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Effect of adenosine and some of its structural analogues on the conductance of NMDA receptor channels in a subset of rat neostriatal neurones

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1 In order to investigate the modulatory effects of adenosine on excitatory amino acid projections onto striatal medium spiny neurones, whole-cell patch clamp experiments were carried out in rat brain slices. The effects of various agonists for P1 (adenosine) and P2 (ATP) purinoceptors and their antagonists were investigated. The A_{2A} receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS 21680; 0.1 μM), the A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA; 10 μM) and the non-selective P1 purinoceptor antagonist 8-(p-sulphophenyl)-theophylline (8-SPT; 100 μM) did not alter the resting membrane potential, the threshold current necessary to elicit an action potential, the amplitude of spikes, their rise time, the amplitude of the afterhyperpolarization (AHP) and the time to peak of the AHP.

2 N-methyl-D-aspartate (NMDA; 1–1000 μM) caused a concentration-dependent inward current which was larger in the absence than in the presence of Mg^{2+} (1.3 mM). In a subset of striatal neurones, the current response to NMDA (10 μM) and the accompanying increase in conductance were both inhibited by CGS 21680 (0.01–1 μM). The effect of CGS 21680 (0.1 μM) persisted in the presence of tetrodotoxin (0.5 μM) or in a Ca^{2+}-free medium, under conditions when synaptically mediated influences may be negligible.

3 The A_3 receptor agonist N^6-2-(4-aminophenyl)ethyladenosine (APNEA; 0.1–10 μM) also diminished the effect of NMDA (10 μM), while the A_1 receptor agonists CCPA (0.01–10 μM) and (2**S**)-N^6-[2-endo-norbornyl]adenosine [**S**(−)-ENBA; 10 μM] as well as the endogenous, non-selective P1 purinoceptor agonist adenosine (100 μM) were inactive. The endogenous non-selective P2 purinoceptor agonist ATP (1000 μM) also failed to alter the current response to NMDA (10 μM). Adenosine (100 μM), but not ATP (1000 μM) became inhibitory after blockade of nucleoside uptake by **S**(4-nitrobenzyl)-6-thioguanosine (NBTG; 30 μM).

4 8-(p-Sulphophenyl)-theophylline (8-SPT; 100 μM), as well as the A_{2A} receptor antagonist 8-(3-chlorostyryl)caffeine (CSC; 1 μM) and the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) at 0.03, but not 0.003 μM abolished the inhibitory action of CGS 21680 (0.1 μM). None of these compounds altered the effect of NMDA (10 μM) by itself. DPCPX (0.03 μM) prevented the inhibition by APNEA (10 μM).

5 There was no effect of CGS 21680 (0.1 μM), when guanosine 5′-*O*-(3-thiodiphosphate (GDP-β-S; 300 μM) was included in the pipette solution in order to block G protein-mediated reactions.

6 In conclusion, adenosine receptors, probably of the A_{2A}-subtype, inhibit the conductance of NMDA receptor channels in a subset of medium spiny neurones of the rat striatum by a transduction mechanism which involves a G protein.

Keywords: Rat striatum; medium spiny neurone; adenosine A_{2A} receptor; NMDA receptor channel; receptor interaction; whole-cell patch clamp

Introduction

Adenosine is a potent CNS depressant which induces sedation and inhibition of psychomotor functions (Williams, 1989). Extracellular adenosine concentrations in the brain may be elevated when the demand on cellular energy exceeds supply, e.g. when neuronal activity is increased and during hypoxic/hypoglycaemic states. Metabolic damage facilitates the intracellular formation of the nucleoside and subsequently its extrusion via the cell membrane into the extracellular space (Meghji, 1993). Under physiological conditions, the breakdown of the central (co)transmitter adenosine 5′-triphosphate (ATP; Edwards *et al*., 1992; Illes *et al*., 1996) may provide another possible source of extracellular adenosine.

Four adenosine receptor (P1 purinoceptors) have been cloned to date, and are referred to as the A_1, A_{2A}, A_{2B} and A_3 subtypes (Fredholm *et al*., 1994). In the brain, the occurrence of A_{2A} receptors is mostly restricted to the striatum (Jarvis & Williams, 1989). They are mainly expressed in the principal neuronal cell type of the striatum, the γ-aminobutyric acid (GABA)ergic medium-sized spiny projection neurones (Schiffmann *et al*., 1991; Schiffmann & Vanderhaeghen, 1993). The striatum receives massive glutamatergic inputs from the cerebral cortex and related structures (Parent, 1990) and also exhibits a high density of excitatory amino acid (EAA) binding sites (Albin *et al*., 1992). It is well established that activity in glutamatergic corticostriatal afferents induces excitation of the medium spiny neurones via postsynaptic N-methyl-D-aspartate (NMDA) and non-NMDA receptors (Calabresi *et al*., 1996). However, the effects of adenosine on these output neurones are incompletely characterized, although the activation of a K^+ outward current (Trussel & Jackson, 1985) and an A_{2A} receptor mediated inhibition of striatal GABA release which is thought to originate partly from recurrent axon collaterals of medium spiny neurones have been described (Kirk & Richardson, 1994).

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Findings at the whole animal level indicate a complex interplay between adenosine, EAA and in addition dopamine receptor-mediated mechanisms in the control of motor behaviour. A_{2A} (Ferré *et al*., 1992) and NMDA receptor agonists (Schmidt & Bury, 1988), as well as dopamine D_2 receptor antagonistic neuroleptic-drugs (Yoshida *et al*., 1991) have been shown to induce catalepsy. D_2 receptor antagonists produce, in addition, a pronounced upregulation of A_{2A} receptor density in the caudate-putamen (Parsons *et al*., 1995). On the other hand, NMDA receptor antagonists which relieve experimentally-induced akinetic symptoms (Yoshida *et al*., 1991), also reduce the basal adenosine outflow in the striatum (Pazzagli *et al*., 1995). Hence, by applying whole-cell patch clamp methods to medium spiny neurones in a rat striatal brain slice preparation, we have investigated whether adenosine and its structural analogues have modulatory influences on NMDA receptor-mediated neurotransmission. A preliminary account of some of the results has appeared in abstract form (Nörenberg *et al*., 1996).

Methods

Brain slice preparation

Young Wistar rats (5–27 days old) were decapitated and their brains were quickly removed and submerged in ice cold artificial cerebrospinal fluid (ACSF) saturated with 95% O_2 and 5% CO_2 of the following composition (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $CaCl_2$ 2.4, $MgCl_2$ 1.3, $NaHCO_3$ 26 and glucose 10; pH 7.4. Thin coronal slices from hemisected forebrains, 150–200 μm in thickness, containing parts of the neocortex-corpus callosum and the neostriatum were prepared by a Lancer vibratome. After being sectioned, 3–5 slices obtained from a single brain were transferred to a holding chamber and stored in oxygenated ACSF at 36°C for at least 1 h before use. Then, single slices were continuously superfused (3 ml min^{-1}) with oxygenated ACSF at room temperature (23–25°C) in a recording bath (300–400 μl volume). The bath solution differed from ACSF used for incubation, in that Mg^{2+} was omitted in order to augment NMDA-induced responses. The slices were left to recover for at least 15 min before the start of individual experiments in Mg^{2+}-free ACSF. In some experiments a Ca^{2+}-free medium (no added Ca^{2+}) was used.

