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Autoradiographic comparison of the potency of several structurally unrelated adenosine receptor antagonists at adenosine A_1 and A_{2A} receptors

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Abstract

We have examined the potency of several adenosine receptor antagonists at adenosine A_1 and A_{2A} receptors using quantitative autoradiography and have compared the results with those of previous studies using the same radioligands in membrane preparations. The agonists [^{3}H]cyclohexyladenosine and [^{3}H]2-[*p*-(2-carbonylethyl)-phenylethylamino]-5′-*N*-ethylcarboxamido adenosine ([^{3}H]CGS 21680) were used as radioligands for the two receptors. The results show that 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX) is almost 1000-fold and 8-chloro-4-cyclohexyl-amino-1-(trifluoromethyl)[1,2,4]triazolo[4,3-*a*] quinoxaline (CP-68,247) about 300-fold more potent at adenosine A_1 receptors in cortex and striatum than at striatal adenosine A_{2A} receptors. Conversely, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo [1,5-*c*]pyrimidine (SCH 58261) is approximately 1000-fold and 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-yl amino]ethyl)phenol (ZM 241,385) about 400-fold more potent at adenosine A_{2A} than at A_1 receptors. Caffeine and its metabolites did not show any selectivity. Other studied antagonists were non-selective or showed a modest (20- to 40-fold) adenosine A_{2A} receptor selectivity. Thus, only a few of the antagonists show such high selectivity that it is not offset by differences in drug distribution and levels of receptor subtype expression.

Keywords: Cortex; Striatum; Autoradiography; Xanthine; Adenosine receptor

1. Introduction

Adenosine is an important neuromodulator in the brain, and there is evidence that it acts in particular at adenosine A_1 and A_{2A} receptors (Fredholm, 1995). The elucidation of the relative roles of these receptors has largely depended on the use of antagonists with higher or lower degrees of selectivity. The degree of selectivity has mostly been determined by binding assays using membranes prepared from brain. The potency at adenosine A_1 receptors is most often determined by displacement of the antagonist radioligand [^{3}H]1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), whereas the potency at adenosine A_{2A} receptors has most often been determined using the agonist [^{3}H]2-[*p*-(2-carbonylethyl)-phenylethylamino]-5′-*N*-ethylcarboxamido adenosine ([^{3}H]CGS 21680) (Jarvis et al., 1989). However, in such membrane preparations, the apparent affinity of [^{3}H]CGS 21680 is much lower than that found when using receptor autoradiography (Parkinson and Fredholm, 1990). Furthermore, in membrane preparations, the binding is affected only little by GTP, whereas it is strongly affected by GTP in autoradiographic experiments (Parkinson and Fredholm, 1990, 1992b). One possible explanation for this difference is that the ability of the receptor to associate with the relevant G protein is much better when the cell membranes are fairly intact, as they are in autoradiographic experiments, than when they are disrupted by homogenization, as in membrane preparations.

Based on these considerations, we have re-examined the selectivity of a number of commonly used adenosine receptor antagonists using quantitative receptor autoradiography. To this end, we have examined their ability to displace an adenosine A_1 receptor agonist [^{3}H]N^6-cyclohexyl adenosine (CHA) and the adenosine A_{2A} receptor agonist [^{3}H]CGS 21680 from their binding sites in rat brain.

