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The Role of Adenosine Receptors on Acetylcholine Release in the Rat Striatum

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As it has been reported that the depolarization induced acetylcholine (ACh) release is modulated by activation of presynaptic A_1 adenosine heteroreceptor and various evidence suggest that indicate the A_2 adenosine receptor is present in the striatum, this study was undertaken to delineate the role of adenosine receptors on the striatal ACh release.

Slices from the rat striatum were equilibrated with [^{3}H]choline and then the release amount of the labelled product, [^{3}H]ACh, which was evoked by electrical stimulation (rectangular pulses, 3 Hz, 2 ms, 24 mA, 5 Vcm^{-1}, 2 min), was measured, and the influence of various agents on the evoked tritium outflow was investigated. And also, quantitative receptor autoradiography and drug-receptor binding assay were performed in order to confirm the presence and characteristics of A_1 and A_2 adenosine receptors in the rat striatum.

Adenosine (10～100 μM) and N^6-cyclopentyladenosine (CPA, 1～100 μM) decreased the [^{3}H]ACh release in a dose-dependent manner without changing the basal rate of release in the rat striatum. The reducing effects of ACh release by adenosine and CPA were abolished by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 2 μM), a selective A_1 adenosine receptor antagonist, treatment. The effect of adenosine was potentiated markedly by 3,7-dimethyl-1-propargylxanthine (DMPX, 10 μM), a specific A_2 adenosine receptor antagonist. 2-P-(2-carboxyethyl)phenethylamimo-5'-N- ethylcarboxamidoadenosine hydrochloride (CGS-21680C), in concentrations ranging from 0.01 to 10 μM, a recently introduced potent A_2 adenosine receptor agonist, increased the [^{3}H]ACh release in a dose related fashion without changing the basal rate of release. These effects were completely abolished by DMPX (10 μM). In autoradiography experiments, [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA) bindings were highly localized in the hippocampus and the cerebral cortex. Additionally, lower levels of binding were found in the striatum. However, [^{3}H]CGS-21680C bindings were highly localized in the striatal region with the greatest density of binding found in the caudate nucleus and putamen. Lower levels of binding were also found in the nucleus accumbens and olfactory tubercle. In drug-receptor binding assay, binding of [^{3}H]CCPA to A_1 adenosine receptors of rat striatal membranes was inhibited by CPA (K_i = 1.6 nM) and N-ethylcarboxamidoadenosine (NECA, K_i = 12.9 nM), but not by CGS-21680C (K_i = 2609.2 nM) and DMPX (K_i = 19,386 nM). In contrast, [^{3}H]CGS-21680C binding to A_2 adenosine receptors was inhibited by CGS-21680C (K_i = 47.6 nM) and NECA (K_i = 44.9 nM), but not by CPA (K_i = 2099.2 nM) and DPCPX (K_i = 19,207 nM).

The results presented here suggest that both types of A_1 and A_2 adenosine heteroreceptors exist and play an important role in ACh release in the rat striatal cholinergic neurons.

Key Words: Adenosine receptors, Acetylcholine, Striatum, Autoradiography

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INTRODUCTION

Two adenosine receptor subtypes, termed A_1 and A_2, have been differentiated based on the pharmacological profile of adenosine agonists and antagonists at each receptor subtype (Daly et al., 1983; Hamprecht and Van calker, 1985). Inhibition by adenosine on the release of various neurotransmitters including acetylcholine (ACh), norepinephrine (NE), 5-hydroxytryptamine and glutamate in the central nervous system has been reported, and the receptor participated in the inhibitory effect was defined as A_1-subtype (Jackish et al., 1985; Fredholm et al., 1986; Fredholm and Lindgren, 1987). Recently, ligand binding studies revealed the existence of A_2 adenosine receptor subtypes with a heterogeneous distribution in the peripheral and central nervous systems (Burnstock and Brown, 1981; Barraco et al., 1991). Although functionally distinct roles for A_1 and A_2 adenosine receptors have been established for many peripheral tissues (Bruns et al., 1986; Barraco et al., 1991), the physiological significance of multiple adenosine receptor subtypes in the central nervous system remains unclear.

It is well known that the striatal cholinergic neurons might play a role in striatal physiology because anti-cholinergic drugs siginficantly improved symptoms of Parkinsonian disease (Brumilk et al., 1964; Bruns et al., 1964). An interaction between cholinergic and doparminergic transmissions in the striatum has been supported by neurochemical data in which potassium evoked release of ACh from striatal slices was attenuated by dopamine D_2 receptor agonists (Stoof et al., 1982). Moreover, anatomical study has demonstrated that dopaminergic terminals are in direct apposition to cholinergic terminals in rat striatum (Pickel and Chan, 1990). Taken together, these observations have suggested that striatal cholinergic transmission might play an important role in striatal physiology, but the exact contribution of the striatal cholinergic neurons to the functioning of the brain is not precisely known.

Both A_1 and A_2 adenosine receptors are functionally relevant in the striatum and there are high affinity A_2 adenosine receptors that stimulate adenylate cyclase (Fredholm, 1977; Premont et al., 1979; Parkinson and Fredholm, 1990). The messenger RNA for these receptors is also enriched in the striatum, suggesting that, unlike A_1 adenosine receptors, A_2 adenosine receptor may largely be localized on intrinsic neurons, in agreement with the results obtained by lesion experiment (Alexander and Reddington, 1989).

Recently, Kirkpatrick and Richardson (1993) have shown that the A_2-selective agonist2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosi ne hydrochloride (CGS-21680C) increased and A_1-selective agonist R-N^6-phenylisopropyladenosine (R-PIA) decreased the veratridine-evoked ACh release from rat striatal synaptosomes. On the other hand, Jin et al (1993) have shown that both A_2-selective agonist CGS-21680C and A_1-selective agonist cyclohexyladenosine (CHA) caused a concentration dependent inhibition of electrically-evoked ACh release from rat striatal slices. However, the characteristics of the A_1 and A_2 adenosine receptors on the cholinergic neurons in the rat striatum remains unclear until now.

