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Evidence for high-affinity binding sites for the adenosine A_{2A} receptor agonist [^{3}H] CGS 21680 in the rat hippocampus and cerebral cortex that are different from striatal A_{2A} receptors

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Abstract The binding of the adenosine A_{2A} receptor selective agonist 2-[4-(2-*p*-carboxyethyl) phenylamino] -5′-*N*-ethylcarboxamidoadenosine (CGS 21680) to the rat hippocampal and cerebral cortical membranes was studied and compared with that to striatal membranes. [^{3}H] CGS 21680, in the concentration range tested (0.2–200 nM), bound to a single site with a K_d of 58 nM and a B_{max} of 353 fmol/mg protein in the hippocampus, and with a K_d of 58 nM and a B_{max} of 264 fmol/mg protein in the cortex; in the striatum, the single high-affinity [^{3}H] CGS 21680 binding site had a K_d of 17 nM and a B_{max} of 419 fmol/mg protein. Both guanylylimidodiphosphate (100 μM) and Na^+ (100 mM) reduced the affinity of [^{3}H] CGS 21680 binding in the striatum by half and virtually abolished [^{3}H] CGS 21680 binding in the hippocampus and cortex. The displacement curves of [^{3}H] CGS 21680 binding with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), N^6-cyclohexyladenosine (CHA), 5′-*N*-ethylcarboxamidoadenosine (NECA) and 2-chloroadenosine (CADO) were biphasic in the hippocampus and cortex as well as in the striatum. The predominant [^{3}H]CGS 21680 binding site in the striatum (80%) had a pharmacological profile compatible with A_{2A} receptors and was also present in the hippocampus and cortex, representing 10–25% of [^{3}H]CGS 21680 binding. The predominant [^{3}H]CGS 21680 binding site in the hippocampus and cortex had a pharmacological profile distinct from A_{2A} receptors: the relative potency order of adenosine antagonists DPCPX, 1,3-dipropyl-8-{4-[(2-aminoethyl)amino]carbonylmethyloxyphenyl} xanthine (XAC), 8-(3-chlorostyryl) caffeine (CSC), and (*E*)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-methylxanthine (KF 17,837) as displacers of [^{3}H] CGS 21680 (5 nM) binding in the hippocampus and cerebral cortex was DPCPX > XAC ≫ CSC ≈ KF 17,837, and the relative potency order of adenosine agonists CHA, NECA, CADO, 2-[(2-aminoethylamino)carbonylethylphenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (APEC), and 2-phenylaminoadenosine (CV 1808) was CHA ≈ NECA ≥ CADO > APEC ≈ CV1808 > CGS 21680. In the presence of DPCPX (20 nM), [^{3}H] CGS 21680 (0.2–200 nM) bound to a site (A_{2A}-like) with a K_d of 20 nM and a B_{max} of 56 fmol/mg protein in the hippocampus and with a K_d of 22 nM and a B_{max} of 63 fmol/mg protein in the cortex. In the presence of CSC (200 nM), [^{3}H]CGS 21680 (0.2–200 nM) bound to a second high-affinity site with a K_d of 97 nM and a B_{max} of 255 fmol/mg protein in the hippocampus and with a K_d of 112 nM and a B_{max} of 221 fmol/mg protein in the cortex. Two pharmacologically distinct [^{3}H]CGS 21680 binding sites were found in synaptosomal membranes of the hippocampus and cortex and in the striatum, one corresponding to A_{2A} receptors and the other to the second high-affinity [^{3}H]CGS 21680 binding site. In contrast, the pharmacology of [^{3}H]CHA binding was similar in synaptosomal membranes of the three brain areas. The present results establish the existence of at least two high-affinity [^{3}H]CGS 21680 binding sites in the CNS and demonstrate that the [^{3}H]CGS 21680 binding site predominant in the hippocampus and cerebral cortex has different binding characteristics from the classic A_{2A} adenosine receptor, which predominates in the striatum.

Key words Adenosine receptors · Hippocampus · Cerebral cortex · Striatum · CGS 21680 · Radioligand assay

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Introduction

Adenosine exerts neuromodulatory effects by interacting with membrane receptors. Several subtypes of adenosine receptors have been recognised by using structural and pharmacological criteria [see Fredholm et al. (1994)]. The adenosine analogue. 2-[4-(2-*p*-carboxyethyl) phenylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) binds with nanomolar affinity to A_{2A} adenosine receptors (Jarvis et al. 1989) and with much lower affinity (micromolar) to other subtypes of adenosine receptors (Jarvis et al. 1989; Stehle et al. 1992; Zhou et al. 1992). CGS 21680 has therefore been used as a radioligand in studies of A_{2A} receptors in the CNS. The first descriptions of the localisation of the A_{2A} receptors using receptor autoradiography and membrane binding studies with [^{3}H] CGS 21680 suggested that the A_{2A} receptors were exclusively located in the striatum (Jarvis and Williams 1989; Jarvis et al. 1989). However, functional studies have shown that, in the hippocampus, low nanomolar concentrations of CGS 21680 increase neuronal excitability (Sebastião and Ribeiro 1992), synaptic transmission (Cunha et al. 1994b) and long-term potentiation (de Mendonça and Ribeiro 1994), as well as increase the evoked release of acetylcholine (Cunha et al. 1994a). The facilitation of acetylcholine release by CGS 21680 in the hippocampus is antagonised by A_{2A}, but not by A_1 antagonists, suggesting that it is mediated by an A_{2A} receptor subtype (Cunha et al. 1995). Further evidence for the existence of A_{2A} receptors in the hippocampus and cerebral cortex has been obtained using morphological and molecular biology approaches (Cunha et al. 1994b). [^{3}H]CGS 21680 binding sites have been detected also in cortical brain membranes (Wan et al. 1990; James et al. 1992). However, the pharmacological profile of [^{3}H] CGS 21680 binding in cortical areas, as assessed by receptor autoradiography, is distinct from the striatal A_{2A} receptor and the classical A_1 receptor (Johansson et al. 1993; Johansson and Fredholm 1995).

The present work was undertaken to characterise further the second, high-affinity binding site of [^{3}H] CGS 21680 in the hippocampus and cerebral cortex of the rat, as well as in the striatum. To evaluate whether the [^{3}H] CGS 21680 binding sites were present in nerve terminals, the binding of [^{3}H]CGS 21680 to whole membranes was compared with that to membranes prepared from synaptosomes of the rat hippocampus, cerebral cortex and striatum. Finally, we compared the pharmacology of [^{3}H] N^6-cyclohexyladenosine (CHA) and [^{3}H]CGS 21680 binding to hippocampal, cortical and striatal membranes to investigate whether regional differences also occur with an A_1 agonist. A brief account of some of the results has been published elsewhere (Cunha et al. 1994c).

