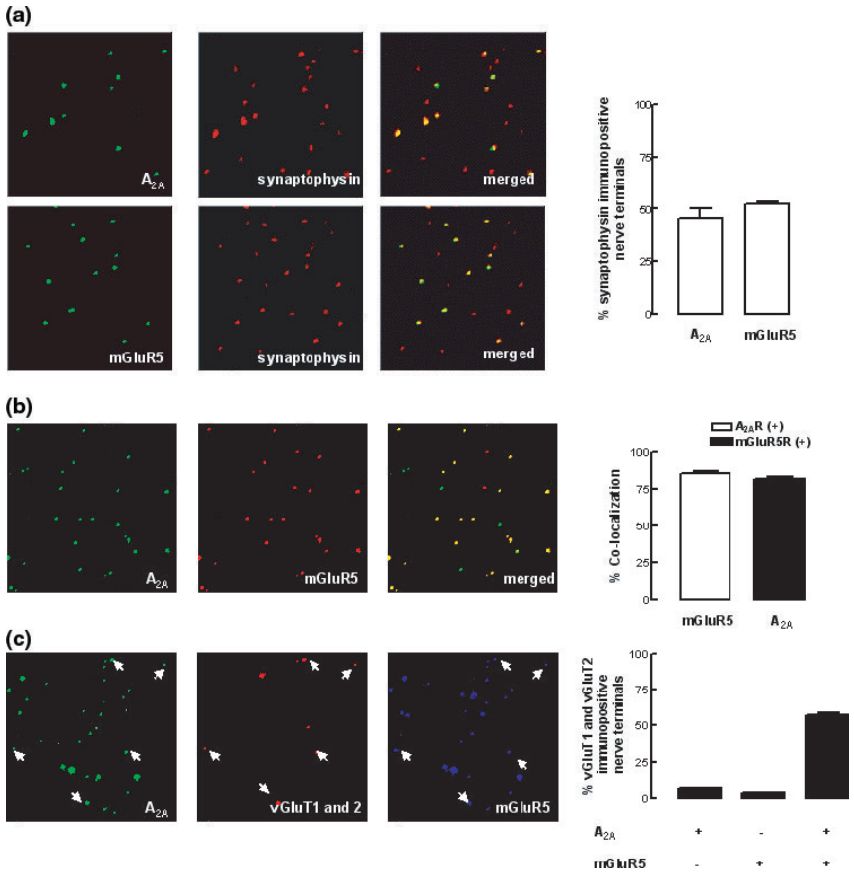

Fig. 4 Co-localization of adenosine A_{2A} receptors and metabotropic glutamate receptor subtype 5 (mGluR5) in glutamatergic nerve terminals of the rat striatum. (a) The immunocytochemical identification of adenosine A_{2A} receptors and mGluR5 (left panels) in the total population of rat striatal nerve terminals (identified as synaptophysin immunoreactive). Merged images (right panels) illustrate that about 45% of striatal nerve terminals are endowed with A_{2A} receptors and near 50% are endowed with mGluR5. (b) The immunocytochemical identification of adenosine A_{2A} receptors (left panel) and mGluR5 receptors (centre panel) and a merged image that illustrates the near complete (84–90%) co-localization of these two receptors in striatal nerve terminals. (c) The immunocytochemical identification of A_{2A} receptors (left panel), mGluR5 (right panel) and vesicular glutamate transporters type 1 and type 2 (vGluT1 and vGluT2, markers of glutamatergic nerve terminals) showing that there is a co-localization of mGluR5 and A_{2A} receptors in nearly 50% of the glutamatergic nerve terminals (identified as vGluT1 and vGluT2 immunoreactive). These fields are representative of three different fields per cover-slip, in experiments carried out three to four times using different synaptosomal preparations from different animals. The data are mean ± SEM of three to four experiments.