Consequently, the aim of the present study was to examine and compare the role of the A_1 and A_2 adenosine receptors in the evoked ACh release in the rat striatum. Moreover, to examine the presence and characteristics of the A_1 and A_2 adenosine receptors, receptor autoradiography and binding assay were performed.

ATERIALS AND METHODS

Release of radiolabelled acetylcholine (ACh)

Slices of 2.5~3.0 mg, 400 μM in thickness, were prepared from the striatum of Sprague-Dawley rats of either sex weighing 250~300 gm with a balzers tissue chopper and were incubated in 2 ml of modified Krebs-Henseleit medium containing 0.1 μmol/L [^{3}H]choline for 30 min at 37℃. Subsequently, the [^{3}H]choline-pretreated slices were superfused with medium containing hemicholinium-3 (10 μM) and atropine (30 nM) for 140 min at a rate of 0.5 ml/min. The composition (mM) of superfusion medium was 118 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 0.57 ascorbic acid, 0.03 Na_2EDTA, and 11 glucose, and the superfusate was continuously aerated with 95% O_2+5% CO_2, the pH adjusted to 7.4.

Collection of 5 min fraction (2.5 ml) of the superfusate began after 50 min of superfusion. Electrical stimulations (3 Hz, 5 Vcm^{-1}, 2 ms, rectangular pulses) for 2 minutes were performed at 60 min (S_1) and 120 min (S_2). Drugs were added between S_1 and S_2 to the

superfusion medium. At the end of superfusion, the slices were solubilized in 0.5 ml tissue solubilizer (0.5 N quaternary ammonium hydroxide in toluene). The radioactivity in the superfusates and solubilized tissues were determined by liquid scintillation counting (Beckman LS 5000TD). The fractional rate of tritium-outflow (5 min^{-1}) was calculated as tritium-outflow per 5 min divided by the total tritium content in the slice at the start of the respective 5 min period (Hertting et al., 1980). As reported previously, the electrical stimulation of brain slices incubated with [^{3}H]choline causes the release of [^{3}H]acetylcholine only (Richardson and Szerb, 1974). Drug effects on the evoked tritium-outflows were evaluated by calculation the ratio of the outflow evoked by S_2 and by S_1 (S_2/S_1).

The following chemicals were used: [methyl-^{3}H] choline chloride (72～78 Ci $mmol^{-1}$, Amersham), adenosine (RBI), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX RBI), atropine sulfate (Sigma), 2-p-(2-carboxyethyl)phenethylamimo-5'-N-ethylcarboxamidoad enosine hydrochloride (CGS-21680C, RBI), N^6-cyclopentyladenosine (CPA, RBI), hemicholinium-3 (Sigma), 3,7-dimetyl-1-propargylxanthine (DMPX, RBI). Drugs was dissolved in the medium except for DPCPX which were initially dissolved in DMSO and then diluted in the medium. The maximum concentration of DMSO used in the experiments (0.1%, vol/vol) did not affect basal tritium outflow or stimulation-evoked [^{3}H]ACh release.

Autoradiography

The rats were killed by decapitation and the brains were rapidly dissected and frozen by immersion in isopentane on powdered dry ice. And the tissues were stored at −20℃ with wet tissue until completely frozen. The tissues were sectioned in a cryostat and 7 μm sagittal brain sections were thaw-mounted onto cold silan-coated microscope slides. The mounted moist sections were then placed in a slide box with silica gel at 4℃ until they were very dry. When the sections were dried at 4℃ conditions, the sections were stored at −20℃ with silica gel in slide boxes until utilized.

On the day of the assay, brain sections were warmed to room temperature and allowed to dry. Sections were pre-incubated for 30 min at room temperature in a 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$, pH 7.4, and thereafter for 25 min at room temperature in the same medium with 2 IU/ml adenosine deaminase (Type VI, Sigma) to remove endogenous adenosine. Sections were rinsed three times by the fresh buffer and perfectly dried. Tissue sections were then incubated in the buffer containing 1 nM [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H] CCPA) or 16 nM [^{3}H]CGS-21680C and/or inhibitor for 120 min at room temperature. Previous experiments with this ligand have demonstrated that binding is fully equilibrated under these conditions (Jarvis, 1988; Lohse et al., 1988; Williams et al., 1989). Non-specific binding (blank) was defined as the binding remaining in consecutive sections incubated in the presence of 20 μM N-ethylcarboxamidoadenosine (NECA). After incubation, the sections were washed by immersion for 5 min in ice-cold buffer to remove unbound ligand and reduce non-specific binding. After washing with buffer, the slides were dipped twice in ice-cold distilled water to remove salt from the sections and rapidly dried under a stream of cold air. The dried sections were put, together with radioactive standards (^{3}H-microscales, Amersham) onto tritium-sensitive film (^{3}H-microscales, Amersham) in a light-tight cassette and they were exposed at -20 ℃. The exposure time was 28 days. Finally, the films were developed in Kodak developer D-19 and fixed.

Quantitative analysis of the resulting autoradiograms was performed using a video-based densitometric process (Jarvis, 1988) with a Image-Pro Plus Image Analyzer (Media Cybernetics, Maryland, USA). Gray values obtained from each brain region were converted to a measure of radioactivity (dpm/mg tissue) based upon internal tritium brain-paste standards (Unnerstall et al., 1982). Saturation parameter (K_d or K_i) for each brain region and data from competition experments were analyzed by non-linear regression with the interactive curve fitting program Ladlig (Cambridge, UK).