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2. Materials and methods

2.1. Chemicals

[^{3}H]CGS 21680 (45 Ci/mmol) and [^{3}H]N^6-cyclohexyl adenosine (CHA; 27 Ci/mmol) were from DuPont (New England Nuclear, Bad Homburg, Germany). 5′-(*N*-Ethyl)-carboxamido adenosine (NECA), 2-chloroadenosine and adenosine deaminase were purchased from Sigma, St. Louis, MO, USA. (*R*)-N^6-Phenylisopropyl adenosine (R-PIA), 5-amino-9-chloro-2-(2-furanyl)-1,2,4-triazolo [1,5-*c*] quinazolinemonomethane sulfonate (CGS 15943), 8-(3-chlorostyryl) caffeine (CSC) and unlabelled CGS 21680 were from Research Biochemicals International. CGS 15493 and CSC were dissolved in dimethyl sulfoxide (DMSO; 10 mM stock solution). 4-(2-[7-Amino-2-(2-furyl) [1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-yl amino]ethyl)phenol (ZM 241,385) was a kind gift from Dr. Simon M. Poucher of Zeneca Pharmaceuticals, Alderley Park, England and was dissolved at 1 mM in 50% DMSO. 1-Ethyl-4-methyl-amino-[1,2,4]triazolo[4,3-*a*]quinoxaline (CP-57,103), 4-amino-8-chloro-1-phenyl-[1,2,4]triazolo[4,3-*a*]quinoxaline (CP-66,713) and 8-chloro-4-cyclohexyl-amino-1-(trifluoro-methyl)[1,2,4]triazolo[4,3-*a*]quinoxaline (CP-68,247) were kind gifts from Drs. Reinhard Sarges and John Stam of Pfizer Central Research, Groton, CT, USA. These drugs were all dissolved in ethanol. 1,3-Dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KF 17837) was a kind gift from Dr. Fumio Suzuki of Kyowa Hakko Kogyo, Shinozuka, Japan. It was dissolved in DMSO.

2.2. Receptor autoradiography

The studies were approved by the local ethical committee on animal experimentation. From brains of male Sprague–Dawley rats (weighing approximately 200 g), 10 μm coronal sections were cut with a cryostat at approximately bregma +1 to +1.2 according to the Paxinos and Watson atlas (Paxinos and Watson, 1986), thaw-mounted on gelatin-coated slides and used for quantitative receptor autoradiography. The mounted sections were stored at −20°C. The sections were pre-incubated in 170 mM Tris–HCl buffer containing 1 mM EDTA and 2 U/ml adenosine deaminase at 37°C for 30 min. Sections were then washed twice for 10 min at room temperature in 170 mM Tris–HCl buffer with 10 mM $MgCl_2$.

2.2.1. [^{3}H]CGS 21680

Incubations were performed using the drop method (0.9 ml solution per slide) for 2 h at room temperature in Tris–HCl buffer containing 0.9 or 2 nM [^{3}H]CGS 21680, 10 mM $MgCl_2$, 2 U/ml adenosine deaminase and appropriate concentrations of competing ligands. Non-specific binding was defined by 20 μM 2-chloroadenosine. Sections were then washed twice for 5 min each in ice-cold Tris–HCl, dipped quickly three times in ice-cold distilled water and dried at 4°C overnight in a stream of air. The binding of [^{3}H]CGS 21680 to rat brain sections has been characterized and validated in previous studies (Parkinson and Fredholm, 1990, 1992b). When used at 2 nM, the binding of [^{3}H]CGS 21680 averaged 133 fmol/mg (ranging from 78 fmol/mg [experiments with caffeine] to 153 fmol/mg [experiments with CP-66,713]). When used at 0.9 nM binding averaged 52 fmol/mg.

2.2.2. [^{3}H]Cyclohexyl adenosine

Sections were incubated in Tris–HCl buffer with 2 U/ml adenosine deaminase (0.9 ml drop on the slide) for 2 h at room temperature with 1 nM [^{3}H]CHA and appropriate concentrations of competing ligands. To define non-specific binding, slides were incubated with 20 μM R-PIA. After incubation, the sections were washed twice in ice-cold Tris buffer for 5 min and dipped three times in water. Since some of the drugs tested had to be dissolved in DMSO or ethanol, the influence of these solvents was also tested. The binding of [^{3}H]CHA to rat brain sections has been characterized and validated in previous studies (Fastbom et al., 1987; Fastbom and Fredholm, 1990). The binding of [^{3}H]CHA averaged 211 fmol/mg in cortex (ranging from 174 fmol/mg [experiments with CSC] to 253 fmol/mg [experiments with KF 17387] and 126 fmol/mg in striatum (ranging from 94 fmol/mg [experiments with CSC] to 176 fmol/mg [experiments with KF 17387]).