Methods

Drugs. [^{3}H] CGS 21680 (specific activity 38.30–42.16 Ci/mmol) and [^{3}H] CHA (specific activity 27.7 Ci/mmol) were obtained from DuPont NEN. Adenosine deaminase (ADA) and guanylylimidodiphosphate [Gpp(NH)p] were from Boehringer Mannheim. The substances 5′-*N*-ethylcarboxamidoadenosine (NECA), CHA, 2-chloroadenosine (CADO) and 2-phenylaminoadenosine (CV 1808) were from Sigma; 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 1,3-dipropyl-8-{4-[(2-aminoethyl)amino]carbonylmethyloxyphenyl} xanthine (XAC) and CGS 21680 were purchased from Research Biochemicals. The substances 2-[(2-aminoethylamino) carbonylethylphenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (APEC) and 8-(3-chlorostyryl) caffeine (CSC) were generous gifts of K.A. Jacobson (NIH, Bethesda) and (*E*)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KF 17,837) was kindly supplied by F. Suzuki (Kyowa Hakko Kogyo, Japan).

Preparation of whole membranes from the striatum, hippocampus and cerebral cortex. Male Sprague-Dawley rats (180–200 g) were killed by decapitation after CO_2 anaesthesia. The brains were removed and the two striata, the two hippocampi and either the left or the right frontal cerebral cortex were dissected out at 4° C. After weighing, each area was homogenised by sonication in 10 volumes of sucrose solution (0.32 M) containing 50 mM TRIS, 2 mM EGTA and 1 mM dithiotreitol, pH 7.6. The resulting homogenates were centrifuged at 1,000 × *g* for 10 min at 4° C. The supernatants were transferred to new tubes and centrifuged at 13, 000 × *g* for 20 min at 4° C. The pellets were then resuspended in a solution containing 50 mM TRIS (pH 7.4 at 23° C), 2 mM EGTA, 1 mM EDTA and, except where otherwise stated, 5 U/ml ADA and incubated for 30 min at 37° C to remove endogenous adenosine. The mixture was centrifuged at 30, 000 × *g* for 10 min at 4° C, and the pellets resuspended in the incubation solution containing 50 mM TRIS, 10 mM $MgCl_2$, pH 7.4 and, except where otherwise stated, 5 U/ml ADA.

Binding assays. [^{3}H] CGS 21680-binding studies were performed by incubation of [^{3}H]CGS 21680 for 4 h at room temperature (23–25° C) with 90–320 µg of membrane protein in a final volume of 300 µl, in a solution containing 50 mM TRIS and 10 mM $MgCl_2$ pH 7.4 and 5 U/ml ADA, except where otherwise stated. When the influence of sodium was studied, the incubation medium contained 100 mM NaCl. When the influence of guanyl nucleotides was studied, the incubation medium contained 100 µM Gpp(NH)p. The specific binding was determined by subtraction of the non-specific binding which was measured in the presence of 100 µM CADO. The binding reactions were stopped by vacuum filtration through glass fibre filters (Skatron), and washing of the filters and reaction chamber with 15 ml of a solution containing 50 mM TRIS and 10 mM $MgCl_2$, pH 7.4, kept at 4° C (Skatron 1719 cell harvester, position 333). The filters were then placed in scintillation vials and 3 ml of scintillation liquid (Ready Safe, Beckman) added. Radioactivity bound to the filters was determined after 12 h or more. The counting efficiency was 55–60%. To ensure a counting error lower than 5% of counts, the samples were counted for 4 min (cortex or striatum) or 10 min (hippocampus). All binding assays were performed in duplicate. The amount of membrane protein was determined according to Spector (1978).

Association curves were performed with 10 nM [^{3}H]CGS 21680 and 12 different incubation times ranging from 15 min to 7 h. The influence of ADA was studied using 10 nM [^{3}H] CGS 21680 and an incubation time of 2 h. Saturation experiments were performed with [^{3}H]CGS 21680 concentrations of 0.2–200 nM. Competition studies were performed with 5 nM [^{3}H]CGS 21680. The competitors tested were DPCPX (10^{-11}–10^{-5} M), XAC (10^{-11}–10^{-5} M), KF 17,837 (10^{-11}–10^{-6} M), CSC (10^{-11}–10^{-6} M), CHA (10^{-11}–10^{-5} M), CADO (10^{-11}–10^{-5} M), NECA (10^{-11}–10^{-5} M), APEC (10^{-11}–10^{-5} M) and CV 1808 (10^{-11}–10^{-5} M).

The pharmacological dissection of [^{3}H]CGS 21680 binding was done using male Wistar rat (180–200 g) membranes, prepared as described above. In each experiment, [^{3}H] CGS 21680 saturation was studied both in the presence of DPCPX (20 nM) and in the presence of CSC (200 nM). The experimental procedures of the binding assay were as described, except that the medium was harvested through Whatman GF/C filters, which were counted with 5 ml of Scintran Cocktail T (BDH).

The data were initially processed in QuattroPro (Borland) to determine the average specific binding. The specific binding from saturation experiments was fitted by non-linear regression using the Raphson-Newton method, performed with commercial software (InPlot, GraphPAD Software, San Diego Calif., USA) to determine the binding parameters (dissociation constant, K_d, and number of binding sites, B_{max}). The binding data of competition experiments were analysed using the LIGAND program. The concentration required for half-maximal inhibition (IC_{50}) was determined upon non-linear fitting of the semi-logarithmic curves derived from the competition curves (Munson and Rodbard 1980). In both cases, an F-test ($P < 0.05$) was used to determine whether the competition curves were fitted best by one or two independent binding sites. The confidence intervals (95%) of the K_i values were determined from the log values.

Binding assays in synaptosomal membranes. Synaptosomes from the hippocampus, cerebral cortex and striatum of the rat were prepared by differential sucrose-Percoll density gradient centrifugations as described previously (Cunha et al. 1994d). The synaptosomes of each of the rat brain regions were then resuspended in a 30 ml solution containing 50 mM TRIS (pH 7.4 at 23° C), 2 mM EGTA, 1 mM EDTA and 5 U/ml ADA and incubated for 30 min at 37° C. The mixture was centrifuged at 30,000 × g for 10 min at 4° C and the pellets resuspended in an incubation solution containing 50 mM TRIS, 10 mM $MgCl_2$ (pH 7.4) and 5 U/ml ADA.