The following chemicals were used: [^{3}H]CCPA (30 ～60 Ci $mmol^{-1}$, Dupont New England Nuclear), [^{3}H]CGS-21680C (30～80 Ci $mmol^{-1}$, Dupont New England Nuclear), adenosine deaminase (Type VI, sigma), NECA (sigma).

Binding assay

Rats were sacrificed by decapitation, and their brains rapidly removed to ice. Striatums were dissected from the remaining brain tissue, collected in Tris-buffer (50 mM Tris HCl, 10 mM $MgCl_2$, pH adjusted to 7.4) and chilled on ice. The tissues were

homogenized with up and down stroke of a motor driven glass-teflon homogenizer on ice in 10 volumes of Tris-buffer. The striatal homogenate was filtered through two layers of gauze and the filtrate centrifuged at 40,000×g for 10 min at 4℃. The resulting pellet was resuspended in 10 volumes of ice-cold buffer and stirred for 30 min at 4℃. After the homogenate was recentrifuged at 40,000×g for 10 min at 4℃, the pellet was resuspended in 20 volumes of Tris-buffer. Adenosine deaminase (2 IU/ml) was added and the suspension was incubated at 37℃ for 30 min to remove endogenous adenosine. The suspension was recentrifuged at 40,000×g for 10 min at 4℃ and the final pellet was resuspended in ice-cold Tris-buffer. The crude membranes were stored frozen at −70℃ at a concentration of 2% weight/volume. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Membranes were thawed at room temperature and homogenized by 10 strokes in a glass-teflon homogenizer. For A_1 adenosine receptor assays, DMPX (3 µM) was added to each tube in order to block A_2 adenosine sites and for A_2 adenosine receptor assays, DPCPX (2 µM) was added to each tube in order to block A_1 adenosine sites. These concentrations of site selective displacing ligands were selected from an analysis of their potencies as displacers of each labeled ligand. After all additions, the final membrane concentration in the assay tube was 1% w/v, corresponding to about 300~500 µg protein per sample. All drug solutions were prepared in Tris-buffer, pH 7.4. Final volume in each tube was 500 µl. Triplicate samples of membrane suspension were preincubated with or without non-radioactive displacer at 37℃. Radiolabeled ligands (1 nM [^{3}H] CCPA or 16 nM [^{3}H] CGS-21680C) were then added and the incubation continued 120 min. The incubation was terminated by the addition of four ml ice-cold buffer and rapid filtration through glass fiber filters (Type G-7; Inotech, Zürich, Switzerland) under reduced pressure by using cell harvester (Inotech). The filters were washed with an additional four ml buffer, transferred to scintillation vials, soaked in an half ml each absolute ethanol, and counted in 3 ml scintillation coctail by liquid scintillation counter (Beckman LS 5000TD). Non-specific binding was defined as the fraction of bound radioligand that remained in the presence of 20 µM NECA. Specific binding to membranes was always less than 10% of added radioligand.

Saturation and displacement data were analyzed by the use of the computer program LIGAND (Munson and Rodbard, 1980). This program utilizes a non-linear least squares curve fitting algorithm, and assumes the simultaneous contribution of one or more independent binding sites.

Satistics

All results are given as Mean±SEM. Significance of difference between the groups was determined by ANOVA and subsequently by Student's t-test.

RESULTS

Effects of A_1 and A_2 Adenosine Receptor Agonists and Antagonists on [^{3}H]ACh Release Evoked by Electrical Stimulation

Striatal slices prelabelled with [^{3}H]choline, a [^{3}H]ACh precursor, were superfused with the medium containing hemicholinium-3 (10 µM), a choline uptake inhibitor. And in order to eliminate the inhibition of ACh release by activating muscarinic autoreceptor, atropine (30 nM), a muscarinic antagonist,

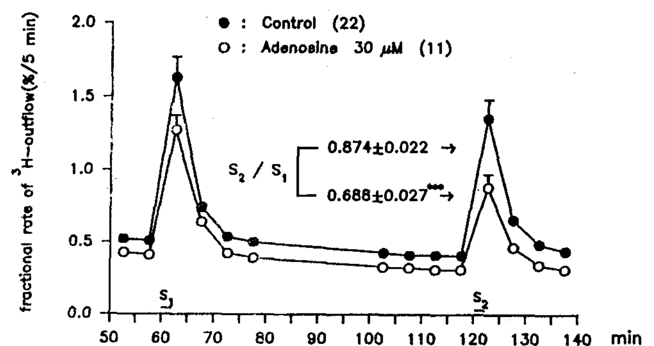

Fig. 1. Effect of 30 µM adenosine on the tritium-outflow from the rat striatal slices preincubated with [^{3}H]choline. The slices were electrically stimulated twice for 2 min after 60 (S_1) and 120 min (S_2) of superfusion, respectively. The drug effect on the stimulation-evoked tritium outflow is expressed by the ratio S_2/S_1. Adenosine was presented 15 min before S_2 onwards. The tritium contents of the tissue at the start of experiments were 1.68 ± 0.14 (●) and 1.95 ± 0.23 (○) pmol. The mean ± SEM of the experiments (n) are given. Asterisks indicate the significant difference from drug free control (***; $p<0.001$).

Table 1. Effect of adenosine (Ade) on the electrically-evoked and basal outflow of tritium from the rat striatal slices preincubated with [^{3}H]choline

Drugs at S_2 (μM)		n	S_2/S_1	b_2/b_1
Ade	none	22	0.8742 ± 0.0222	0.7914 ± 0.0133
	10	10	0.8641 ± 0.0272	0.8374 ± 0.0253
	30	11	0.6882 ± 0.0274***	0.7614 ± 0.0232
	100	10	0.6481 ± 0.0449***	0.8370 ± 0.0354

After preincubation, the slices were superfused with medium containing hemicholinium-3 (10 μM) and atropine (30 nM), and then stimulated twice (S_1, S_2). Drugs were present from 15 min before S_2 onwards at the concentrations indicated. Drug effects on basal outflow are expressed at the ratio b_2/b_1 between fractional rates of outflow immediately before S_2 (95 ~ 100 min) and before S_1 (55 ~ 60 min). Mean ± SEM from number (n) of observations are given. Significant differences from the drug-free control (none) are marked with asterisks (***; p<0.001).