2.2.3. Analysis

The dried sections, together with plastic tritium standards (Amersham) were apposed to Hyperfilm-^{3}H (Amersham) for 1.5 to 5 weeks. The autoradiograms were analyzed with an M4 Imaging Device (Imaging Research, St. Catharines, Canada). Optical densities were converted to binding density (fmol/mg gray matter) using the plastic standards and the specific activity of the radioligands. Results were analyzed according to procedures in GraphPad Prism (2.01). This program package was also used to analyze dose–response curves. Statistical hypotheses were tested using analysis of variance procedures.

3. Results

Several of the antagonists used in this study are very poorly water soluble and had to be dissolved in ethanol or DMSO. In one control study, we therefore examined the potency of the water soluble antagonist theophylline in the presence of increasing concentrations of ethanol or DMSO. The solvents had no major effects. For example, the IC_{50} for displacement of CGS 21680 in this experiment was 16.7 (9.7–28.9) μM in the controls (mean and 95% confidence interval); 12.8 (6.3–26.1) μM in the presence of

2.5% ethanol; 9.6 (6.9–13.1) μM in the presence of 1% DMSO and 36.1 (13.1–98.7) μM in the presence of 2.5% DMSO. In a similar experiment where theophylline was used to displace [^{3}H]CHA, the corresponding values were in controls 13.6 (2.7–68) μM; with 1% ethanol 16.6 (5.8–23.7) μM; with 2.5% ethanol 28 (5.0–165) μM; with 1% DMSO 27.7 (17.1–45.0) μM and with 2.5% DMSO 23.6 (10.8–51.6) μM. There were, however, changes in the maximal binding. DMSO decreased [^{3}H]CGS 21680 binding by 0.4% at a concentration of 0.5%; by 2.1% at 1%; by 4.7% at 2% and by 7.6% at a DMSO concentration of 3%. At the highest concentration of ethanol used (1%) the decrease was 2.1%. [^{3}H]CHA binding decreased 1.4% at a DMSO concentration of 0.5%; not at all at 1%; by 7% at 2%; by 10.9% at 2.5%; by 8.4% at 3%; by 9% at 3.5% and by 19.3% at a DMSO concentration of 5%. At 2% ethanol (the highest concentration used), a 4.8% decrease in [^{3}H]CHA binding was seen. In all experiments, corrections were made for these effects of the solvent.

The results obtained with six of the studied adenosine receptor antagonists are shown in Fig. 1. The results for all the compounds investigated are summarized in Table 1. In the table, we have also included results from experiments we have carried out using the identical technique and reported elsewhere (Parkinson and Fredholm, 1990; Fredholm et al., 1998; Svenningsson et al., 1999). From an examination of the data, it is apparent that the ability of these drugs to displace [^{3}H]CHA binding from cortex and striatum is virtually identical, despite the fact that binding in cortex was about twice that seen in striatum. Hence, the mean value was used to calculate adenosine A_1/A_{2A} receptor selectivity.

It can be seen in Table 1 that caffeine and its metabolites theophylline, paraxanthine and theobromine, the non-xanthine CGS 15943, as well as the agonist NECA, are virtually unselective for adenosine A_1 or A_{2A} receptors using the autoradiographic assay. It is also clear that DPCPX is about 750-fold selective for the adenosine A_1 over the A_{2A} receptor and CP-68,247 about 300-fold selective for the A_1 receptor. The converse high selectivity is exhibited by ZM 241,385 and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo [1,5-*c*]pyrimidine (SCH 58261; 400- to 1000-fold). As reported earlier CP-66,713 was about 30-fold selective for adenosine A_{2A} receptors. By contrast, two compounds reported to be several hundred-fold selective for adenosine