terminals. Furthermore, the activation of A_{2A} or mGluR5 receptors facilitated the evoked release of glutamate from superfused striatal nerve terminals, these effects being prevented by selective antagonists of each receptor. The strength of this main conclusion is reinforced by our choice to use purified nerve terminals, which excludes the possible involvement of indirect, circuit-mediated effects and allows ascribing the observed effects as direct effects on glutamatergic nerve terminals. It should be pointed out that, although we are emphasising the novelty of the present finding that A_{2A} and mGluR5 receptors are located in glutamatergic nerve terminals, we observed that the majority of each of these receptors is located outside nerve terminals in striatal tissue, namely in the postsynaptic density, in accordance with previous reports (Hettinger *et al*. 2001; Paquet and Smith 2003). This confirms the role of A_{2A} and mGluR5 receptors in the control of the responsiveness of medial spiny neurons (Ferré *et al*. 2002; Nishi *et al*. 2003). However, the observation that there was a lower density of A_{2A} and mGluR5 receptors located in glutamatergic nerve terminals does not necessarily mean that the modulation of glutamate release by A_{2A} and mGluR5 receptors might represent a minor action of these receptors in the striatum. In fact, it should be noted that the volume and amount of protein associated with nerve terminals is expected to be several-fold lower than other neuronal compartments like dendrites (Rusakov *et al*. 1998; Itzev *et al*. 2001). Therefore, when considering receptors that are located both pre- and postsynaptically, it is always expected that they should be more abundant postsynaptically because of the larger volume

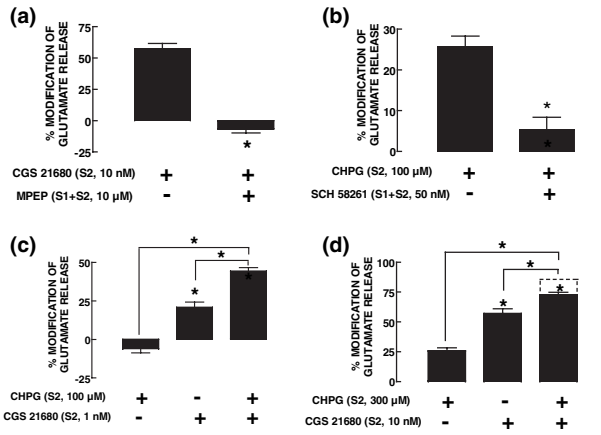

Fig. 5 Interaction between the adenosine A_{2A} receptor agonist, CGS 21680, and the metabotropic glutamate receptor subtype 5 (mGluR5) agonist, CHPG, in the facilitation of the evoked release of [^{3}H]glutamate from rat striatal nerve terminals. $^*p < 0.05$. The dashed bar in (d) illustrates the arithmetic sum of the facilitatory effects of 10 nM CGS 21680 and 300 μM CHPG, if these effects were purely additive. The results are mean ± SEM of four to six experiments.

occupied by dendrites. Also, the population of nerve terminals which are glutamatergic is low in the striatum (20%), so the relative amount of A_{2A} and mGluR5 receptors associated with these particular subtypes of nerve terminals actually represent a robust density of these receptors in glutamatergic nerve terminals, given that only 50% of the glutamatergic terminals are equipped with these receptors. Finally, it should be stressed that glutamate is actually the chief signal to drive rather than to modulate striatal circuits (as occurs with postsynaptic metabotropic receptors) and any modulatory effect on the release of glutamate is expected to have profound consequences in the functioning of striatal circuits.

Previous studies by others have documented the ability of A_{2A} receptor ligands to affect the outflow of glutamate in the striatum (Corsi *et al*. 2000). However, these studies were carried out using *in vivo* microdialysis and do not allow us to distinguish between a direct control of glutamate release and indirect effects as a result of the change of firing of the circuitry controlling glutamate release, as pointed out by the authors (Corsi *et al*. 2000, 2003). In fact, these studies observed that activation of A_{2A} receptors had an opposite effect on the spontaneous and K^+-evoked release of glutamate (Corsi *et al*. 2000), which supports multiple superimposable mechanisms of control by A_{2A} receptors of glutamate release that do not allow us to ascribe direct effects of A_{2A} receptors on glutamatergic nerve terminals. Likewise, it was also shown that activation of mGluR5 receptors enhances striatal glutamate levels (Pintor *et al*. 2000), but again it was not possible to define whether these effects were indirectly circuit-mediated or direct effects on glutamatergic terminals, an issue that is still debatable in other brain regions (cf. Sistisaga *et al*. 1998; Reid *et al*. 1999). Thus, the present results allow us to conclude that both adenosine A_{2A} and mGluR5 receptors are located in glutamatergic nerve terminals and their activation facilitates the evoked release of glutamate in the striatum.