Competition studies of [^{3}H]CGS 21680 or of [^{3}H] CHA binding were performed by incubation of 30 nM [^{3}H]CGS 21680 or of 2 nM [^{3}H]CHA for 4 h at room temperature (23–25° C) with 0.180–0.605 mg of membrane synaptosomal protein in a final volume of 300 µl in a solution containing 50 mM TRIS, 10 mM $MgCl_2$ (pH 7.4) and 5 U/ml ADA. The competitors tested were CSC (200 nM), KF 17, 837 (200 nM), DPCPX (20 nM), XAC (100 nM), CGS 21680 (30 nM) and CHA (100 nM). Specific binding was determined by subtracting the non-specific binding, measured in the presence of 100 µM CADO. The binding reactions were stopped by vacuum filtration through glass fibre filters (Skatron), and washing of the filters and reaction chamber with 15 ml of a solution containing 50 mM TRIS and 10 mM $MgCl_2$, pH 7.4, kept at 4° C (Skatron 1719 cell harvester, position 333). Radioactivity bound to the dry filters and membrane protein were determined as described above. All binding assays were performed in triplicate. [^{3}H]CGS 21680 and [^{3}H]CHA specific binding to synaptosomal membranes increased linearly with increasing amount of protein in the assay between 0.1 and 1 mg.

Results

We first examined the time course of [^{3}H]CGS 21680 binding to membranes of the rat hippocampus, cerebral cortex and striatum by incubation with 10 nM [^{3}H] CGS 21680 for periods of 15 min to 7 h. In the three brain regions, an apparent equilibrium was reached after 3–4 h of incubation at room temperature (23–25° C) (data not shown), and binding remained virtually unchanged up to 7 h. For the remaining experiments, the incubation time was thus fixed at 4 h.

Table 1 Mean binding parameters K_d (in *parentheses* the 95% confidence limits calculated from the log values) and B_{max} (± SEM) of [^{3}H]2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS 21680) to membranes of the cerebral cortex, hippocampus and striatum, derived from saturation experiments. In *italics* are shown the binding parameters obtained upon pharmacological isolation of each of the two [^{3}H]CGS 21680 binding sites. The results were obtained in three to five experiments performed in duplicate. The specific binding of [^{3}H]CGS 21680 corresponded to about 47% of total binding in the hippocampus, 38% in the cortex and 78% in the striatum, for concentrations of [^{3}H]CGS 21680 near the K_d; at concentrations of [^{3}H]CGS 21680 near 1/4 of the K_d and at concentrations of [^{3}H]CGS 21680 of 200 nM, the specific binding of [^{3}H]CGS 21680 was 48% and 32% of total binding in the hippocampus, 42% and 21% in the cortex and 85% and 54% in the striatum respectively (*DPCPX* 1,3-dipropyl-8-cyclopentylxanthine, *CSC* 8-(3-chlorostyryl)caffeine)

	K_d (nM)	B_{max} (fmol/mg protein)
Cortex	58 (31–109)*	264 ± 75
+ *DPCPX (20 nM)*	*22 (16–31)*	*63 ± 10*
+ *CSC (200 nM)*	*112 (99–126)*	*221 ± 43*
Hippocampus	58 (30–115)*	353 ± 89
+ *DPCPX (20 nM)*	*20 (11–30)*	*56 ± 11*
+ *CSC (200 nM)*	*97 (65–144)*	*255 ± 14*
Striatum	17 (9–28)	419 ± 36
+ *DPCPX (20 nM)*	*15 (10–22)*	*297 ± 25*
+ *CSC (200 nM)*	*37 (32–43)*	*140 ± 15*

* $P < 0.05$ vs. striatum in control conditions (Student's t-test)

To determine if differences in the binding of [^{3}H] CGS 21680 to membranes of the hippocampus, cortex and striatum could be attributed to differences in the amounts of endogenous adenosine, we investigated the effect of ADA (0.1–10 U/ml), an enzyme that catabolises adenosine, on [^{3}H]CGS 21680 binding to membranes from these regions. The specific binding of 10 nM [^{3}H]CGS 21680 with 1 U/ml of ADA in the assay corresponded to 75% (striatum), 67% (cortex) and 67% (hippocampus) of the specific binding with 5 U/ml of ADA in the assay. The specific binding of 10 nM [^{3}H]CGS 21680 was maximal at an ADA concentration of 5 U/ml in the three brain regions and, therefore, all subsequent binding assays were performed in the presence of 5 U/ml ADA.

Saturation curves with [^{3}H] CGS 21680 (0.2–200 nM) were performed in each brain area. The fitting of the saturation isotherm obtained for each brain area showed a single binding site for [^{3}H]CGS 21680 in each of the brain areas. The data were not better fitted with a two -independent -site model in any of the individual experiments ($P > 0.05$) . The averaged binding parameters are shown in Table 1. The affinity for [^{3}H]CGS 21680 in the hippocampus and cortex was one-third of that in the striatum (Table 1). The B_{max} in the hippocampus and cortex was slightly lower than in the striatum (Table 1).

Differential effect of Sodium and Gpp(NH)p

The presence of either Gpp(NH)p (100 μM) or Na^+ (100 mM) virtually abolished the specific binding of [^{3}H]CGS 21680 (0.2–200 nM) to hippocampal or cortical membranes in three out of four experiments; in one experiment there was residual [^{3}H]CGS 21680 binding at concentrations greater than 20 nM. In contrast, in the striatum, the effect of Gpp(NH)p (100 μM) or Na^+ (100 mM) on [^{3}H]CGS 21680 binding was less pronounced: Gpp(NH)p (100 μM) increased the K_d of [^{3}H]CGS 21680 binding to 30 ± 8 nM ($n = 4$) but B_{max} was virtually unchanged. Addition of Na^+ (100 mM) also increased the K_d for [^{3}H]CGS 21680 binding to 32 ± 1 nM ($n = 4$), leaving B_{max} virtually unchanged.

Competition experiments with antagonists of adenosine receptors

We compared the relative potency of four adenosine receptor antagonists, KF 17,837, CSC, DPCPX and XAC, as competitors of [^{3}H]CGS 21680 binding in membranes of the three brain regions. KF 17,837 and CSC were chosen because they behave as potent and selective A_{2A} receptor antagonists in the striatum (Shimada et al. 1992; Jacobson et al. 1993); DPCPX because it is a potent and selective A_1 antagonist (Bruns et al. 1987) and finally XAC because it is a potent, non-selective A_1/A_2 antagonist [e.g. Bruns et al. (1986) Jarvis et al. (1989)].