Tight seal whole-cell recording

To record membrane currents and membrane potentials of medium spiny neurones in striatal brain slices, procedures similar to those described previously were used (Edwards *et al*., 1989). Experiments were performed and cells were visualized directly with an upright differential interference contrast microscope with a ×40 water immersion objective (Axioscope; Carl Zeiss, Oberkochen, Germany). Patch pipettes, produced from borosilicate glass capillaries (Hilgenberg; Malsfeld, Germany) having an outer diameter of 2 mm, were filled with intracellular solution of the following composition (mM): potassium gluconate 140, NaCl 10, $MgCl_2$ 1, *N*-2-hydroxyethylpiperazine-*N*′-ethane-sulphonic acid (HEPES) 10, ethylene glycol-*bis*-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) 11, MgATP 1.5 and GTP 0.3; pH 7.3 adjusted with KOH. In some experiments guanosine 5′-*O*-(β-thiodiphosphate) (GDP-β-S, 0.3 mM) was used instead of GTP. No cleaning procedure (Edwards *et al*., 1989) was necessary to achieve seals having a resistance of 4–7 GΩ. The liquid junction potential (V_{LJ}) between the bath- and pipette solution measured according to the method of Neher (1992) was 13.7 ± 0.1 mV ($n = 6$). Therefore, all membrane potential values given in this study are corrected for V_{LJ} and cells were voltage clamped at a holding potential of −80 mV taking into account this potential.

After whole cell access had been achieved, the system was left for 5–10 min to allow for the settling of a diffusion equilibrium between the patch pipette and the cell interior. Then, whole-cell recordings of about 30 min in duration could be routinely achieved. However, in some cases longer lasting experiments (1–2 h) were also obtained. The membrane properties of the cells were stable throughout. When the input resistance was measured at the beginning (5–10 min after gaining whole-cell access) and at the end of the experiments (25–30 min later) no differences were found (1039.7 ± 92.5 MΩ and 1013.8 ± 93.4 MΩ, respectively; $n = 23$).

Pipette resistances were in the range of 3 to 7 MΩ, when filled with the intracellular solution, giving rise in the whole-cell configuration to a series resistance (Rs) of 27.5 ± 3.2 MΩ associated with a membrane time constant of 646.5 ± 62.6 μs ($n = 36$), as calculated from the capacitance compensation settings of the patch amplifier (EPC-7; List Electronic, Darmstadt, Germany). In neurones contained in brain slices, current analysis is complicated by extensive dendritic arborization, which is likely to escape the somatic voltage clamp. These space clamp limitations may be aggravated by Rs-related problems as shown, for example, with fast synaptic currents, the amplitude of which was markedly underestimated in the absence of Rs-compensation (Llano *et al*., 1991). In our experiments, Rs compensation values ranging from 60 to 75% could be achieved, thereby minimizing Rs to a residual value of 10.5 ± 0.9 MΩ ($n = 36$). Under these conditions, NMDA (10 μM)-induced inward currents (177.8 ± 22.6 pA; $n = 36$) had amplitudes similar to those measured in another cell population without Rs compensation (173.4 ± 23.9 pA; $n = 36$). Therefore, and to avoid the risks of accidental overcompensation, this procedure was not generally used. However, the occurrence of action currents during the application of NMDA was taken as an indication for improper space clamp and these recordings were discarded from data evaluation.

Data were filtered at 3–10 kHz with the inbuilt filter of the EPC-7, digitized at 0.15–3 kHz (Model 1401, Cambridge Electronic Devices, Cambridge, U.K.) and stored on a laboratory computer. Data analysis was performed by use of commercially available patch and voltage clamp software (Cambridge Electronic Design, Cambridge, U.K.).

Selection of cells

The principal cell type of the striatum is the GABAergic medium spiny projection neurone (90–95% of the total population; Bishop *et al*., 1982). These cells are characterized by a small soma (about 13 μm diameter), a resting membrane potential negative to −60 mV and under *in vitro* conditions, a lack of spontaneous activity (Calabresi *et al*., 1987). The residual cell population consists of at least 4 types of interneurones which can be differentiated by morphological, immunohistochemical and electrophysiological criteria (Kawaguchi, 1993; Kawaguchi *et al*., 1995). Only cells having a diameter smaller than 14 μm and a membrane potential more negative than −60 mV were included in this study. All cells were neurones as judged from the firing of action potentials during the establishment of the gigaseal. This was probably due to mechanical irritation, since after whole-cell access had been achieved no spontaneous activity was seen. Furthermore, at the end of the experiments action currents could be elicited by depolarizing voltage jumps. Hence, the vast majority of the patched cells might belong to the medium-sized spiny type.

Application of drugs

Different drugs were applied by changing the superfusion medium by means of three-way taps according to protocols indicated in the text. At the constant flow rate of 3 ml min^{-1} about 20 s were required until the drugs reached the bath.

P1 purinoceptor antagonists (except in experiments when the membrane properties and the neuronal excitability were measured; see above) or compounds interfering with the cellular uptake of adenosine were present in the bath solution 15 min before the start of and throughout the experiments.

Materials

The following drugs were used: N-methyl-D-aspartate sodium salt (NMDA), adenosine disodium salt, guanosine 5′-triphosphate lithium salt (GTP), guanosine 5′-*O*-(3-thiodiphosphate) (GDP-β-S), adenosine 5′-triphosphate magnesium salt (MgATP), adenosine 5′-triphosphate disodium salt (ATP) (Sigma, Deisenhofen, Germany); N^6 - 2 - (4 - aminophenyl)-ethyladenosine (APNEA), 2-chloro-N^6-cyclopentyladenosine (CCPA), 2 - p - (2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680), 8-(p-sulphophenyl) - theophylline (8 - SPT), 8 - (3 - chlorostyryl)caffeine (CSC), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), (2**S**)-N^6-[2-endo-norbornyl]adenosine [**S**(−)-ENBA], **S**(4-nitrobenzyl)-6-thioguanosine (NBTG) (RBI, Natick, MA, U.S.A.).

Stock solutions (1–100 mM) of drugs were prepared with distilled water or dimethyl sulphoxide (APNEA, CSC) and aliquots were stored at −20°C. Further dilutions were made daily with the extra- or intracellular solution as appropriate. Equivalent quantities of the solvent had no effect.