This observed presynaptic localization and marked facilitatory effects of A_{2A} and mGluR5 receptors in striatal glutamatergic nerve terminals may be of relevance for our understanding of the mechanisms by which antagonists of either type of receptors acts as neuroprotective or anti-Parkinsonian agents. In fact, imbalances in the amount of released glutamate are a common cause of neurodegeneration in a variety of acute or chronic noxious brain conditions (Lipton and Rosenberg 1994). Also, because glutamate is the signal that actually triggers, rather than modulates, striatal circuits (Calabresi *et al*. 1996), any modulatory effect on this chief signal is expected to have a stronger impact on striatal circuitry. Either A_{2A} receptor antagonists (Schwarzschild *et al*. 2002) or mGluR5 receptor antagonists (Ossowska *et al*. 2001) are currently being explored as potential anti-parkinsonian drugs. The anti-parkinsonian effects of these two types of antagonists has so far been thought to result from their ability to control postsynaptic responsiveness selectively in medial spiny neurons of the indirect pathway (Ferré *et al*. 2003), which is hyperfunctioning in Parkinson's disease (Obeso *et al*. 2000). The present observation that these two receptor systems are also located in glutamatergic nerve terminals and enhance the release of glutamate, which is actually the trigger of striatal pathways, raises the hypothesis that the anti-parkinsonian effects of these antagonists might also involve the attenuation of the glutamatergic drive of striatal pathways. If A_{2A} and mGluR5 receptors would be located in glutamatergic terminals impinging on medial spiny neurons of the direct pathway, blockade of these facilitatory receptors would contribute to decreasing the drive of this already depressed pathway in situations of Parkinson's disease, i.e. A_{2A} and mGluR5 antagonists would actually contribute to worsen the balance of striatal circuitry, the opposite of that which is experimentally observed (Ossowska *et al*. 2001; Schwarzschild *et al*. 2002). For the control of glutamate release by A_{2A} and mGluR5 receptors to make sense in the realm of their anti-parkinsonian effects, one needs to assume the hypothesis that these receptors would be located mostly in the glutamatergic bouttons that trigger the indirect rather than the direct pathway. It should be made clear that this is a working hypothesis and that there is no experimental evidence to support this hypothesis. However, there is a proof of concept for the selective localization of a presynaptic neuromodulatory system (operated by ATP P2X2 receptors) only in synapses with particular targets within the

same neuron in hippocampal circuits (Khakh *et al*. 2003). It is also striking that only half of the glutamatergic nerve terminals in the striatum are equipped with A_{2A} and mGluR5 receptors. Further work based on electrophysiological recordings of cortico-thalamic striatal excitatory transmission will hopefully allow the testing of this current hypothesis that A_{2A} and mGluR5 receptors might only be located in the glutamatergic nerve terminals that selectively innervate medial spiny neurons of the indirect pathway question.

Another issue that arises from the present results is the interest of either A_{2A} (Fredholm *et al*. 2003) or mGluR5 antagonists (Bruno *et al*. 2000; Battaglia *et al*. 2002) as neuroprotective agents in different neurotoxic situations that involve glutamatergic excitotoxicity. In light of the observed cross-inhibitory effects of mGluR5 and A_{2A} receptor antagonists now observed to occur in the control of glutamate release, is would be interesting to test whether there is also a lack of additivity in the neuroprotective effects of the antagonists of these two modulatory systems. In fact, if there is an additivity of protective effects caused by antagonists of A_{2A} and mGluR5 receptors, then one should search for sites of action of these receptors systems other than the control of glutamate release or the control of the responsiveness of the indirect pathway. In contrast, if there is no additive protective effects with the simultaneous administration of the antagonists of A_{2A} and mGluR5 receptors, then one is faced with a very favourable situation where a combined subdosage of each antagonist could yield maximal neuroprotection, while decreasing the eventual risk of side-effects because of each receptor system operating in different brain regions or organs. The same rationale should hold true for anti-parkinsonian effects for which studies resulting from the co-administration of A_{2A} and mGluR5 antagonists are warranted.