In membranes from the hippocampus, cortex and striatum, the displacement curves of [^{3}H] CGS 21680 (5 nM) binding by KF 17,837 (10^{-11}–10^{-6} M), CSC (10^{-11}–10^{-6} M) and XAC (10^{-11}–10^{-5} M) were monophasic (Fig. 1). The K_i values for these adenosine receptors antagonists are shown in Table 2, where it can be concluded that KF 17,837 was 10 times less potent in the hippocampus and cortex than in the striatum; CSC was also less potent (15–25 times) in the hippocampus and cortex than in the striatum. XAC was slightly more potent (3–4 times) in the hippocampus and cortex than in striatum. In some of the experiments in the hippocampus and cortex, the displacement curves of [^{3}H]CGS 21680 binding by KF 17,837 or by CSC showed a tendency to be biphasic with the presence of a second site where [^{3}H]CGS 21680 was more potently displaced (26–74 nM). However, this was significant ($P < 0.05$) in only three out of eight experiments. DPCPX (10^{-11}–10^{-5} M) yielded a biphasic curve against [^{3}H] CGS 21680 (5 nM) binding in the hippocampus, cortex and striatum (Fig. 1). Most of the [^{3}H]CGS 21680 binding in the hippocampus ($88 \pm 3\%$) and cortex ($89 \pm 5\%$) showed high affinity for DPCPX. The binding site for [^{3}H]CGS 21680 with lower affinity for DPCPX had a K_i of 860 nM in the hippocampus and 3790 nM in the cortex. This binding site represented $12 \pm 2\%$ of the total [^{3}H] CGS 21680 (5 nM) binding in the hippocampus and $11 \pm 2\%$ in the cortex. In contrast, in the striatum, the predominant [^{3}H]CGS 21680 binding site ($85 \pm 6\%$) had low affi-

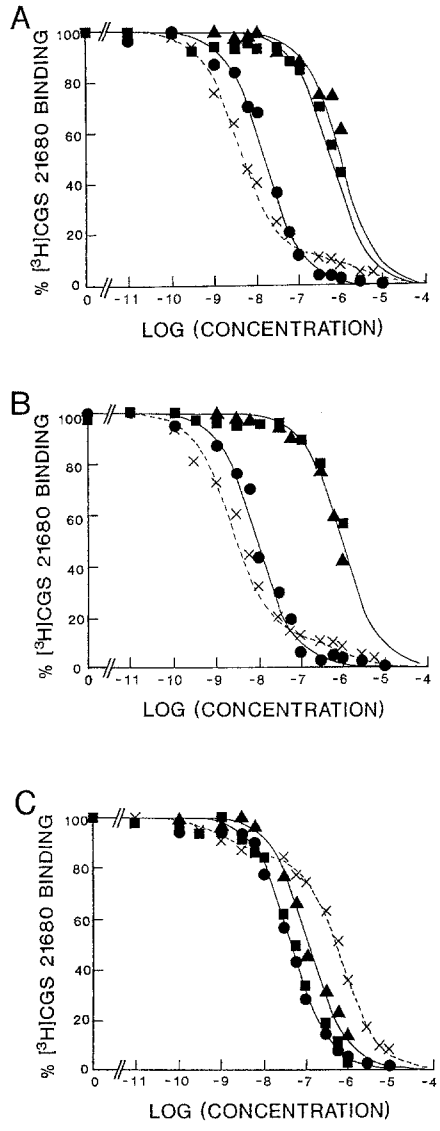

Fig. 1 Inhibition of [^{3}H] 2-[4-(2-*p*-carboxyethyl)phenylamino] 5′-*N*-ethylcarboxamidoadenosine [^{3}H]CGS 21680, (5 nM) binding to whole membranes of the cerebral cortex (**A**), hippocampus (**B**) and striatum (**C**) by the adenosine receptor antagonists, (*E*)-1,3-dipropyl-8(3,4-dimethoxystyryl)-7-methylxanthine (KF 17,837 ▲), 8-(3-chlorostyryl xanthine) (CSC ■), 1,3-dipropyl-8-{4-[2-(aminoethyl) amino]carbonylmethyloxyphenyl} xanthine (XAC ●) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX **X**). The ordinates represent the specific binding of [^{3}H]CGS 21680 obtained upon subtraction of the non-specific binding, determined in the presence of 100 μM 2-chloroadenosine (CADO), from the total binding. The specific binding of [^{3}H]CGS 21680 corresponded to about 49% of total binding in the hippocampus, 42% in the cerebral cortex and 78% in the striatum. *Curves* were generated from the average binding parameters obtained upon fitting by nonlinear regression assuming a single binding site (*solid lines*), or a two independent binding sites model (*dashed line*). Results are the mean of four experiments performed in duplicate, and the SEM were 2.3–12.9% of the means

Table 2 Inhibition constants (K_i in nanomoles per litre) of several adenosine receptor antagonists of [^{3}H]CGS 21680 binding. The values in *parentheses* represent the 95% confidence intervals of three to five experiments. The values in brackets [ ] represent the relative contribution of the binding site to the total [^{3}H]CGS 21680 specific binding. The data were obtained in n experiments performed in duplicate (*KF* 1 *7,837* (*E*)-1,3-dipropyl-8(3,4-dimethoxystyryl)-7-methylxanthine, *XAC* 1,3-dipropyl-8-{4[2-(aminoethyl)amino]carbonylmethyloxyphenyl}xanthine

	DPCPX ($n = 5$)	KF 17,837 ($n = 4$)	CSC ($n = 3$)	XAC ($n = 4$)
CORTEX				
high affinity [89%]	3.5 (0.9–14)	910*	530*	14*
low affinity [11%]	3790 (2130–5550)	(790–1050)	(230–1200)	(9–24)
HIPPOCAMPUS				
high affinity [89%]	2.1 (0.5–8.7)	890*	900*	9*
low affinity [11%]	860 (150–4970)	(630–1250)	(220–3630)	(5–17)
STRIATUM				
high affinity (15%)	0.8 (0.1–6)	93	36	37
low affinity [85%]	570 (310–1040)	(65–136)	(25–52)	(29–48)

* $P < 0.05$ vs. the striatum (Student's t-test) for the confidence limits at $P < 0.05$ (see text)

nity for DPCPX. In this brain region, the binding site for [^{3}H]CGS 21680 with higher affinity for DPCPX represented 15 ± 4% of the total number of [^{3}H]CGS 21680 binding sites.