Data evaluations and statistics

NMDA was applied at first alone (T_1) and 10 min later in the presence of various P1 purinoceptor agonists (T_2). Since amplitudes of NMDA-induced currents showed great variability (20–1000 pA for 10 μM NMDA), the effects of these agonists at T_2 were expressed as percentage inhibition of the NMDA-induced currents measured at T_1. Mean values ±s.e.mean of n experiments are given throughout. Kruskal-Wallis ANOVA on ranks followed by the Mann-Whitney test was used for comparisons of the means and for comparison of the means with zero. For multiple comparisons between independent values. Kruskal-Wallis ANOVA followed by a t test with Bonferroni's correction was employed instead. In the case of repeated measurements on the same preparations Friedman repeated measures ANOVA on ranks followed by a Wilcoxon-signed rank test was used.

Results

Membrane properties of striatal medium spiny neurones

The membrane potential measured in 346 cells, having a diameter of 12.6±0.1 μm, was −69.9±0.5 mV. From 33 cells investigated under current clamp for a longer period (up to 20 min), none showed spontaneous activity. However, current injection (500 ms pulses) elicited de- and hyperpolarizing electrotonic potentials, depending on current polarity (Figure 1a). Action potentials could be triggered by depolarizing currents of sufficient strength (Figure 1a). In 27 cells the threshold current for a single action potential was 13.4±1.8 pA. Spikes triggered by these current pulses were preceded by depolarizing prepotentials and followed by hyperpolarizing afterpotentials (Figure 1a). The amplitude of the fast spikes (rise time from the onset of upstroke to peak 1.8±0.1 ms) was 81.3±2.5 mV with an 11.5±1.8 mV overshoot. Current pulses of stronger intensity triggered trains of action potentials showing little accommodation during current injection (inset in Figure 1a). The time to peak of afterhyperpolarizations (AHPs) having an amplitude of −14.0±1.4 mV was 36.9±3.4 ms (both measured relative to the onset of the action potential).

Lack of effect of P1 purinoceptor agonists and antagonists on passive membrane properties and excitability of medium spiny neurones

A possible purinergic influence on passive membrane properties of medium spiny neurones was tested by measuring the membrane potential under current clamp conditions. No change in resting membrane potential was observed with time (Figure 1b). The same was true after 6 min of exposure to the A_{2A} receptor selective agonist 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS 21680; 0.1 μM), the A_1 receptor selective agonist 2-chloro-N^6-cyclopentyladenosine (CCPA; 10 μM) and the non-selective P1 purinoceptor antagonist 8-(p-sulphophenyl)-theophylline (8-SPT; 100 μM), respectively (Figure 1b).

In addition, a possible effect of P1 purinoceptor ligands on the excitability of medium spiny neurones was investigated by means of current injection (500 ms pulses), in order to elicit action potentials (Figure 1a). When compared with drug-free controls (n=7), no change was observed after 6 min of superfusion with either CGS 21680 (0.1 μM; n=7), CCPA (10 μM; n=7) or 8-SPT (100 μM; n=6) in the threshold current necessary to elicit a single action potential, in the amplitude and rise time of spikes, as well as in the amplitude and rise time of AHPs (not shown). Hence, it seems unlikely that adenosine receptor-mediated effects are involved in the control of vol-

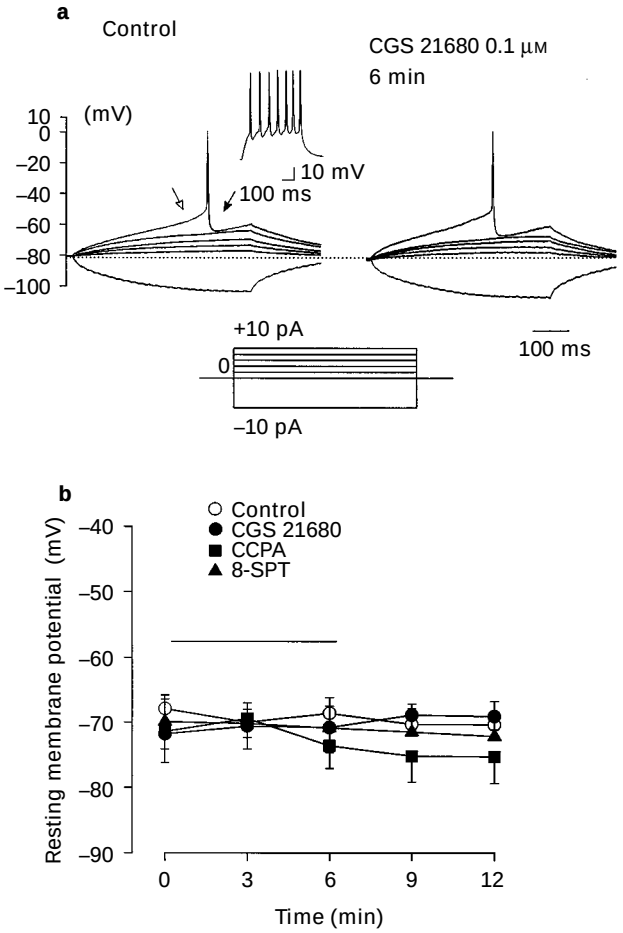

Figure 1 Membrane properties of medium spiny neurones of the rat and lack of effect of P_1 purinoceptor ligands on the resting membrane potential. (a) Electrotonic potentials (upper panel) recorded in a cell which had a resting membrane potential of −80 mV. Current pulses, 500 ms in duration (lower panel) were applied every 10 s in 2 pA increments. Step range was from −10 to +10 pA. Depending on current polarity, de- or hyperpolarizing electrotonic potentials were evoked. Injection of a +10 pA current gave rise to a single action potential. Spikes were preceded by depolarizing prepotentials (open arrow) and followed by afterhyperpolarizations (solid arrow). Injection of currents with stronger intensity (+20 pA in this case), induced trains of action potentials, showing no accommodation during the pulse (inset). A 6 min superfusion period with CGS 21680 (0.1 μM) had no effect on the spiking behaviour of the cells. (b) The resting membrane potential of medium spiny neurones, was measured every 3 min over a 12 min recording period. No changes were observed when after the first measurement drug-free ACSF (control, n=7), or medium containing CGS 21680 (0.1 μM; n=7), CCPA (10 μM; n=7) and 8-SPT (100 μM; n=6) were present for 6 min (horizontal bar).