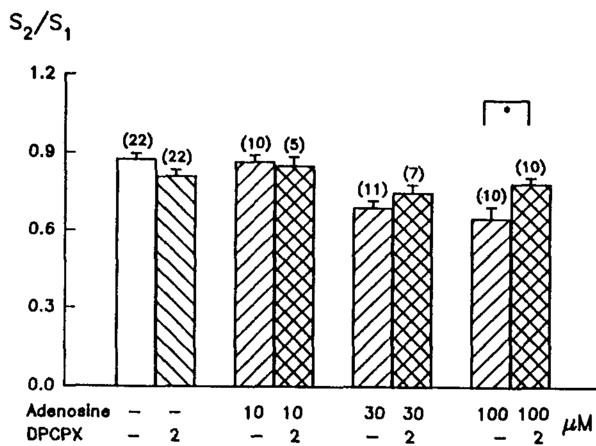

Fig. 2. Influence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on the effect of adenosine on the electrically-evoked tritium outflow from the rat striatal slices. In parentheses are the number of experiments. Both drugs were added to the medium 15 min before S_2. Asterisks indicate the significant difference (*; p<0.05) between groups. Other legends are the same as in Fig. 1.

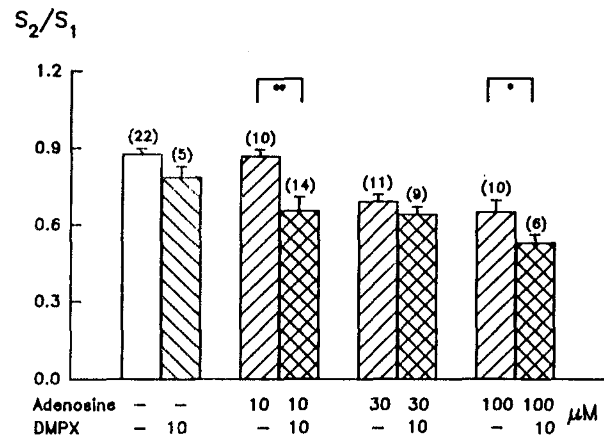

Fig. 3. Influence of 3,7-dimethyl-1-propargylxanthine (DMPX) on the effect of adenosine on the electrically-evoked tritium outflow from the rat striatal slices. Both drugs were added to the medium 15 min before S_2. Asterisks indicate the significant difference (**; p<0.01) between groups. Other legends are the same as in Fig. 2.

was added in the superfusion medium. During superfusion, the tissue was electrically stimulated twice.

As shown in Fig. 1, 30 μM adenosine decreased the electrically-evoked outflow of tritium (S_2/S_1 = 0.6882 ± 0.0274, n=11), but there was no change in the basal release. Adenosine, in doses ranging from 10 to 100 μM, decreased the electrically-evoked [^{3}H]ACh release in a concentration-dependent manner (Table 1).

To ascertain the interaction between adenosine and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective A_1 adenosine receptor antagonist (Bruns et al., 1987), or 3,7-dimethyl-1-propargylxanthine (DMPX), a selective A_2 adenosine receptor antagonist (Sebastião and Ribeiro, 1989), the effects of adenosine were observed in the presence of the 2 μM DPCPX (control: b_2/b_1 = 0.7914 ± 0.0133, S_2/S_1 = 0.8742 ± 0.0222, n = 22, 2μM DPCPX: b_2/b_1 = 0.8281 ± 0.0196, S_2/S_1 = 0.8081 ± 0.0259, n = 22) or 10 μM DMPX (control: b_2/b_1 = 0.7914 ± 0.0133, S_2/S_1 =

Table 2. Effect of N^6-cyclopentyladenosine (CPA) on the electrically-evoked and basal outflow of tritium from the rat striatal slices preincubated with [^{3}H]choline

Drugs at S_2 (μM)	n	S_2/S_1	b_2/b_1
none	22	0.8742 ± 0.0222	0.7914 ± 0.0133
CPA			
1	11	0.6958 ± 0.0247***	0.8371 ± 0.0381
3	13	0.6741 ± 0.0286***	0.8608 ± 0.0684
10	9	0.6310 ± 0.0317***	0.7256 ± 0.0417
30	11	0.5804 ± 0.0441***	0.7885 ± 0.0357
100	7	0.6661 ± 0.0336***	0.7844 ± 0.0433

Legends are the same as in Table 1.

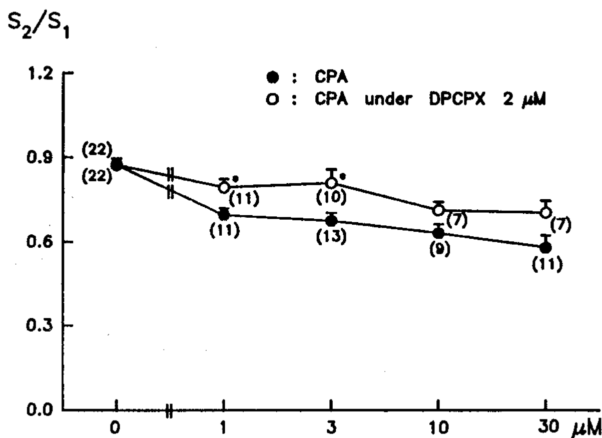

Fig. 4. Influence of DPCPX on the effect of N^6-cyclopentyladenosine (CPA) on the electrically-evoked tritium outflow from the rat striatum. Each point denotes mean ± SEM. In parentheses are the number of experiments. Other legends are the same as in Fig. 2.