Competition experiments with agonists of adenosine receptors

We compared the relative potency of five adenosine receptor agonists, APEC, CHA, NECA, CADO and CV 1808, as competitors of [^{3}H]CGS 21680 binding in membranes of the three brain regions. APEC is a potent and selective A_{2A} receptor agonist in the striatum (Barrington et al. 1989); CHA is a potent and selective A_1 agonist (Bruns et al. 1980); NECA and CADO are considered non-selective A_1/A_2 agonists [e.g. Bruns et al. (1986)], and CADO is the chemically closer analogue of adenosine; CV 1808 is an A_2 ligand (Bruns et al. 1986) that in low nanomolar concentration binds to a form of A_{2A} receptor (Luthin and Linden 1995) that has been termed A_4 receptor (Cornfield et al. 1992).

Figure 2 shows the displacement curves of [^{3}H] CGS 21680 (5 nM) binding by the agonists. In all brain regions, the displacement by APEC (10^{-11}–10^{-5} M) and CV 1808 (10^{-11}–10^{-5} M) was monophasic. The displacement curve of [^{3}H]CGS 21680 (10 nM) by CGS 21680 (10^{-11}–10^{-5} M) was also monophasic and the K_i values obtained in the three brain areas (Table 3) are similar to the K_d values of [^{3}H]CGS 21680 binding in the saturation experiments (Table 1). As shown in Table 3, APEC was slightly more potent in the hippocampus and cortex than in the striatum. CV 1808 was more than 10 times more potent inhibiting [^{3}H] CGS 21680 binding to hippocampal and cortical membranes than to striatal membranes. The displacement of [^{3}H] CGS 21680 (5 nM) by CHA (10^{-11}–10^{-5} M), CADO (10^{-11}–10^{-5} M) and NECA (10^{-11}–10^{-5} M) was biphasic in the hippocampus, cortex and striatum (Fig. 2). However, as can be concluded from the data shown in Table 3, the [^{3}H]CGS 21680 binding site with higher affinity for CHA, NECA and CADO was predominant in the hippocampus and cortex, representing 75–93% (hippocampus) and 75–88% (cortex) of the total [^{3}H]CGS 21680 (5 nM) binding. In contrast, in the striatum, the predominant [^{3}H]CGS 21680 binding site (74–89%) had a lower affinity for CHA, NECA and CADO. In the hippocampus and cerebral cortex the [^{3}H]CGS 21680 binding site with lower affinity for CHA, NECA and CADO was also present but only represented 7–25% of the total [^{3}H] CGS 21680 (5 nM) binding. In contrast, in the striatum, the less abundant [^{3}H]CGS 21680 binding site (11–26%) had a higher affinity for CHA, NECA and CADO.

Pharmacological dissection of [^{3}H]CGS 21680 binding

We tried to isolate the two [^{3}H] CGS 21680 binding sites in hippocampal and cortical membranes using pharmacological tools. As shown in Table 1, in the presence of DPCPX (20 nM), [^{3}H]CGS 21680 (0.2–200 nM) bound to a site with a K_d of 20 nM and a B_{max} of 56 fmol/mg protein in the hippocampus ($n = 5$) and to a site with a K_d of 22 nM and a B_{max} of 63 fmol/mg protein in the cortex ($n = 4$) . In the presence of CSC (200 nM), [^{3}H]CGS 21680 (0.2–200 nM) bound to a site with a K_d of 97 nM and a B_{max} of 225 fmol/mg protein in the hippocampus ($n = 3$) and to a site with a K_d of 112 nM and a B_{max} of 221 fmol/mg protein in the cortex ($n = 4$) . Some of the experiments performed in the hippocampus and in the cortex could not be analysed because of large data scatter and low [^{3}H] CGS 21680 binding. In striatal membranes, the pharmacological isolation of the two [^{3}H]CGS 21680

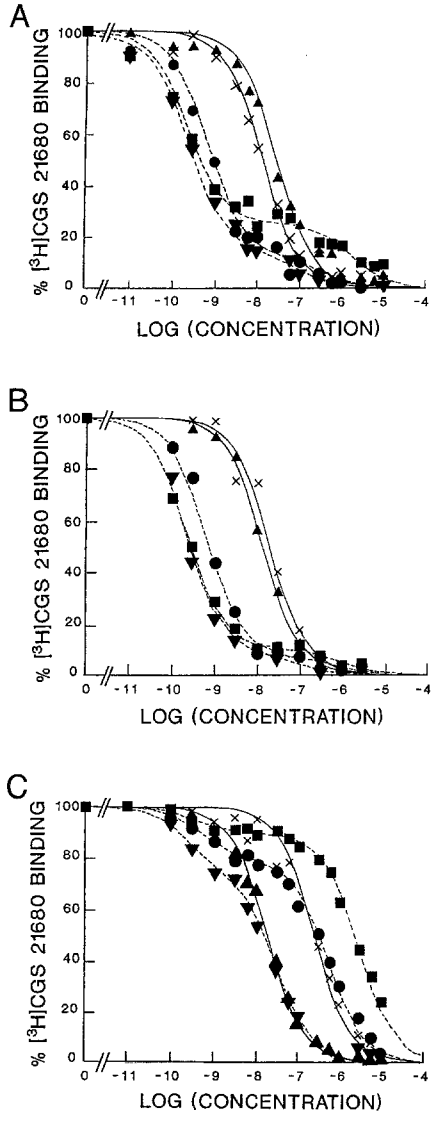

Fig. 2 Inhibition of [^{3}H]CGS 21680 (5 nM) binding to whole membranes of the cerebral cortex (**A**), hippocampus (**B**) and striatum (**C**) by the adenosine receptor agonists, 2-[(2-aminoethylamino) carbonylethylphenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (APEC ▲), N^6-cyclohexyladenosine (CHA ■), 5′-*N*-ethylcarboxamidoadenosine (NECA ▼), 2-chloroadenosine (CADO ●) and 2-phenylaminoadenosine (CV 1808 **X**). The *ordinates* represent the specific binding of [^{3}H]CGS 21680 obtained upon subtraction of the non-specific binding, determined in the presence of 100 μM CADO, from the total binding. The specific binding of [^{3}H]CGS 21680 corresponded to about 52% of total binding in the hippocampus, 39% in the cerebral cortex and 74% in the striatum. *Curves* were generated from the average binding parameters obtained upon fitting by nonlinear regression assuming a single binding site (*solid lines*), or a two independent binding sites model (*dashed lines*). Results are the means of four experiments performed in duplicate, and the SEMs were 1.9–16.8% of the means

binding sites was not so clear-cut. [^{3}H]CGS 21680 (0.2–200 nM) bound to a site with a K_d of 15 nM and a B_{max} of 297 fmol/mg protein in the presence of DPCPX (20 nM, $n = 4$) and to a site with a K_d of 37 nM and a B_{max} of 140 fmol/mg protein in the presence of CSC (200 nM, $n = 4$).