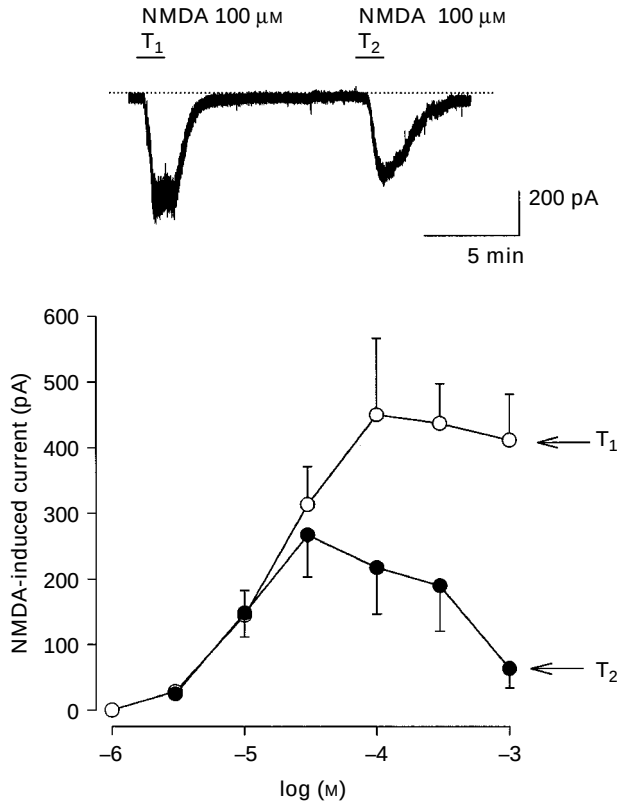

Figure 2 Concentration-response relationship for NMDA-induced inward currents in medium spiny neurones elicited at a holding potential of −80 mV. In every neurone the same concentration of NMDA was superfused twice for 1.5 min (T_1, T_2), with 10 min intervals (example given in the inset for 100 μM NMDA; the dotted line in this and all subsequent recordings, denotes the zero current level). Experiments were carried out in Mg^{2+}-free ACSF. Data points represent means of 5–9 cells; vertical lines show s.e.mean.

tage-dependent channels contributing either to the resting membrane potential or to the excitability of striatal medium spiny neurones.

Effects of N-methyl-D-aspartate (NMDA) on striatal medium spiny neurones

The effects of NMDA on medium spiny neurones were investigated in the concentration range of 1–1000 μM. All the following experiments were made in a Mg^{2+}-free medium except when the amplitude of the NMDA-induced current was compared in the presence and absence of external Mg^{2+}. When superfused for 1.5 min at a holding potential of −80 mV, NMDA activated inward currents in striatal neurones (Figure 2). The lowest effective concentration was 3 μM and the maximum response was achieved with 100 μM NMDA. Fitting a Hill equation ($I/I_{max} = 1/\{1+(K_D/[NMDA])^{n_H}\}$) to the data normalized with respect to the maximum response at 100 μM, revealed an EC_{50} value of 16.5±1.2 μM with a Hill coefficient of 1.6 ($n=41$). Under current-clamp conditions 10 μM NMDA gave rise to a membrane depolarization of 34.0±5.5 mV accompanied by bursts of action potentials ($n=6$; not shown).

In a single series of experiments, the effect of Mg^{2+} was investigated on the NMDA-induced current and was found to be inhibitory. NMDA (30 μM) evoked an inward current amplitude of 313.6±57.5 pA in Mg^{2+}-free and of 61.4±33.2 pA in Mg^{2+} (1.3 mM)-containing medium, respectively ($n=5$, each; $P<0.05$).

In order to investigate the reproducibility of NMDA-induced responses, single concentrations were applied twice for 1.5 min (T_1, T_2), being separated by a superfusion period of 10 min with drug-free ACSF. Under these conditions, NMDA-evoked current amplitudes of stable size were recorded up to a concentration of 10 μM (145.0±37.8 pA at T_1 and 148.3±36.4 pA at T_2; $n=7$). However, a pronounced reduction of the second current was found at higher NMDA concentrations (30 μM–1 mM) (Figure 2).

Effect of CGS 21680 on NMDA-induced current and membrane conductance change in a population of striatal neurones

Possible actions of purinoceptor agonists on NMDA-induced inward currents were evaluated by the following protocol: NMDA (10 μM) was superfused twice for 1.5 min (T_1, T_2) to the same cell with a drug-free interval of 10 min. NMDA-evoked currents were again stable (Figure 4a and c). However, when the A_{2A}-receptor selective agonist CGS 21680 (0.1 μM) was present in the bath 5 min before and during the second application of NMDA (10 μM; T_2), a pronounced inhibition was observed.

In the present study, the A_{2A} receptor agonist CGS 21680 (0.1 μM) was applied to a total population of 69 cells. In another 36 cells NMDA (10 μM) was applied twice (T_1, T_2) in the absence of this compound. In these latter experiments, the second NMDA-evoked current amplitude did not change with respect to the first (−1.3±4.9% decrease; $P>0.05$). When a level of at least 15% inhibition (3 times s.e.mean of the control experiments) was selected as a significant effect, 54 out of 69 neurones (78%) reacted to CGS 21680 (0.1 μM) with depression of the NMDA-induced inward currents (45.5±2.7% inhibition; $P<0.05$), whereas 15 (22%) did not (−3.2±1.9% inhibition; $P>0.05$).

In a series of experiments, the input resistance of 12 striatal medium spiny neurones was monitored by applying hyperpolarizing voltage steps (10–20 mV, 100 ms, every 10 s) in the presence of NMDA (10 μM) alone or together with CGS 21680 (0.1 μM; Figure 3). The experimental protocol was the same as used previously. Mean input resistance values were calculated from current amplitudes evoked by 3 hyperpolarizing voltage steps at the following time points: immediately before the first application of NMDA (R_1), during the peak of this response (R_2 at T_1), immediately before the second application of NMDA in the presence of CGS 21680 (R_3), and during the peak of this response (R_4 at T_2; Figure 3a). In 8 out of 12 cells the CGS 21680 (0.1 μM)-induced inhibition of the second NMDA (10 μM)-current at T_2 was 44.8±7.1% ($n=8$). The input resistance of these cells was similar, either in the absence (R_1) or presence (R_3) of CGS 21680 (0.1 μM; Figure 3b). Due to the opening of NMDA receptor channels the input resistance decreased dramatically in the presence of NMDA (10 μM). However, this decrease was clearly less pronounced upon coapplication of NMDA (10 μM) and CGS 21680 (0.1 μM) than during the application of NMDA (10 μM) alone (Figure 3a,b).

In the residual 4 cells out of the total population of 12, CGS 21680 (0.1 μM) did not alter the NMDA (10 μM)-induced current (12.2±10.2% inhibition at T_2; $P>0.05$). The input resistance values in the absence of NMDA were again similar, irrespective of whether CGS 21680 (0.1 μM) was present or not (Figure 3c). In addition, these values were in the same range as in CGS 21680-sensitive cells. However, in contrast to the CGS 21680-sensitive cell population, the conductance of NMDA receptor channels was not influenced by the A_{2A} receptor agonist. This is indicated by the fact that the decrease in membrane resistance was the same irrespective of whether NMDA (10 μM) alone or NMDA plus CGS 21680 (0.1 μM) were present in the bath (Figure 3c).

This differential sensitivity of striatal neurones may be attributed to the different neuronal cell types present in the striatum (medium spiny projection neurones and at least 4 types of interneurones). However, comparison of cell size, resting membrane potential and input resistance of the neurones revealed no obvious differences between the responsive

and non-responsive cell population. In consequence, non-responsive cells were not included in the calculation of means (current amplitude or its inhibition at T_2) given in this or in subsequent paragraphs.