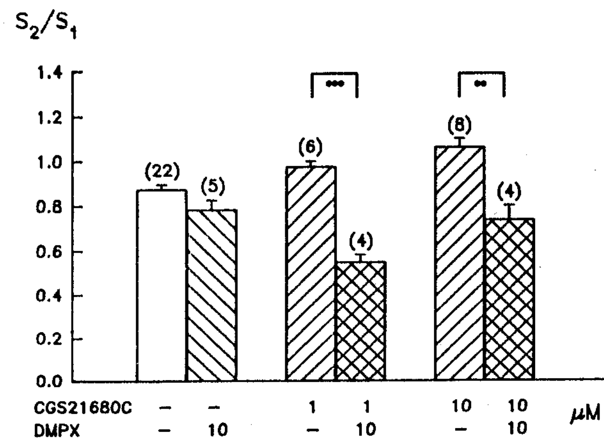

Fig. 5. Influence of DMPX on the effect of 2-p-(2-carboxyethyl)phenethylamino-5'-N- ethylcarboxamidoadenosine hydrochloride (CGS-21680C) on the electrically-evoked tritium outflow from the rat striatum. Both drugs were added to the medium 15 min before S_2. Other legends are the same as in Fig. 3.

0.8742 ± 0.0222, n = 22, 10 μM DMPX: b_2/b_1 = 0.8536 ± 0.0426, S_2/S_1 = 0.7835 ± 0.0427, n = 5). All drugs were added to the superfusion medium 15 min before S_2. Fig. 2 and Fig. 3 depicted the effects of adenosine on DPCPX- and DMPX-treated slices as compared with those of non-treated group. The decrements of tritium outflow were significantly inhibited by DPCPX (Fig. 2) and potentiated by DMPX (Fig. 3).

N^6-cyclopentyladenosine (CPA), a selective A_1 adenosine receptor agonist (Williams et al., 1986), in doses ranging from 1 to 100 μM, decreased the electrically-evoked [^{3}H]ACh release in a dose-dependent manner without changing the basal rate of release (Table 2). In addition, the effect of CPA was significantly inhibited by DPCPX (2 μM) treatment and the dose-response curve were shifted to the right (Fig. 4).

In order to study the role of A_2 adenosine receptor on ACh release in the rat striatum, the effects of 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarbo xamino-adenosine hydrochloride (CGS-21680C), a recently introduced potent A_2 adenosine receptor agonist (Hutchison et al., 1989), were examined. As shown in Table 3, CGS-21680C, in doses ranging from 0.01 to 10 μM, increased the electrically-evoked [^{3}H]ACh release in a dose-related fashion without

Table 3. Effect of 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcar- boxamidoadenosine hydrochloride (CGS-21680C) on the electrically-evoked and basal outflow of tritium from the rat striatal slices preincubated with [^{3}H]choline

Drugs at S_2 (μM)		n	S_2/S_1	b_2/b_1
	none	22	0.8742 ± 0.0222	0.7914 ± 0.0133
CGS-21680C				
	0.01	11	0.9317 ± 0.0456	0.7870 ± 0.0291
	0.1	6	0.9382 ± 0.0294	0.8440 ± 0.0343
	1.0	6	0.9741 ± 0.0252**	0.8768 ± 0.0400
	10.0	8	1.0599 ± 0.0400***	0.7302 ± 0.0536

Significant differences from the drug-free control (none) are marked with asterisks (**; p<0.01). Other legends are the same as in Table 1.

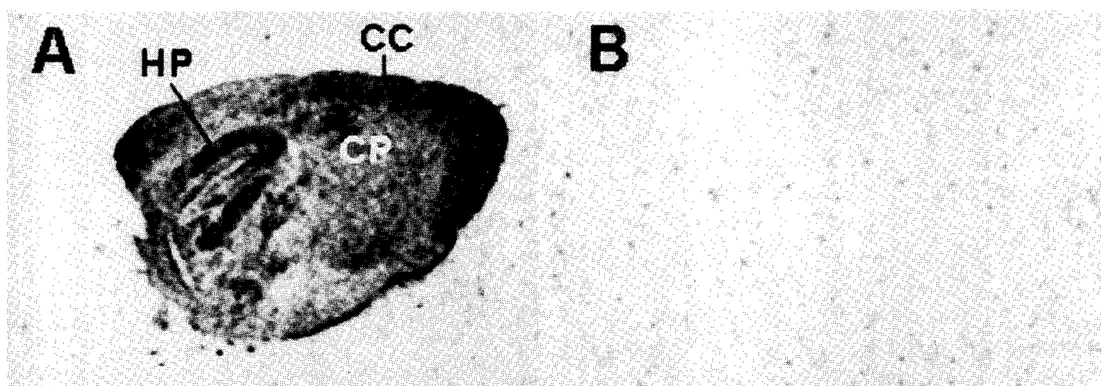

Fig. 6. Linearized autoradiographic image of specific 16 nM [^{3}H]2-chloro-N^6- cyclopentyladenosine ([^{3}H]CCPA) binding (A) to a thickness of 7 μm in sagittal section of the rat brain. Specific binding images were obtained by digital subtraction autoradiography where the image of non-specific binding for radioligand (B), determined from adjacent brain sections, were subtracted from the images of total binding. Non-specific binding was determined in the presence of 20 μM N-ethylcarbox-amidoadenosine (NECA). [^{3}H]CCPA binding was evaluated in four individual rats with the greatest density of binding (expressed in units of fmol/mg protein) found in the cerebral cortex (12.33±1.65), caudate-putamen (5.26±0.63) and hippocampus (12.86±1.36). Abbreviations: CC (cerebral cortex), CP (caudate- putamen), HP (hippocampus).

changing the basal rate of release. The increments of evoked [^{3}H]ACh release by CGS-21680C were completely inhibited by treatment with 10 μM DMPX (Fig. 5).