Comparison of [^{3}H] CGS 21680 and [^{3}H]CHA binding to synaptosomal membranes

The specific binding of [^{3}H]CGS 21680 (30 nM) to synaptosomal membranes was 51.4 ± 9.7 fmol/mg protein ($n = 5$) in the cortex, 83 ± 17 fmol/mg protein ($n = 5$) in the hippocampus and 276 ± 16 fmol/mg protein ($n = 5$) in the striatum. These values (normalised per mg of protein) were similar to those found in whole membranes in each of the regions (65 ± 25 fmol/mg protein, $n = 4$, in the cortex; 111 ± 26 fmol/mg protein, $n = 4$, in the hippocampus; 270 ± 17 fmol/mg protein, $n = 4$, in the striatum).

As shown in Fig. 3, the pharmacology of [^{3}H] CGS 21680 (30 nM) displacement by adenosine receptor agonists and antagonists was similar in the hippocampus and cerebral cortex, but distinct from that observed in the striatum: CSC (200 nM) and KF 17,837 (200 nM) inhibited [^{3}H]CGS 21680 binding in the striatum ($P < 0.05$) but not in the hippocampus ($P > 0.05$) and cortex ($P > 0.05$). DPCPX (20 nM) and CHA (100 nM) inhibited [^{3}H]CGS 21680 binding in the hippocampus ($P < 0.05$) and cortex ($P < 0.05$) but not in the striatum ($P > 0.05$) . XAC (100 nM) inhibited ($P < 0.05$) [^{3}H] CGS 21680 binding to membranes of the three brain regions. In contrast, the binding of the A_1 agonist, [^{3}H] CHA (2 nM), was equally displaced in synaptosomal membranes of the three brain regions (Fig. 3). Both DPCPX (20 nM) and XAC (100 nM) inhibited ($P < 0.05$) [^{3}H]CHA binding in all three brain regions. CSC (200 nM), KF 17,837 (200 nM) and CGS 21680 (30 nM) did not inhibit ($P > 0.05$) [^{3}H]CHA binding appreciably in any of the regions (Fig. 3).

Discussion

The present work shows that [^{3}H] CGS 21680 binds to two high-affinity binding sites in cortical, hippocampal and striatal membranes of the rat. The first high-affinity binding site predominates in the striatum, but is also present in the cortex and hippocampus and has binding characteristics compatible with A_{2A} receptors. The second high-affinity binding site predominates in the cortex and hippocampus, but is also present in the striatum.

The greatest pharmacological differences between the binding of [^{3}H]CGS 21680 to hippocampus and cortex compared with its binding to striatum were observed with the selective A_{2A} antagonists, KF 17,837 and CSC and the selective A_1 antagonist, DPCPX. KF 17,837 and CSC inhibited most of the [^{3}H]CGS 21680 binding to striatal membranes, as previously shown

Table 3 Inhibition constants (K_i in nanomoles per litre) of several adenosine receptor agonists of [^{3}H]CGS 21680 binding. The values in *parentheses* represent the 95% confidence intervals of three or four experiments. The values in *brackets* [ ] represent the relative contribution of the predominant binding site to the total [^{3}H]CGS 21680 specific binding. The data was obtained in *n* experiments performed in duplicate (*CHA* N^6-cyclohexyladenosine, *NECA* 5′-*N*-ethylcarboxamidoadenosine, *CADO* 2-chloroadenosine, *APEC* 2-[2-(aminoethylamine)carbonylethylphenylethylamine]-5′-*N*-ethylcarboxamidoadenosine)

	CHA ($n = 4$)	NECA ($n = 4$)	CADO ($n = 4$)	APEC ($n = 4$)	CV1808 ($n = 3$)	CGS21680 ($n = 3$)
CORTEX						
high affinity [77–88%]	0.20 (0.10–0.39)	0.21 (0.06–0.71)	0.60 (0.28–1.27)	18	12*	63*
low affinity [12–25%]	1580 (1240–2010)	799 (59–106)	350 (190–650)	(12–27)	(10–16)	(37–106)
HIPPOCAMPUS						
high affinity [75–88%]	0.21 (0.09–0.48)	0.21 (0.07–0.64)	0.59 (0.36–0.96)	12*	14*	62*
low affinity [12–25%]	860 (190–3960)	87 (51–148)	650 (210–2020)	(5–27)	(9–21)	(54–71)
STRIATUM						
high affinity [11–26%]	0.27 (0.10–0.74)	0.19 (0.13–2.8)	0.38 (0.10–1.4)	24	190	12
low affinity [74–89%]	2260 (1010–5090)	24 (13–43)	320 (250–410)	(19–30)	(180–200)	(8–16)

* $P < 0.05$ vs. the striatum (Student's *t* test) for the confidence limits at $P < 0.05$ (see text)

(Shimada et al. 1992; Jacobson et al. 1993a). However, KF 17,837 and CSC were considerably less potent in inhibiting most of the [^{3}H]CGS 21680 binding in the hippocampus and cortex, suggesting that at least the xanthine locus of the [^{3}H]CGS 21680 binding sites in the hippocampus and cortex is different from that in the striatum (see Table 4). This idea is strengthened by the different potency of DPCPX as an inhibitor of [^{3}H]CGS 21680 binding in the three regions. The displacement of [^{3}H]CGS 21680 binding by DPCPX was biphasic in all the three studied brain regions. We observed the presence of two components, one with a K_i in the low nanomolar range and a second with a K_i in the submicromolar or micromolar range. In the striatum, the more abundant [^{3}H]CGS 21680 binding site had low affinity for DPCPX and had a pharmacological profile compatible with its classification as an A_{2A} receptor (Jarvis et al. 1989; Shimada et al. 1992). We also observed the presence of a second, high-affinity binding site for [^{3}H]CGS 21680 in the striatum, which was displaced by low nanomolar concentrations of DPCPX. This second binding site in the striatum, representing 15% of the total [^{3}H]CGS 21680 binding, has not previously been described [but see James et al. (1992) and Luthin et al. (1995)], although there is immunological evidence for subtypes of A_{2A} receptors (Palmer et al. 1992). However, in the hippocampus and cerebral cortex, most (88–89%) of the [^{3}H]CGS 21680 binding was displaced by DPCPX at low nanomolar concentrations. Using human brain membranes, it has been observed also that DPCPX has higher affinity for the [^{3}H]CGS 21680 binding sites in the cortex than in the striatum (James et al. 1992).