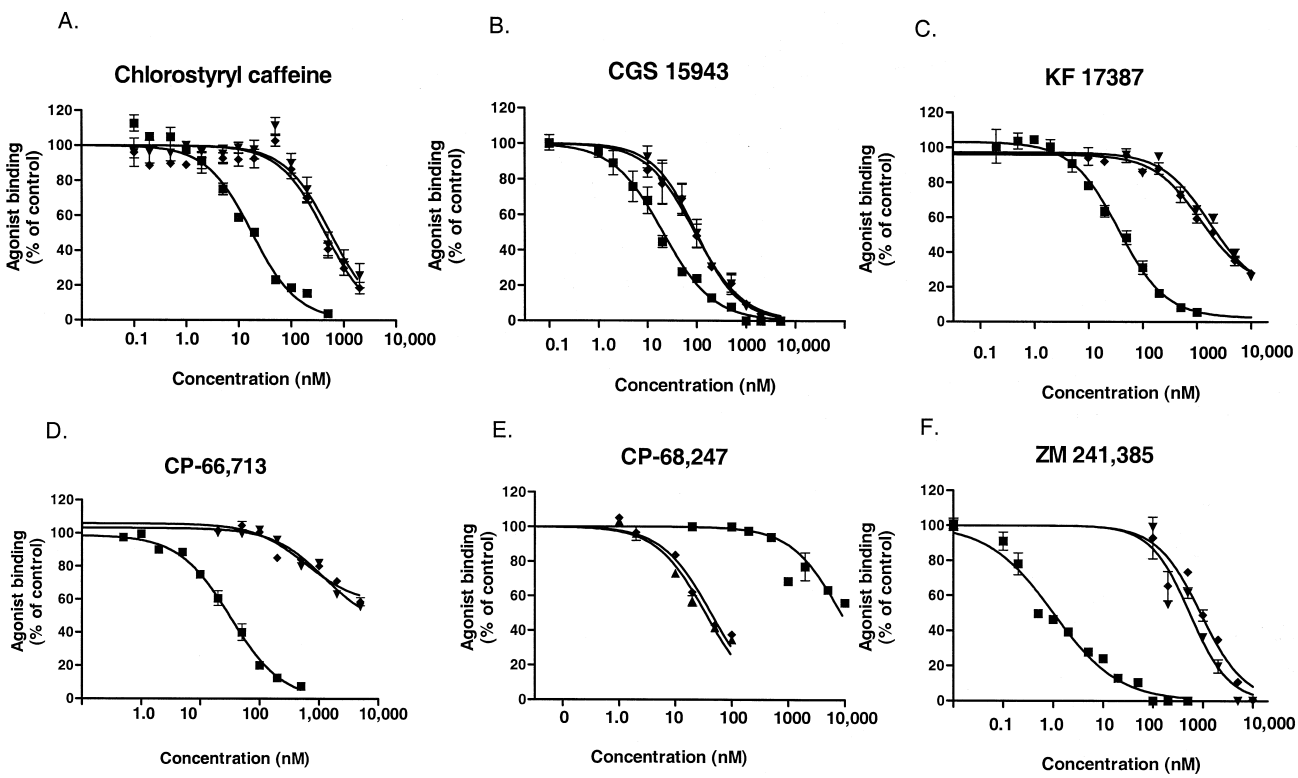

Fig. 1. Displacement of the adenosine A_1 receptor agonist [^{3}H]CHA from cerebral cortex (♦) and striatum (▼) and of the adenosine A_{2A} agonist [^{3}H]CGS 21680 from striatum (■). (Panel A) Displacement by chlorostyrylcaffeine. [^{3}H]CHA, duplicate experiments; [^{3}H]CGS 21680, quadruplicate experiments. (Panel B) Displacement by CGS 15943. [^{3}H]CHA and [^{3}H]CGS 21680, six experiments. (Panel C) Displacement by KF 17387. [^{3}H]CHA and [^{3}H]CGS 21680, duplicate experiments. (Panel D) Displacement by CP-66,713. [^{3}H]CHA and [^{3}H]CGS 21680, duplicate experiments. (Panel E) Displacement by CP-68,247. [^{3}H]CHA and [^{3}H]CGS 21680, duplicate experiments. (Panel F) Displacement by ZM 241,385. [^{3}H]CHA and [^{3}H]CGS 21680, duplicate experiments. The concentration of [^{3}H]CHA was 1 nM in all experiments. The concentration of [^{3}H]CGS 21680 was 2 nM in the experiments presented in panels A, B, C, D, and E and 0.9 nM in the experiments presented in panel F.

Table 1
Potency of some adenosine receptor antagonists to displace CGS 21680 from adenosine A_{2A} receptors in striatum or CHA from adenosine A_1 receptors in striatum or cortex. Results are given as K_i values[a] in nM (mean and 95% confidence interval). In addition, the A_1/A_{2A} selectivity was calculated using the mean of the A_1 data in cortex and striatum. Displacement by CP-57,103, caffeine, theophylline, paraxanthine and theobromine (was performed in duplicate). Further experimental details are given in the legend to Fig. 1.

Drug	A_{2A} striatum	A_1 striatum	A_1 cortex	Ratio A_1/A^a_{2A}
CSC	61 (47–79)	1493 (722–3084)	3143 (1649–5914)	38