Autoradiographic Studies

[^{3}H]CGS-21680C and [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA) bound to rat brain sagittal section with specific binding amounting to approximately 95% of total binding, respectively (Fig. 6, 7). At a concentration of 16 nM the greatest density of [^{3}H]CGS-21680C binding was observed in the caudate-putamen (87.2±1.0 fmol/mg protein). Lower levels of [^{3}H]CGS-21680C binding were also observed in the olfactory tubercle (68.4±4.9 fmol/mg protein) and nucleus accumbens (40.8±6.7 fmol/mg protein). However, no significant amounts of specific [^{3}H]CGS-21680C binding were observed in any other region (Fig. 6).

The localization of A_2 adenosine receptors labelled with [^{3}H]CGS-21680C was markedly different from the regional distribution of A_1 adenosine receptor labelled with 1 nM [^{3}H]CCPA. [^{3}H]CCPA binding sites were highly concentrated in the cerebral cortex

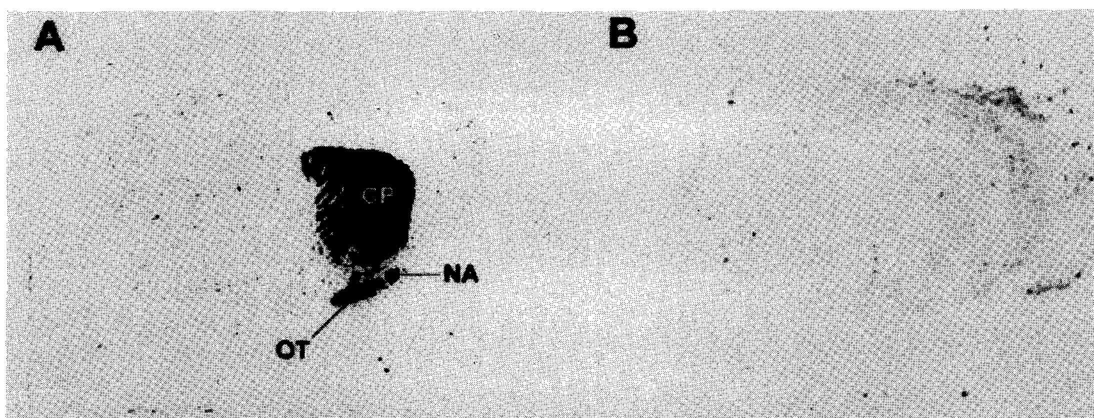

Fig. 7. Linearized autoradiographic image of specific 16 nM [^{3}H]CGS-21680C binding (A) to a thickness of 7 μm in sagittal section of the rat brain. Specific binding images were obtained by digital subtraction autoradiography where the image of non-specific binding for radioligand (B), determined from adjacent brain sections, were subtracted from the images of total binding. Non-specific binding was determined in the presence of 20 μM NECA. [^{3}H]CGS-21680C binding was evaluated in four individual rats with the greatest density of binding (expressed in units of fmol/mg protein) found in the caudate-putamen (87.2 $\pm$ 1.0), nucleus accumbens (40.8 $\pm$ 6.7) and olfactory tubercle (68.4 $\pm$ 4.9). Total [^{3}H]CGS-21680C binding in other brain regions did not significantly exceed non-specific binding levels. Abbreviations: CP (caudate-putamen), NA (nucleus accumbens) and OT (olfactory tubercle).

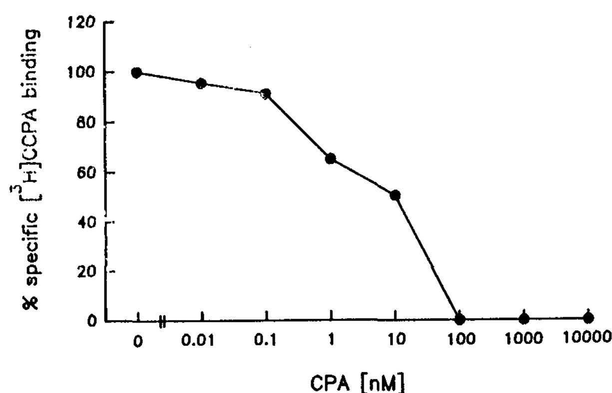

Fig. 8. Competition of cold CPA with [^{3}H]CCPA for binding to sagittal rat striatal sections. Binding was measured in the presence of increasing concentrations of competitive inhibitor (CPA). Non-specific binding was determined in the presence of 20 μM NECA.

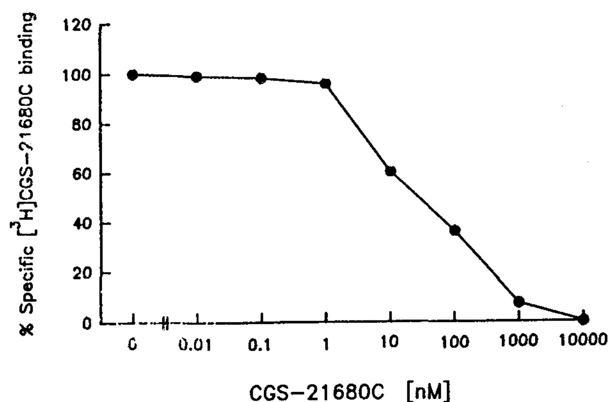

Fig. 9. Competition of cold CGS-21680C with [^{3}H] CGS-21680C for binding to sagittal rat striatal sections. Binding was measured in the presence of increasing concentrations of competitive inhibitor (CGS-21680C). Non-specific binding was determined in the presence of 20 μM NECA.