The presence of two, independent, high-affinity binding sites for [^{3}H]CGS 21680 in the three brain regions was also inferred from the biphasic curves obtained with the A_1 agonist, CHA, and the mixed A_1/A_2 agonists CADO and NECA. As with DPCPX, these adenosine agonists were very potent inhibitors of [^{3}H]CGS 21680 binding in the hippocampus and cortex (K_i in the sub-nanomolar range), while in the striatum their K_i values were at least 100 times higher. In the hippocampus and cerebral cortex there was also a less abundant [^{3}H]CGS 21680 binding site (7–25% of total binding) with a pharmacology compatible with an A_{2A} receptor, i.e. displaced by DPCPX, CHA and CADO concentrations of the order of hundreds of nanomoles per litre. The existence of two high-affinity binding sites for [^{3}H]CGS 21680 was further confirmed by the pharmacological isolation of two binding sites for [^{3}H]CGS 21680. In the hippocampus and cortex the predominant binding site has a K_d of around 100 nM (in the presence of 200 nM CSC). There was also an A_{2A}-like binding site with a K_d of around 20 nM (in the presence of 20 nM DPCPX), which only accounts for nearly 1/5 of the total number of [^{3}H]CGS 21680 binding sites in the cortex and hippocampus.

The existence of two high-affinity [^{3}H]CGS 21680 binding sites in rat cortical membranes has been reported elsewhere (Kirk and Richardson 1995), and interpreted as mixture of binding to putative A_1 and A_{2A} receptors. These authors used 1 U/ml ADA, which we have shown to be insufficient to reveal the second high-affinity binding site for [^{3}H]CGS 21680, and performed their study at low pH. Since the pH

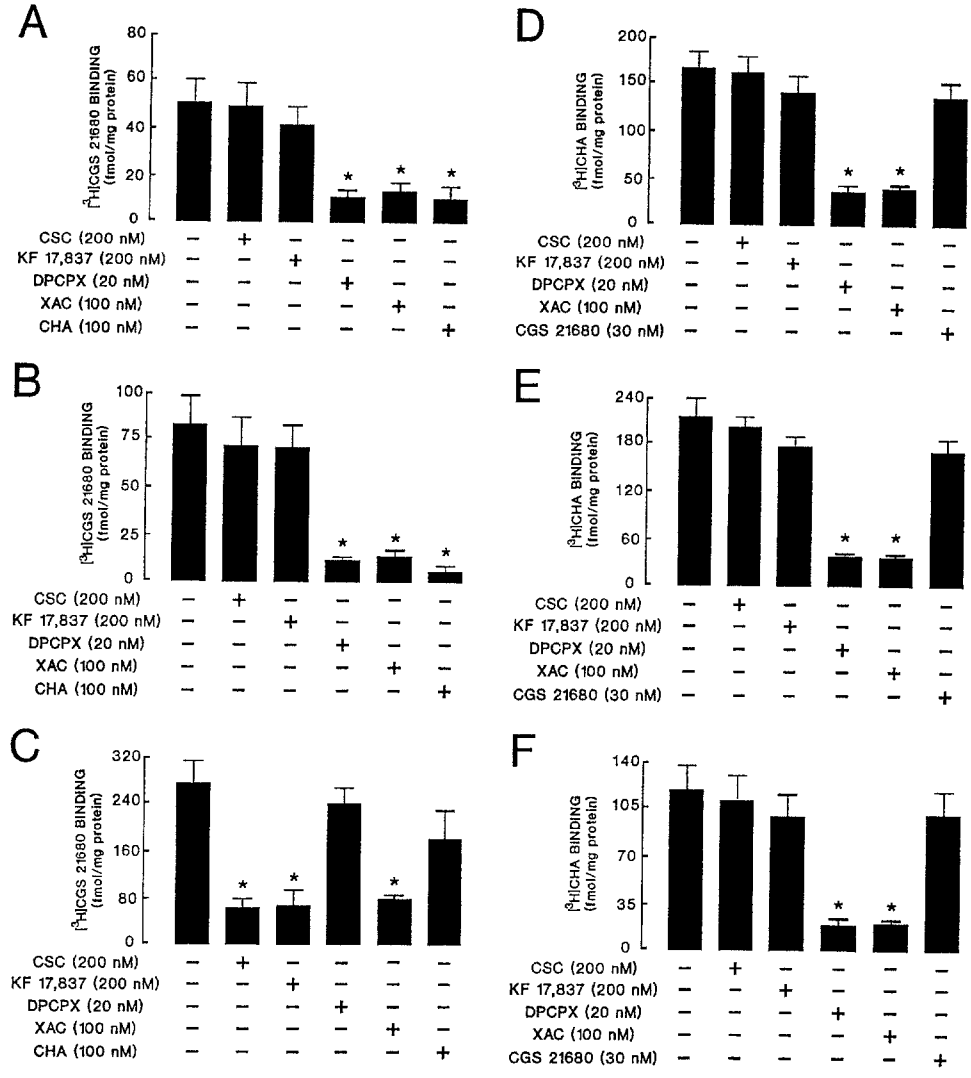

Fig. 3 Inhibition of [^{3}H]CGS 21680 binding (**A**–**C**) and inhibition of [^{3}H] CHA binding (**D**–**F**) to synaptosomal membranes of the cerebral cortex (**A**, **D**), hippocampus (**B**, **E**) and striatum (**C**, **F**) by different adenosine receptor ligands. Synaptosomal membranes (0.180–0.605 mg of protein) were incubated with 30 nM [^{3}H]CGS 21680 or with 2 nM [^{3}H] CHA for 4 h at room temperature in a final volume of 300 µl. The *ordinates* represent the specific binding of [^{3}H]CGS 21680 or of [^{3}H]CHA obtained upon subtraction of the non-specific binding, determined in the presence of 100 µM CADO, from the total binding. The specific binding of [^{3}H]CGS 21680 corresponded to about 37% of total binding in the hippocampus, 29% in the cerebral cortex and 59% in the striatum. The specific binding of [^{3}H]CHA corresponded to about 97% of total binding in the hippocampus, 95% in the cerebral cortex and 93% in the striatum. The presence (+) or absence (−) of each of the drugs is indicated below each *bar*. Results are mean ± SEM of four experiments performed in triplicate
$P < 0.05$ vs control (absence of inhibitors)