Effect of P1 and P2 purinoceptor agonists on NMDA-induced currents

The inhibition of the current response to NMDA (10 μM) at T_2 depended on the concentration of CGS 21680 (0.01–1 μM; Figure 4c). The A_3 receptor agonist N^6-2-(4-aminophenyl)-ethyladenosine (APNEA) acted in a similar manner to CGS 21680. However, it required at least ten fold higher concentrations for comparable effects (Figure 4c). In contrast to CGS 21680 and APNEA, the A_1 receptor selective agonists CCPA (0.01–10 μM) and (2**S**)-N^6-[2-endo-norbornyl]adenosine [S(−)-ENBA; 10 μM] were ineffective (Figure 4c). Finally, we searched for possible effects of two putative endogenous ligands at striatal purinoceptors. However, neither adenosine (P1 purinoceptors) nor adenosine 5′-triphosphate (ATP; P2 purinoceptors) in the concentrations of 100 and 1000 μM, respectively, showed any inhibitory action on NMDA-induced currents (Figure 4c).

Direct or indirect effect of CGS 21680

In order to elucidate whether the inhibitory action of CGS 21680 was attributable to a direct or indirect (synaptically mediated) action on striatal neurones, experiments with CGS 21680 (0.1 μM) similar to those described above were carried out in Mg^{2+}-free medium, either containing tetrodotoxin (TTX; 0.5 μM) or no added Ca^{2+}. The necessity of doing this may be underlined by frequent observations of spontaneous synaptic currents which, due to the slow sampling frequency (0.15–0.5 kHz), were only occasionally resolved in the recordings shown. An example of such a synaptic event is given at the end of trace b in Figure 4b. The omission of Ca^{2+} or the application of TTX had no major effect on the amplitude of NMDA (10 μM)-induced inward currents as judged from amplitudes at T_1 which were 200.0 ± 35.3 pA ($n=11$), 203.3 ± 62.5 pA ($n=8$), and 190.1 ± 53.2 pA ($n=7$) under standard conditions (Ca^{2+} 2.4 mM), when Ca^{2+} was omitted and when Ca^{2+} and TTX (0.5 μM) were both present. In all cases, CGS 21680 (0.1 μM) was able to inhibit significantly NMDA-evoked responses (Figure 4c, inset), although in a Ca^{2+}-free medium or in the presence of TTX synaptically mediated influences may be negligible. The percentage inhibi-

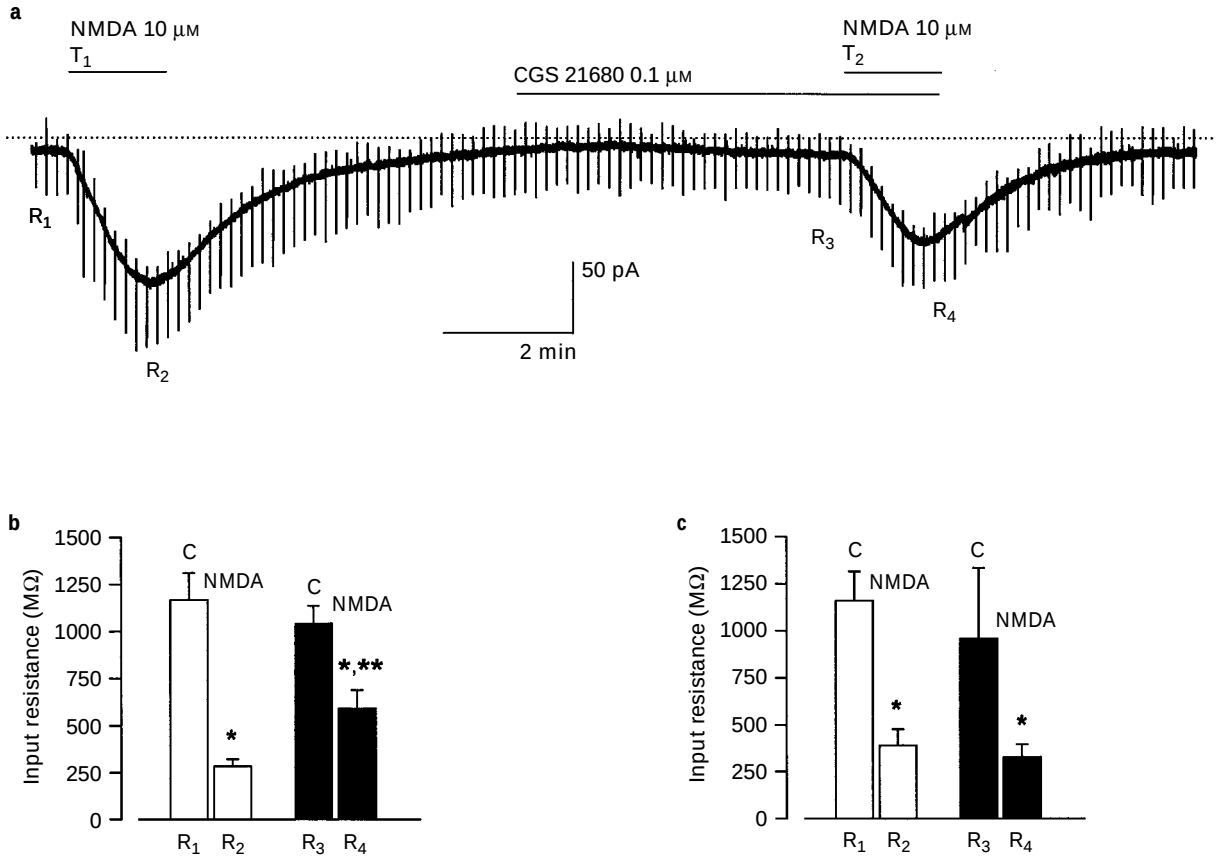

Figure 3 CGS 21680 inhibited the conductance of NMDA receptor channels. The holding potential was in this and all subsequent experiments −80 mV. (a) Experimental procedure to assess possible effects of CGS 21680 on the conductance of NMDA-activated channels. The input resistance of striatal medium spiny neurones was monitored by applying hyperpolarizing voltage steps, 10 to 20 mV in amplitude, and 100 ms in duration every 10 s. The input resistance was measured 4 times: immediately before the first application of NMDA (10 μM; R_1 before T_1), during the maximum response to NMDA (10 μM; R_2 during T_1), immediately before the second challenge with NMDA (10 μM) in the presence of CGS 21680 (0.1 μM; R_3 before T_2), and during the maximum response to NMDA (10 μM) in the presence of CGS 21680 (0.1 μM; R_4 during T_2). (b) Input resistance values in 8 neurones sensitive to CGS 21680. Means of 3 current responses at R_1, R_2, R_3 and R_4, respectively were obtained according to the scheme in (a), either in the absence (open columns) or in the presence of CGS 21680 (0.1 μM; solid columns). $^*P<0.001$, significant differences from the respective controls (c) in the absence of NMDA (R_2 compared with R_1 and R_4 compared with R_3, respectively); $^{**}P<0.05$, significant difference between NMDA alone and NMDA plus CGS 21680 (R_2 and R_4). (c) Input resistance in 4 medium spiny neurones which did not respond to CGS 21680. Here, the NMDA (10 μM)-evoked increase in membrane conductance was uninfluenced by CGS 21680 (0.1 μM; compare R_2 with R_4). $^*P<0.001$, significant differences from the respective controls in the absence of NMDA (10 μM).

tion was larger in a nominally Ca^{2+}-free medium than under standard conditions and was unaltered in the presence of TTX (0.5 μM) (Figure 4c, inset). Finally, CGS 21680 (0.1 μM) did not produce a current response of its own under any of these conditions.