Acknowledgements

This work was supported by Fundação para a Ciência e Tecnologia (POCTI/44740/2002). The authors thank J. O. Malva and P. C. Pinheiro for helping in the subcellular fractionation experiments and R. Franco and F. Ciruela for fruitful discussions.

References

Battaglia G., Fornai F., Busceti C. L., Aloisi G., Cerrito F., de Blasi A., Melchiorri D. and Nicoletti F. (2002) Selective blockade of mGlu5 metabotropic glutamate receptors is protective against methamphetamine neurotoxicty. *J. Neurosci.* **22**, 2135–2141.

Breitwieser G. E. (2004) G protein-coupled receptor oligomerization. Implications for G protein activation and cell signalling. *Circ. Res.* **94**, 17–27.

Breysse N., Baunez C., Spooren W., Gasparini F. and Amalric M. (2002) Chronic but not acute treatment with a metabotropic glutamate 5 receptor antagonist reverses the akinetic deficits in a rat model of parkinsonism. *J. Neurosci.* **22**, 5669–5678.

Bruno V., Ksiazek I., Battaglia G., Lukic S., Leonhardt T., Sauer D., Gasparini F., Kuhn R., Nicoletti F. and Flor P. J. (2000) Selective blockade of metabotropic glutamate receptor subtype 5 is neuroprotective. *Neuropharmacology* **39**, 2223–2230.

Calabresi P., Pisan A., Mercuri N. B. and Bernardi G. (1996) The corticostriatal projection: from synaptic plasticity to dysfunctions of the basal ganglia. *Trends Neurosci.* **19**, 19–24.

Corsi C., Melani A., Bianchi L. and Pedata F. (2000) Striatal A_{2A} adenosine receptor antagonism differentially modifies striatal glutamate outflow *in vivo* in young and aged rats. *Neuroreport* **11**, 2591–2595.

Corsi C., Pinna A., Gianfriddo M., Melani A., Morelli M. and Pedata F. (2003) Adenosine A_{2A} receptor antagonism increases striatal glutamate outflow in dopamine-denervated rats. *Eur. J. Pharmacol.* **464**, 33–38.

Cunha R. A. (1998) On slices, synaptosomes and dissociated neurones to study *in vitro* ageing physiology. *Trends Neurosci.* **21**, 286.

Diáz-Hernández M., Pereira M. F., Pintor J., Cunha R. A., Ribeiro J. A. and Miras-Portugal M. T. (2002) Modulation of the rat hippocampal dinucleotide receptor by adenosine receptor activation. *J. Pharmacol. Exp. Ther.* **301**, 441–450.

Ferré S., Karcz-Kubicha M., Hope B. T. *et al.* (2002) Synergistic interaction between adenosine A_{2A} and glutamate $mGlu_5$ receptors: implications for striatal neuronal function. *Proc. Natl Acad. Sci. USA* **99**, 11 940–11 945.

Ferré S., Ciruela F., Woods A. S. *et al.* (2003) Glutamate $mGlu_5$-adenosine A_{2A}–dopamine D_2 receptor interactions in the striatum. Implications for drug therapy in neuro-psychiatric disorders and drug abuse. *Curr. Med. Chem.* **3**, 1–26.

Fredholm B. B., Cunha R. A. and Svenningsson P. (2003) Pharmacology of adenosine A_{2A} receptors and therapeutic applications. *Curr. Top. Med. Chem.* **3**, 413–426.

Hettinger B. D., Lee A., Linden J. and Rosin D. L. (2001) Ultrastructural localization of adenosine A_{2A} receptors suggests multiple cellular sites for modulation of GABAergic neurons in rat striatum. *J. Comp. Neurol.* **431**, 331–346.

Itzev D., Lolova I., Lolov S. and Usunoff K. G. (2001) Age-related changes in the synapses of the rat's neaostriatum. *Arch. Physiol. Biochem.* **109**, 80–89.

Kenakin T. (2002) Drug efficacy at G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* **42**, 349–379.