KF 17387	17 (13–22)	438 (268–716)	643 (473–874)	31.8
CP-66,713	16 (13–20)	244 (146–407)	389 (189–800)	19.8
CP-68,247	41,561 (29,858–57,854)	87 (70–108)	204 (170–246)	0.0035
CP-57,103	3878 (1878–8010)	283 (155–518)	462 (343–618)	0.096
Caffeine[b]	8556 (6468–11,312)	20,490 (11,809–34,837)	16,643 (9871–27,962)	2.17
Paraxanthine[b]	7575 (4135–13,161)	5024 (4338–5835)	9823 (3834–24,750)	0.98
Theophylline[b]	5048 (2376–10,717)	4658 (3258–6929)	9830 (2408–40,139)	1.4
Theobromine[b]	109,048 (19,483–577,561)	96,505 (66,964–158,474)	197,000 (167,600–234,300)	1.35
CGS 15943	0.9 (0.5–1.5)	2.5 (1.9–3.2)	3.8 (2.9–4.8)	3.59
SCH 58261[c]	1.2 (0.6–2.4)	740 (148–3696)	1608 (620–4236)	1021
ZM 241,385	0.67 (0.51–0.95)	138 (100–193)	374 (269–510)	382
DPCPX	682 (243–1949)[d]	0.6 (0.3–1.0)	1.24 (1.12–1.34)	0.00135
NECA	1.9 (0.9–5.6)[d]	3.2 (2.6–4.0)	4.9 (3.4–6.3)	2.12

[a] Based on the previous determinations of K_D values for CHA [0.34 (0.15–0.80) in striatum and 0.68 (0.62–0.75) nM in cortex (Parkinson and Fredholm, 1992a; Johansson et al., 1993a)] and for CGS 21680 [1.7 (1.3–3.1) and 2.1 (1.8–2.35) nM (Parkinson and Fredholm, 1990; Johansson et al., 1993a)]. K_i values were calculated from the IC_{50} values according to the Cheng and Prussoff equation. The ratio between these estimates was used to calculate receptor selectivity.
[b] Some of these data have been presented elsewhere (Svenningsson et al., 1999).
[c] Some of these data have been presented previously (Fredholm et al., 1998).
[d] Data from Parkinson and Fredholm (1990).

A_{2A} vs. A_1 receptors, CSC and KF 17387, in our assay showed only a 20- to 40-fold selectivity.