Dependence of the adenosine and ATP effects on the blockade of nucleoside transporters

As already mentioned, adenosine (100 μM) by itself had no effect on NMDA (10 μM)-induced inward currents (Figure 5a and c). This may be due to the rapid enzymatic degradation of adenosine and/or to its effective clearance from the extracellular space by uptake mechanisms. Since enzymes involved in nucleoside metabolism (adenosine-deaminase and adenosine-kinase) are mainly located in the cytosol (Meghji, 1993), the significance of the cellular uptake may be more important. Hence, in the presence of the adenosine transport inhibitor **S**(4-nitrobenzyl)-6-thioguanosin (NBGT; 30 μM; added to the bathing medium 15 min before the start and throughout the experiments) adenosine (100 μM) inhibited the second NMDA (10 μM)-evoked current amplitude (T_2; Figure 5b and c). NBTG (30 μM) on its own, when it was superfused for 5 min before and during T_2, had no effect (Figure 5c). In contrast to adenosine (100 μM), ATP (1000 μM) did not became active in the presence of NBTG (30 μM).

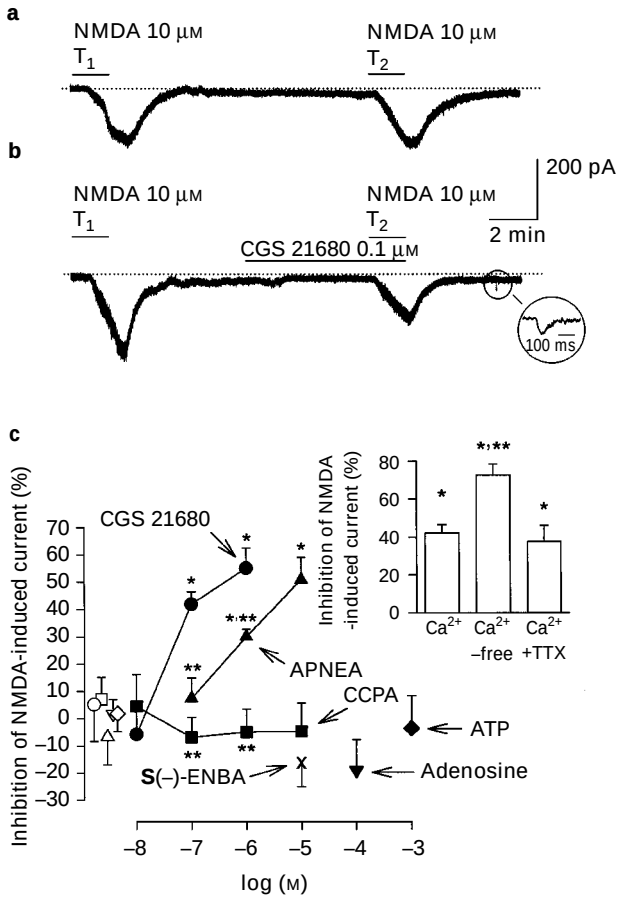

Figure 4 Effects of purinoceptor agonists on NMDA-induced currents. (a) NMDA (10 μM) was applied twice (T_1, T_2) for 1.5 min with a 10 min interval between applications. (b) Five minutes before and during the second application of NMDA (10 μM; T_2) CGS 21680 (0.1 μM) was added to the medium. An example of spontaneous synaptic currents is given at the end of the trace in (b). (c) Concentration-response relationships for inhibitory effects of purinoceptor agonists on NMDA (10 μM)-evoked currents. Data shown represent the decrease of the second NMDA-induced response (T_2) with respect to that obtained at T_1 and are expressed as percentage inhibition. Control experiments (open symbols) were performed according to the scheme shown in (a) in the absence of purinoceptor agonists. When the effects of purinoceptor agonists were tested on the NMDA-current, the experimental schedule was similar to that in (b). Means from $n=5-13$ experiments are shown. Both CGS 21680 (0.01 – 1 μM) and APNEA (0.1 – 10 μM) concentration-dependently inhibited inward currents elicited by NMDA (10 μM). CCPA (0.01 – 10 μM), **S**(−)-ENBA (10 μM), adenosine (100 μM) and ATP (1000 μM) were ineffective. $^*P<0.001$, significant differences from respective control experiments. $^{**}P<0.01$, significant differences from the effect of CGS 21680 at 0.1 and 1 μM, respectively. Inset: CGS 21680 (0.1 μM) inhibited the NMDA (10 μM-evoked current in a standard Ca^{2+} (2.4 mM)-containing medium ($n=7$), in the absence of added Ca^{2+} (Ca^{2+}-free; $n=6$) or in the presence of tetrodotoxin (TTX, 0.5 μM; $n=8$). $^*P<0.05-0.001$, significant differences from zero; $^{**}P<0.05$, significant difference from the effect of CGS 21680 in a standard medium.

Interactions between the A_{2A} receptor agonist CGS 21680 and P1 purinoceptor antagonists were investigated by the following