Khakh B. S., Gittermann D., Cockayne D. A. and Jones A. (2003) ATP modulation of excitatory synapses onto interneurons. *J. Neurosci.* **23**, 7426–7437.

Lipton S. A. and Rosenberg P. A. (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *N. Engl. J. Med.* **330**, 613–622.

Lopes L. V., Cunha R. A., Kull B., Fredholm B. B. and Ribeiro J. A. (2002) Adenosine A_{2A} receptor facilitation of hippocampal synaptic transmission is dependent on tonic A_1 receptor inhibition. *Neuroscience* **112**, 319–329.

Nishi A., Liu F., Matsuyama S., Hamada M., Higashi H., Nairn A. C. and Greengard P. (2003) Metabotropic mGlu5 receptors regulate adenosine A_{2A} receptor signalling. *Proc. Natl Acad. Sci. USA* **100**, 1322–1327.

Obeso J. A., Rodríguez-Oroz M. C., Rodríguez M., Lanciego J. L., Artieda J., Gonzalo N. and Olanow C. W. (2000) Pathophysiology of the basal ganglia in Parkinson's disease. *Trends Neurosci.* **23**, S8–S19.

Ossowska K., Konieczny J., Wolfarth S., Wieronska J. and Pilc A. (2001) Blockade of the metabotropic glutamate receptor subtype 5 (mGluR5) produces antiparkinsonian-like effects in rats. *Neuropharmacology* **41**, 413–420.

Paquet M. and Smith Y. (2003) Group I metabotropic glutamate receptors in the monkey striatum: subsynaptic association with glutamatergic and dopaminergic afferents. *J. Neurosci.* **23**, 7659–7669.

Phillips G. R., Huang J. K., Wang Y., Tanaka H., Shapiro L., Zhang M., Gordon R. E., Gawinowicz M. A., Zhao Y. and Colman D. R. (2001) The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. *Neuron* **32**, 1–20.

Pintor A., Pèzzola A., Regio R., Quarta D. and Popoli P. (2000) The mGlu5 receptor agonist CHPG stimulates striatal glutamate release: posible involvement of A_{2A} receptors. *Neuroreport* **11**, 3611–3614.

Raiteri L. and Raiteri M. (2000) Synaptosomes still viable after 25 years of superfusion. *Neurochem. Res.* **25**, 1265–1274.

Rebola N., Sebastião A. M., de Mendonça A., Oliveira C. R., Ribeiro J. A. and Cunha R. A. (2003a) Enhanced adenosine A_{2A} receptor facilitation of synaptic transmission in the hippocampus of aged rats. *J. Neurophysiol.* **90**, 1295–1303.

Rebola N., Pinheiro P. C., Oliveira C. R., Malva J. O. and Cunha R. A. (2003b) Subcellular localization of adenosine A_1 receptors in nerve terminals and synapses of the rat hippocampus. *Brain Res.* **987**, 49–58.

Reid M. E., Toms N. J., Bedingfield J. S. and Roberts P. J. (1999) Group I mGlu receptors potentiate synaptosomal [^{3}H]glutamate release independently of exogenously applied arachidonic acid. *Neuropharmacology* **38**, 477–485.

Rusakov D. A., Harrison E. and Stewart M. G. (1998) Synapses in the hippocampus occupy only 1–2% of cell membranes and are spaced less than half-micron apart: a quantitative ultrastructural analysis with discussion of physiological implications. *Neuropharmacology* **37**, 513–521.

Schwarzschild M. A., Chen J. F. and Ascherio A. (2002) Caffeinated clues and the promise of adenosine A_{2A} antagonists in PD. *Neurology* **58**, 1154–1160.

Sistisaga A., Herrero I., Conquet F. and Sanchez-Prieto J. (1998) The metabotropic glutamate receptor 1 is not involved in the facilitation of glutamate release in cerebrocortical nerve terminals. *Neuropharmacology* **37**, 1485–1492.

Svenningsson P., Le Moine C., Fisone G. and Fredholm B. B. (1999) Distribution, biochemistry and function of striatal adenosine A_{2A} receptors. *Prog. Neurobiol.* **59**, 355–396.