4. Discussion

The present results provide a comparison of several commonly used adenosine receptor antagonists and show that only a few of the compounds tested are highly selective for one receptor subtype. Despite the fact that these compounds have been widely used, no comparative study of all these compounds appears to have been published. The results obtained with the present method are in good agreement with some, but not all published data.

The results with the CP-compounds are in good agreement with the results reported previously (Sarges et al., 1990) despite the fact that the adenosine A_{2A} receptor potency was examined using a non-selective agonist radioligand in the earlier study. The results we present here for CGS 15943, DPCPX, SCH 58261, ZM 241,385 and the classical xanthines also agree with most published reports (Williams et al., 1987; Baraldi et al., 1994; Nonaka et al., 1994; Poucher et al., 1995; Ongini et al., 1999). There are, however, some discrepancies between the present results and reports on the selectivity for some compounds.

In our experiments, we found that KF 17387 was only some 30-fold selective for adenosine A_{2A} over A_1 receptors. This contrasts with data obtained using a membrane binding assay, where a potency difference of 190-fold was found (Shimada et al., 1992). That publication, by contrast, reported a much lower selectivity for DPCPX at adenosine A_1 receptors (90-fold) than that observed here (740-fold). In fact, the ratio between KF 17387 and DPCPX agreed very well between the two studies (17,100- vs. 22,200-fold). A later study from the same laboratory (Nonaka et al., 1994) reported a selectivity (62-fold) more similar to the one we observed here and in that study, the selectivity for CGS 15943 and NECA also agrees closely with the one we report. Another difference between the present data and those published previously concerns CSC, which was reported to be 520-fold selective for adenosine A_{2A} receptors in studies using a membrane binding assay (Jacobson et al., 1993), whereas we found the selectivity to be only 38-fold. Interestingly, that study also found that caffeine was 41-fold adenosine A_{2A} receptor selective, whereas we found it to be only 2.2-fold selective. Thus, there is agreement between the two studies that CSC is some 10–20 fold more adenosine A_{2A} receptor selective than caffeine. From this, we conclude that even in the instances where there is an apparent discrepancy between the present data and those published earlier, the difference is not with regard to the relative selectivity of drugs examined, but with regard to the numerical value of the adenosine A_1/A_{2A} receptor selectivity.

For most of the compounds, the calculated pseudo-Hill slope was close to 1. However, the displacement curve for the two non-xanthines CGS 15943 and ZM 241385 in striatum were rather shallow (pseudo-Hill slope 0.51–1.09

and 0.55–0.89, respectively). This might indicate that [^{3}H]CGS 21680 binds to two types of binding sites in the striatum at which CGS 15943 and ZM 241385 has widely different affinities. Although there is evidence for multiple binding sites for CGS 21680, the contribution of the low affinity, non-A_{2A} site, to overall binding at the concentration of the radioligand used is small (Johansson et al., 1993b; Cunha et al., 1996).