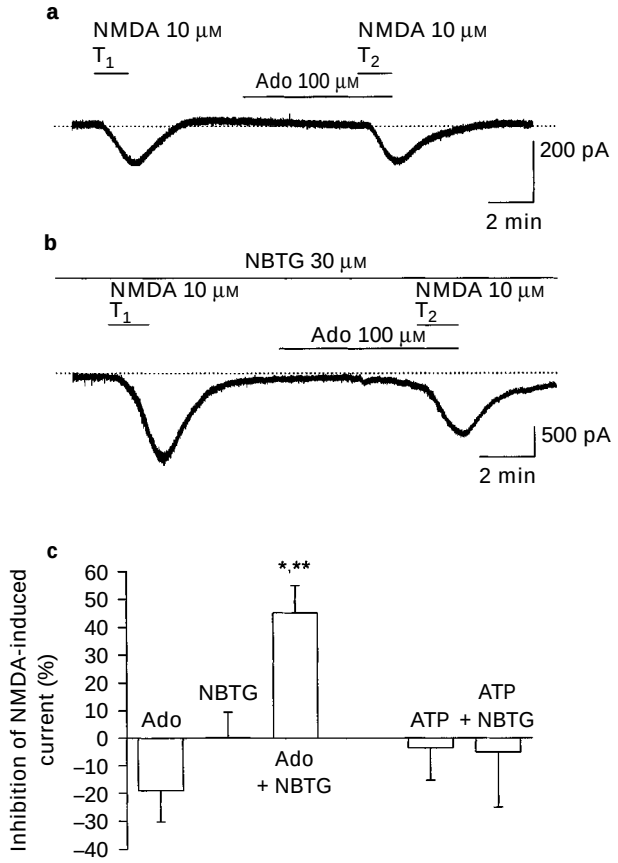

Figure 5 The lack of effect of adenosine, but not ATP is probably due to effective cellular uptake mechanisms for adenosine. (a) NMDA (10 μM) was applied twice for 1.5 min each and with a 10 min interval between applications. Adenosine (Ado, 100 μM) was superfused for 5 min before and during T_2. (b) Similar experiment as shown in (a), except that the adenosine uptake inhibitor **S**(4-nitrobenzyl)-6-thioguanosin (NBTG, 30 μM) was present in the bathing medium 15 min before the start and throughout the experiment. (c) Effects of adenosine (100 μM) alone (same protocol as in (a); $n=13$), NBTG (30 μM) alone (superfused 5 min before and during T_2; $n=7$), and adenosine (100 μM) plus NBTG (30 μM) (same protocol as in (b); $n=9$) on NMDA (10 μM)-induced currents. Effects of ATP (1000 μM) alone (same protocol as in (a); $n=7$) and ATP (1000 μM) plus NBTG (30 μM) (same protocol as in (b); $n=7$) on NMDA (10 μM)-induced currents. The decrease of the current amplitudes from T_1 to T_2 is expressed as percentage inhibition. $^*P<0.05$, significant difference from zero; $^{**}P<0.001$, significant difference from adenosine alone.

method: NMDA (10 μM) was superfused 2–3 times for 1.5 min (T_1-T_2 or T_3). CGS 21680 (0.1 μM) was present in the bathing medium for 5 min before and during T_2 (Figure 6a). Alternatively, similar experiments were carried out under conditions where the non-selective P1 purinoceptor antagonist 8-(p-sulphophenyl)-theophylline (8-SPT; 100 μM), the A_{2A} receptor selective antagonist 8-(3-chlorostyryl)caffeine (CSC; 1 μM) or the A_1-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.003 and 0.03 μM), were present at least 15 min before the start and throughout the experiment (Figure 6b). In a first series of experiments, we tested whether the effect of CGS 21680 (0.1 μM) recovered on washout (Figure 6a). In 11 control experiments CGS 21680 (0.1 μM) depressed the second current amplitude (T_2) by $38.3\pm6.0\%$ (Figure 6c). In 6 out of these 11 cells it was possible to apply NMDA (10 μM) for a third time after a 10 min washout of CGS 21680 (T_3, example given in Figure 6a). It is noteworthy that even after removal of the A_{2A} agonist, NMDA (10 μM)-induced currents remained depressed ($49.8\pm10.7\%$ inhibition at T_3 with respect to T_1; $n=6$; $P<0.05$). However, this was not due to a desensitization of NMDA receptor channels, since in experiments, where NMDA (10 μM) alone could also be applied for a third time, no such depression was observed ($-10.4\pm19.7\%$ inhibition at T_3 with respect to T_1; $n=7$; $P>0.05$).

P1 purinoceptor antagonists had no effects of their own on the current amplitudes elicited by NMDA (10 μM) as judged from current amplitudes at T_1. These were 200.0 ± 35.3 pA ($n=11$), 165.0 ± 30.1 pA ($n=7$; $P>0.05$), 167.5 ± 40.9 pA ($n=9$; $P>0.05$), 220.4 ± 27.3 pA ($n=9$; $P>0.05$) and 233.8 ± 49.1 pA ($n=6$; $P>0.05$) either in the absence of antagonists, or in the presence of 8-SPT (100 μM), CSC (1 μM) and DPCPX (0.003 and 0.03 μM), respectively. However, the inhibitory effect of CGS 21680 (0.1 μM) was abolished by 8-SPT (100 μM), CSC (1 μM) and the higher concentration of DPCPX (0.03 μM) (Figure 6c). The lower concentration of DPCPX (0.003 μM) did not antagonize the effect of CGS 21680 (0.1 μM). In addition, DPCPX (0.03 μM) abolished the inhibitory effect of APNEA (10 μM; inset in Figure 6c). Difficulties in evaluating results obtained in the presence of P1 purinoceptor antagonists may arise from the fact that, under these conditions, it was no longer possible to exclude from the total neuronal population cells not responding to CGS 21680 (about 20% under control conditions). Hence, an underestimation of P1 purinoceptor agonist potency in the presence of antagonists due to the inclusion of non-responsive cells into the evaluation is possible. However, it is most unlikely that only CGS 21680 (and APNEA)-insensitive cells were investigated when the antagonism by 8-SPT, CSC and DPCPX was tested.

Signal transduction mechanism of the P1 purinoceptor responsible for the inhibition of NMDA-evoked currents

Receptors for adenosine belong to the G protein-coupled superfamily (reviewed by Fredholm *et al*., 1994). In order to verify the likely involvement of G proteins in the inhibitory action of CGS 21680, guanosine-5′-triphosphate (GTP, 300 μM), being a constituent of the standard intracellular solution, was replaced by guanosine-5′-O-(2-thiodiphosphate) (GDP-β-S, 300 μM). This replacement had no consequence for the amplitudes of NMDA (10 μM)-induced currents at T_1 (131.0 ± 28.5 pA with GDP-β-S (300 μM) and 128.2 ± 27.1 pA with GTP (300 μM; $n=6$ each; $P>0.05$). However, the CGS 21680 (0.1 μM)-induced inhibition of the second NMDA (10 μM)-current (T_2) found under standard conditions ($57.6\pm6.5\%$; $n=6$) was no longer detectable in cells internally microperfused with GDP-β-S (300 μM) ($3.8\pm5.7\%$; $n=6$; $P<0.05$).