sensitivity of this second [^{3}H]CGS 21680 binding site is not known, we worked at physiological pH (7.4) and used a five fold higher concentration of ADA. Under these conditions, and by using a large range of concentrations of agonists and antagonists, we were able to separate out a component that corresponds to the A_{2A} receptor as well as a second high-affinity [^{3}H] CGS 21680 binding site. This second site exhibited a pharmacology which is in no way representative of a mixture of adenosine A_1 and A_{2A} receptors (see Table 4). It is unlikely that the second high-affinity [^{3}H]CGS 21680 binding site corresponds to classical A_1 receptors since firstly, the affinity of CGS 21680 for A_1 receptors is much lower [K_i of 2600 nM in relation to [^{3}H]CHA binding to whole-brain membranes, see Jarvis et al. (1989)], secondly, CGS 21680 (30 nM) did not inhibit appreciably [^{3}H]CHA binding to synaptosomal membranes prepared from the hippocampus, cortex or striatum, thirdly, adenosine receptor agonists and antagonists show different potencies in displacing [^{3}H]CHA and [^{3}H]CGS 21680 binding, fourthly, the A_{2A}/A_4 agonist CV 1808 (Cornfield et al. 1992; Luthin and Linden 1995), or the selective A_{2A} agonist APEC (Barrington et al. 1989), which have low affinities for A_1 receptors (see Table 4), displace [^{3}H]CGS 21680 binding to hippocampal and cerebral cortical membranes in the low nanomolar range and finally, [^{3}H]

Table 4 Inhibition constants (K_i in nanomoles per litre) of several adenosine receptor antagonists and agonists at different adenosine receptors and binding site

	A_1 [a]	A_{2A} [b]	A_X [c]
CSC	28000	36	530–900
KF17, 837	390–580	93	890–910
DPCPX	0.46–0.9	570	2.1–3.5
XAC	1.2–4	37	9–14
CHA	1.3–2.3	2260	0.20–0.21
NECA	6.0–10	24	0.21
CADO	9.3–14	320	0.59–0.60
APEC	240	24	12–18
CGS21680	2600	12	62–63
CV1808	560–1100	190	12–14

[a] Values for A_1 receptors are from Bruns et al. (1987), Jacobson et al. (1985), Jacobson et al. (1986), Jacobson et al. (1993b), Nonaka et al. (1993), Jarvis et al. (1989), Bruns et al. (1986), Hutchison et al. (1989), Jacobson et al. (1989)

[b] Values for A_{2A} receptors are for inhibition of [^{3}H]CGS 21680 binding to rat striatal membranes as obtained in the present study

[c] A_X values represent the major binding site for [^{3}H]CGS 21680 in cortical and hippocampal membranes in the present study

CGS 21680 binding to hippocampal and cortical membranes is virtually abolished by Gpp(NH)p and by sodium, which contrasts with the mild inhibition of A_1 agonist binding by these agents [e.g. Goodman et al. (1982)]. The second high-affinity [^{3}H]CGS 21680 binding site also has binding characteristics different from the originally reported A_{2A} receptors present in the striatum (Jarvis et al. 1989), such as (1) sensitivity to low nanomolar concentrations of DPCPX, CHA and CADO; (2) greater sensitivity to Gpp(NH)p and sodium; (3) higher K_d for CGS 21680; and (4) densities in the cortex and hippocampus greater than those reported with other A_{2A} ligands (Nokada et al. 1994). The possibility that this [^{3}H]CGS 21680 binding site might be the A_{2A} receptor subtype that preferentially binds [^{3}H]2-phenylaminoadenosine (Luthin and Linden 1995), initially named A_4, can be also excluded, since this putative A_4 binding site is insensitive to CGS 21680 and to Gpp(NH)p (Cornfield et al. 1992). This binding site also did not behave as an A_{2B} receptor, since [^{3}H]CGS 21680 does not activate (Hide et al. 1992; Yakel et al. 1993) or bind to (Stehle et al. 1992) A_{2B} receptors in low nanomolar concentrations. Finally, it is also distinct from the recently cloned rat A_3 receptor, which is rather insensitive to the xanthines used in the present work as well as to CGS 21680 (Zhou et al. 1992). In summary, the present results substantiate the idea that, in the cortex and hippocampus, [^{3}H]CGS 21680 binds predominantly to an hithertho unrecognised binding site, as suggested from receptor autoradiography studies (Johansson et al. 1993; Johansson and Fredholm 1995).

We observed that the sensitivity of [^{3}H]CGS 21680 binding to Gpp(NH)p was much greater in the hippocampus and cerebral cortex than in the striatum, as previously observed for this second binding site by receptor autoradiography (Johansson et al. 1993), and it is also much greater than the sensitivity of A_1 [e.g. Goodman et al. (1982)] and of A_{2A} (Johansson et al. 1992) receptors to GTP analogues. In the cortex and hippocampus the presence of 100 mM NaCl abolished the binding of [^{3}H]CGS 21680, while binding to A_1 (Goodman et al. 1982) and to A_{2A} receptors [see also Johansson et al. (1992)] is only moderately sensitive to sodium. Thus, the interaction between guanine nucleotide-binding proteins (G-proteins) and the second high-affinity [^{3}H]CGS 21680 binding site seems to be different from the interaction of G-proteins with striatal-like A_{2A} or with A_1 receptors.

It is interesting to note that the specific binding of [^{3}H]CGS 21680 (normalised per milligram of protein) to synaptosomal membranes and to whole membranes from the same brain region is quantitatively and pharmacologically similar, suggesting the presence of [^{3}H]CGS 21680 binding sites on both hippocampal and cortical nerve terminals. However, the lack of significant enrichment of the specific binding of [^{3}H]CGS 21680 upon preparation of synaptosomal membranes also suggests that the [^{3}H]CGS 21680 binding sites in the hippocampus and cortex are not confined to nerve terminals. In the synaptosomal membranes from the hippocampus, cortex and striatum, the inhibition of [^{3}H]CHA binding by CSC, KF 17,837, DPCPX, XAC and CGS 21680 was similar and compatible with the binding to an A_1 receptor. This suggests that the presynaptic adenosine A_1 receptors are pharmacologically identical in these three brain areas, in contrast to the variation found in relation to the [^{3}H]CGS 21680 binding sites.

In summary, the present work describes the existence of two, separate, high-affinity [^{3}H]CGS 21680 binding sites coupled to G-proteins, not only in the striatum but also in the hippocampus and cerebral cortex. The marked dependency of [^{3}H]CGS 21680 binding on the concentration of ADA suggests that adenosine is the endogenous ligand at both these binding sites. Whether the predominant, second, high-affinity [^{3}H]CGS 21680 binding site, or the less-abundant, but typical, adenosine A_{2A} receptor, are involved in the stimulatory effects of low concentrations of CGS 21680 (Sebastião and Ribeiro 1992; Cunha et al. 1994b) and adenosine (Okada et al. 1992) on synaptic transmission in the hippocampus and on the inhibitory effect on GABA release from the ischaemic cerebral cortex (O'Reagan et al. 1992) needs to be investigated.

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