It is important in this context to point out that the selectivities for DPCPX, caffeine, SCH 58261, CGS 15943 and ZM 241385 that we report here agree well with that found when binding studies are performed using cloned receptors in a defined cellular background even if other radioligands are used (see Klotz et al., 1998; Ongini et al., 1999). The apparent discrepancies that were noted above *may* therefore be due to problems with the use of agonist radioligands in binding assays on membrane preparations. The affinity of [^{3}H]CGS 21680 binding to striatal membranes is markedly lower than to intact striatal sections, and this in turn might relate to the fact that in striatal sections the binding is almost entirely to a high affinity state (Parkinson and Fredholm, 1990), whereas binding to striatal membranes is largely insensitive to GTP (Jarvis et al., 1989). It is known that this high affinity binding is dependent on the presence of magnesium ions (Jarvis et al., 1989; Parkinson and Fredholm, 1990, 1992a,b; Johansson et al., 1992). However, there is no major difference in this respect between autoradiographic studies and studies using brain homogenates, and most studies have used adequate levels of magnesium in the binding assays. As mentioned in Section 1, adenosine A_{2A} receptors may be better coupled to the relevant G protein when studies are performed on relatively intact membranes as in autoradiographic experiments than when the studies are performed on membranes prepared by homogenization. It is generally believed that unlike agonists, most antagonists do not show any preference for either the G protein-coupled or uncoupled state of the receptor. Thus, the ability of an antagonist to bind — and hence, to compete with the agonist — may be similar in the two types of preparation. The markedly lower potency of the agonist, however, means that the K_i value that is calculated could differ markedly. This factor, we think, could explain much of the discrepancy between the data noted above. The very considerable importance of the estimate of the K_D value can also be deduced from the data of Table 1. Here, it is seen that the somewhat different estimates of the K_D for the adenosine A_1 receptor agonist in striatum and cortex translate into sometimes non-overlapping estimates for antagonist K_i values in these two regions, despite the very close similarity in the IC_{50} values (see Fig. 1). Another factor of unknown importance for naturally occurring receptors is that some antagonists may show preferential binding to a G protein uncoupled state and thus, show inverse antagonism. Recent data on adenosine A_1 receptors over-expressed in CHO cells indicate that some of the antagonists, including DPCPX and CGS 15943, may exhibit this type of behavior (Shryock et al., 1998).

Thus, many of the compounds that have been used to define the receptor subtype responsible for a given adenosine receptor action are much less than 100-fold selective. This can lead to considerable difficulties in interpretation since the antagonists are competitive and since the potency of the agonist differs very much with the density of receptors (see e.g., Kenakin, 1997). For example, we have shown that a doubling of adenosine A_1 receptor number is translated into an almost three-fold higher potency of agonists in DDT_1 MF-2 cells (Gerwins and Fredholm, 1991), and that a 20-fold decrease in adenosine A_{2A} receptor number leads to a 15-fold decrease in potency of an agonist (Arslan et al., 1999). There could easily be similar differences in the number of adenosine A_1 and A_{2A} receptors in brain regions. For example, A_{2A} receptors in striatum are more abundant than A_1 receptors (Fastbom and Fredholm, 1990; Parkinson and Fredholm, 1992a; Fredholm et al., 1998; Svenningsson et al., 1999). Moreover, the adenosine A_{2A} receptors are almost exclusively found on striatopallidal medium-sized spiny neurons (Svenningsson et al., 1997), which comprise less than half of the neuron population, whereas A_1 receptors are present not only on all the neurons (in particular on cholinergic neurons) (Ferré et al., 1996), but also, and in largest number, on glutamatergic and dopaminergic input neurons as well as on glial cells. Thus, the density of the receptors at a given cell membrane could easily differ by orders of magnitude. The possible consequence of this is that it may be easier — with a poorly A_{2A}-selective and competitive antagonist — to block the responses due to activation of a small number of adenosine A_1 receptors, than the responses due to activation of abundant adenosine A_{2A} receptors.

In conclusion, we have compared the effect of several adenosine receptor antagonists used in studies of the role(s) of adenosine A_1 and A_{2A} receptors in neuronal function using an autoradiographic binding assay. With this assay, caffeine and its metabolites have been confirmed to be non-selective receptor antagonists, and DPCPX and SCH 58261 highly selective adenosine A_1 and A_{2A} receptor antagonists, respectively. These studies confirmed the marked adenosine A_{2A} receptor selectivity of ZM 241,385 and the limited A_{2A} receptor selectivity of CP-66,713. On the other hand, CSC and KF 17387 were found to be almost one order of magnitude less selective for adenosine A_{2A} receptors than commonly stated. Interpretation of results obtained with more poorly selective antagonists may be complicated by differences in receptor number and also by an uneven distribution of the antagonist between different biologically relevant compartments. Since the interpretation of results with antagonists is much easier when selectivity is high, it is therefore recommended that only the most selective compounds are used in future studies.

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