(12.33 $\pm$ 1.65 fmol/mg protein) and the hippocampus (12.86 $\pm$ 1.36 fmol/mg protein). But, lower levels of [^{3}H]CCPA binding were detected in the striatal region (5.26 $\pm$ 0.63 fmol/mg protein) (Fig. 7). In competition experiments, the effects of CPA on the binding of [^{3}H]CCPA to A_1 adenosine receptor and the effects of CGS-21680C on the binding of [^{3}H]CGS-21680C to A_2 adenosine receptor were shown in Fig. 8 and Fig. 9, respectively. K_i values for CPA were 27.35 nM against [^{3}H]CCPA binding and K_i values for CGS-21680C were 24.89 nM against [^{3}H]CGS-21680C binding.

Receptor Binding Characteristics of A1 and A2 Adenosine Ligands in Rat Striatal Membranes

After incubation for 120 min at 25℃, 1 nM [^{3}H]CCPA and 16 nM [^{3}H]CGS-21680C bound to rat striatal membranes with specific binding amounting to approximately 85 to 95% of total binding. In rat

Table 4. Comparison of the activity of several adenosine agonists and antagonists in inhibiting the binding of [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA) and [^{3}H]CGS-21680C to rat striatal membranes

Compound	K_i (nM)	
	A_1 Adenosine receptor [^{3}H]CCPA	A_2 Adenosine receptor [^{3}H]CGS-21680C
CGS-21680C	2609.2	47.6
CPA	1.6	2099.2
DMPX	19,386	ND
DPCPX	ND	19,207
NECA	12.9	44.9

K_i values were derived by the computer program LIGAND. Each value represented the mean of two or more independent experiment with varying SEM less than ±11%. All experiments were carried out using Tris-buffer (50 mM Tris HCl, 10 mM $MgCl_2$, pH adjusted to 7.4). ND: Not determined, CGS-21680C: 2-p-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride, CPA: N^6-cyclopentyladenosine, DMPX: 3,7-dimethyl-1-propargylxanthine, DPCPX: 8-cyclopentyl-1,3-dipropylxanthine, NECA: N- ethylcarboxamidoadenosine.

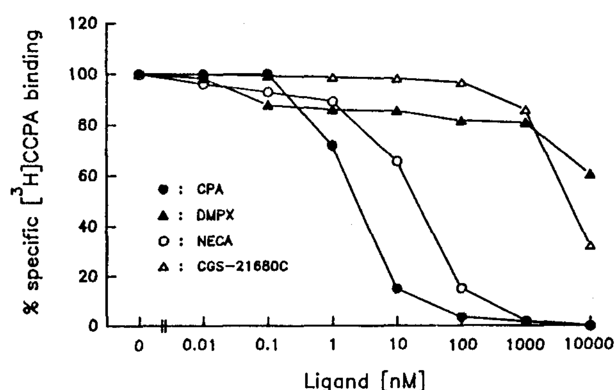

Fig. 10. Comparison of inhibitory potency of CPA, DMPX, NECA and CGS-21680C on the [^{3}H]CCPA binding in the presence of 3 μM DMPX as a blocking ligands for A_2 adenosine receptors in rat striatal membranes.

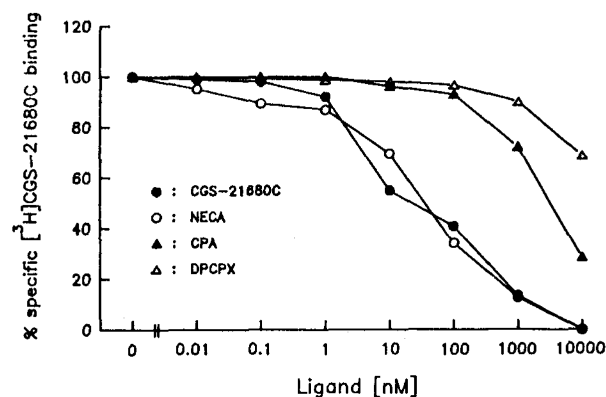

Fig. 11. Comparison of inhibitory potency of CPA, DPCPX, NECA and CGS-21680C on the [^{3}H]CGS-21680C binding in the presence of 2 μM DPCPX as a blocking ligands for A_1 adenosine receptors in rat striatal membranes.

striatal membrane preparations, when A_2 adenosine receptors were blocked by 3 μM DMPX, [^{3}H]CCPA binding was inhibited by CPA with a K_i value of 1.6 nM (B_{max} = 169 fmol/mg protein). Competitions by several agonists and antagonists for [^{3}H]CCPA binding were measured to confirm the [^{3}H]CCPA binds to an A_1 adenosine receptor. [^{3}H]CCPA binding was inhibited by NECA, but not by CGS-21680C and DMPX (Fig. 10, Table 4).

When A_1 adenosine receptors were blocked by 2 μM DPCPX, CGS-21680C and NECA inhibited the binding of the A_2 adenosine receptor-selective agonist [^{3}H]CGS-21680C to rat striatal membranes with a K_i value of 47.6 nM (B_{max} = 268 fmol/mg protein) and 44.9 nM (B_{max} = 297 fmol/mg protein), respectively. In contrast, binding of [^{3}H]CGS-21680C to A_2 adenosine receptors of rat striatal membrane was not inhibited by CPA and DPCPX (Fig. 11, Table 4).

DISSCUSSION

In the present study, the electrically evoked release of [^{3}H]ACh from the rat striatal slices was inhibited by adenosine or CPA. These results are in accordance with other reports that the N^6-cyclohexyladenosine (CHA) (Jin et al., 1993) and R-N^6-(2-phenyliso-

propyl)adenosine (R-PIA) (Kirkpatrick and Richardson, 1993; Kirk and Richardson, 1994) decrease the electrically evoked release of ACh in rat striatum. Moreover, DPCPX, a selective A_1 adenosine receptor antagonist, inhibited the effects of adenosine and CPA. These findings suggest that A_1 adenosine receptors play an important role in ACh release in rat striatum.

Barraco et al. (1991) have shown that the effect of A_2 adenosine receptor different from that of A_1 adenosine receptor in peripheral nervous system. Several researchers demonstrated that both A_1 and A_2 adenosine receptors were funtionally relevant in the striatum of the central nervous system (Kirkpatrick and Richrdson, 1993; Kirk and Richardson, 1994). Kirkpatrick and Richardson (1993) have reported that the A_1 adenosine receptor agonist R-PIA decreased but A_2 adenosine receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680C) increased the veratridine-evoked ACh release from rat striatal synaptosomes, and also, Kirk and Richardson (1994) have shown that the R-PIA decreased and CGS-21680C increased the KCl-evoked ACh release from rat striatal synaptosomes. On the other hand, Jin et al. (1993) have shown that the A_1 and A_2 adenosine receptor agonist activation caused a concentration-dependent inhibition of electrically-evoked ACh release from rat striatal slices, and they insisted that the any exitatory effect did not occur through A_1 and A_2 adenosine receptor in the rat striatum.

The present study showed that CGS-21680C increase the electrically-evoked ACh release from the rat striatal slices, and these effects are completely abolished by the A_2 adenosine receptor antagonist DMPX, but not affected by DPCPX. These results are in accordance with other reports (Kirkpatrick and Richrdson, 1994), indicating that the increasing effect of ACh release by CGS-21680C is mediated by A_2 adenosine receptor in rat striatum.

An alternative means of localizing the sites of synaptic action of putative neurotransmitters is to visualize their receptors at a microscopic level by autoradiography using the elegant techniques developed by Young and Kuhar (1979). It has been possible to label A_1 adenosine receptors with agonist such as [^{3}H]CHA (Bruns et al., 1980), [^{3}H]2-chloroadenosine (Williams and Risley, 1980), [^{3}H]R-PIA (Schwabe and Trost, 1980) and [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA) (Klotz et al., 1989). While the A_1 adenosine receptor has been extensively studied in both membrane homogenate and autoradiographic studies, characterization of the A_2 adenosine receptor has been limited due to the lack of available radioligand with high affinity and selectivity for this receptor subtype (Williams, 1987). In the presence of 50 nM CPA, which is utilized to block binding to the A_1 adenosine receptor, the binding of the non-selective agonist, [^{3}H]N-ethylcarboxamidoadenosine ([^{3}H]NECA), has been specifically localized in the rat striatum with such binding being pharmacologically consistent with the labelling of high affinity A_2 adenosine receptors (Bruns et al., 1986; Jarvis et al., 1989). Recently, the first highly A_2 adenosine receptor-selective agonist radioligand, [^{3}H]CGS-21680C, has been shown to specifically label the A_2 adenosine receptor without the need to block binding to A_1 adenosine receptors (Williams et al., 1989). In this study, quantitative receptor autoradiography and drug-receptor binding assay were performed in order to confirm the presence of A_1 and A_2 adenosine receptors and to examine the characteristics of A_1 and A_2 adenosine receptors by using [^{3}H]CCPA and [^{3}H]CGS-21680C in rat striatum.

In the autoradiography, a highly selective localization of [^{3}H]CCPA and [^{3}H]CGS-21680C recognition sites were observed in rat brain sagittal sections. These results are consistent with the previous autoradiographic localization of high affinity A_1 adenosine receptors obtained with [^{3}H]CHA (Goodman and Snyder, 1982) and A_2 adenosine receptors obtained with [^{3}H]NECA, in the presence of 50 nM CPA to block binding to the A_1 adenosine receptors (Jarvis, 1988; Jarvis et al., 1989) in the rat striatal region. However, there is a marked difference in the regional distributions of adenosine receptor subtypes in the rat brain, the high densities of A_1 adenosine receptors were detected in cerebral cortex, hippocampus and striatum, and the high densities of A_2 adenosine receptors were detected in the striatum, olfactory tubecle and nucleus accumbens in this study.

In the drug-receptor binding assay, [^{3}H]CCPA and [^{3}H]CGS-21680C were found to bind to rat striatal membranes with a pharmacological profile consistent with the specific labelling of A_1 and A_2 adenosine receptors, respectively. CPA was found to have approximately 1300-fold greater activity in inhibiting

the binding of [^{3}H]CCPA (K_i = 1.6 nM) to A_1 adenosine receptors as compared to its activity against [^{3}H]CGS-21680C binding (K_i = 2099.2 nM) to A_2 adenosine receptors. In contrast, CGS-21680C was found to have approximately 50-fold less activity at the A_1 adenosine receptors labelling with [^{3}H]CCPA (K_i = 2609.2 nM) as compared to its activity with [^{3}H]CGS-21680C (K_i = 47.6 nM) at the A_2 adenosine receptors. These findings maybe considered to be in line with the views of Lohse et al. (1988) and Jarvis et al. (1989) that CPA has higher binding activity against to [^{3}H]CCPA (K_i = 0.4 nM) than [^{3}H]CGS-21680C (K_i = 890 nM) and CGS-21680C has higher binding activity against to [^{3}H]CGS-21680C (K_i = 14 nM) than [^{3}H]CCPA (K_i = 3,900 nM), that both A_1- and A_2-adenosine receptors existed in the rat striatum.

Overall, the results of the present study suggested that the decrement of the evoked ACh release is mediated by A_1- and the increment of the evoked ACh release is mediated by A_2-adenosine heteroreceptor in the rat striatal cholinergic neurons. Conclusively, these facts unequivocally demonstrate a specific localization of adenosine receptors in the striatal region of the rat brain and provide further support for a specific neuromodulatory role of adenosine in basal ganglia function.

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