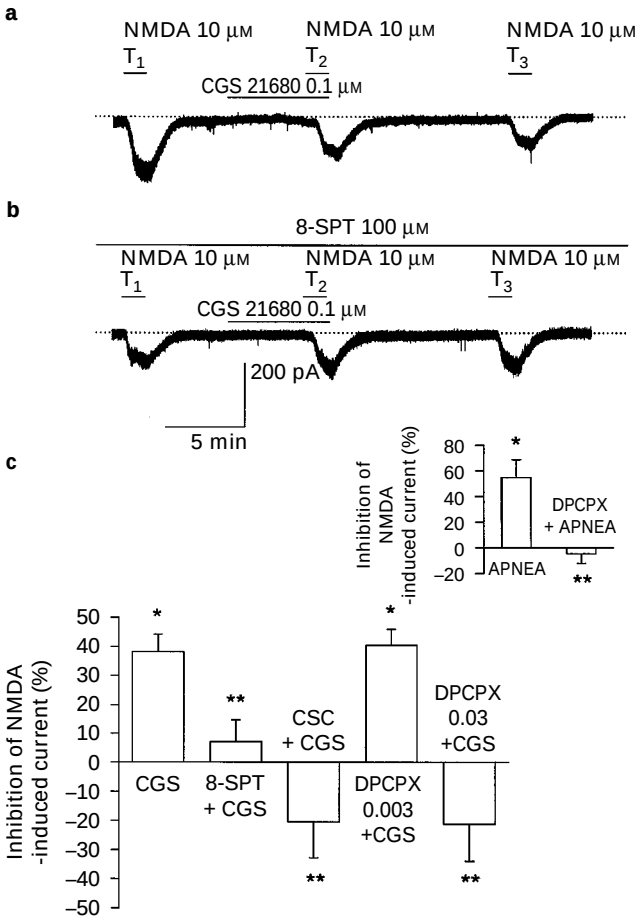

Figure 6 Antagonism of the inhibitory action of CGS 21680 on NMDA-induced currents by P1 purinoceptor antagonists. (a) NMDA (10 μM) was applied three times (T_1-T_3) for 1.5 min each and with 10 min intervals between applications. CGS 21680 (CGS; 0.1 μM) was present 5 min before and during the second administration (T_2) of NMDA (10 μM). The CGS 21680-induced depression of the NMDA response persisted, even after 10 min of washout (T_3). (b) Effects of P1 purinoceptor antagonists were investigated according to the protocol used in (a), except that antagonists (8-SPT; 100 μM in this case) were present at least 15 min before the start and throughout the experiments. (c) Inhibitory effects of CGS 21680 (0.1 μM) either alone ($n=11$) or in the presence of 8-SPT (100 μM; $n=7$), CSC (1 μM; $n=9$), and DPCPX (0.03 μM; $n=6$ or 0.003 μM; $n=7$). The effects of CGS 21680 (0.1 μM) on the NMDA (10 μM)-induced current are expressed as percentage inhibition of T_1 at T_2. The inset shows that DPCPX (0.03 μM) antagonized the effect of APNEA (10 μM; $n=7$ both for APNEA alone and APNEA plus DPCPX, respectively) as well. *$P<0.001$, significant differences from zero; **$P<0.05-0.001$, significant differences from the actions of CGS 21680 (0.1 μM) and APNEA (10 μM) in the absence of antagonists.

Discussion

This work provides evidence that the conductance through NMDA receptor-gated channels in medium spiny neurones of the rat striatum is decreased by adenosine and some of its structural analogues by acting at G protein-coupled adenosine receptors, probably of the A_{2A} subtype. The highest densities of adenosine A_{2A} binding sites in the rat brain are found in the striatum (Jarvis & Williams, 1989), although in this area lower densities of A_1, A_{2B} and A_3 sites were also observed (Bruns *et al*., 1986; Zhou *et al*., 1992; Johansson *et al*., 1993). However, neither the resting membrane potential nor various parameters of action potentials evoked by depolarizing current injection were changed by the A_{2A} receptor agonist CGS 21680 (Jarvis *et al*., 1989), the A_1 receptor agonist CCPA (Lohse *et al*., 1988) and the non-selective P1 purinoceptor antagonist 8-SPT (Fredholm *et al*., 1994). It is interesting to note that dopamine D_1 (Calabresi *et al*., 1987) and D_2 receptor agonists (the latter only after depletion of the endogenous pools of dopamine; Calabresi *et al*., 1988) both increased the firing threshold of rat

neostriatal neurones, although none of these compounds altered the resting membrane potential.

The observation that purinoceptor agonists lack effects on the resting membrane potential of striatal neurones contrasts with data from a previous study showing that adenosine hyperpolarizes striatal neurones cultured from the brain of embryonic mice, probably via the activation of an A_1 receptor (Trussel & Jackson, 1985). A number of reasons may be responsible for this discrepancy. Firstly, cultured cells showed spontaneous spiking, which is unusual for medium spiny neurones in brain slices (Calabresi *et al*., 1987); secondly, the properties of medium spiny neurones are known to change with age (Misgeld *et al*., 1986); and thirdly, striatal neurones of mice may possess another type of adenosine receptor different from those of rats. Hence, alterations in the transduction mechanism of the adenosine receptor may be due to tissue culture conditions, developmental influences or species differences.

The A_{2A} receptor agonist CGS 21680 exerted a strong inhibitory effect on NMDA-induced inward currents in striatal neurones. APNEA, an agonist at A_3 receptors (Zhou *et al*., 1992), also attenuated NMDA-responses, but at 10 times higher concentrations than CGS 21680. The highly selective A_1 receptor agonists CCPA and **S**(−)-ENBA (Trivedi *et al*., 1989) as well as the P2 purinoceptor agonist ATP were inactive. The lowest effective concentration of CGS 21680 was 0.1 μM in the present study. This is rather high when compared with the affinity of CGS 21680 for A_{2A} sites in binding studies ($K_D = 16$ nM; Jarvis *et al*., 1989). Recently it was proposed that an additional adenosine receptor of the A_4 type may be present in the rat striatum; CGS 21680 appeared to exhibit a lower affinity for A_4 than to A_{2A} sites (Cornfield *et al*., 1992). However, A_4 and A_{2A} receptors turned out to be the same molecular entities (Luthin & Linden, 1995). Hence, the discrepancy between functional and binding data may be explained by a decrease in A_{2A} receptor affinity in the absence of Mg^{2+} (Parkinson & Fredholm, 1992).

Both 8-SPT (100 μM), which is known to be antagonistic at A_1 and A_2 (Fredholm *et al*., 1994) but not at A_3 receptors (Fozard & Carruthers, 1993), and CSC, used in a concentration (1 μM) which is selective for A_{2A} receptors (Jacobson *et al*., 1993), abolished the inhibitory effect of CGS 21680. The A_1 receptor antagonist DPCPX (0.03 μM; Lohse *et al*., 1987) inhibited the effects of both CGS 21680 and APNEA. The interaction with APNEA argues against the involvement of A_3 receptors, since DPCPX even at higher concentrations than used in this study (>1 μM) had no affinity for this receptor-subtype (Zhou *et al*., 1992). Hence, the high concentrations of APNEA may act at A_{2A} rather than A_3 receptors of striatal neurones.

The antagonistic potency of DPCPX against CGS 21680 does not necessarily implicate the involvement of A_1 receptors; this compound has affinities for both A_1 and A_{2A} receptors in the submicromolar range (Lohse *et al*., 1987; Fredholm *et al*., 1994). In fact, DPCPX at 0.003 μM in contrast to 0.03 μM did not alter the effect of CGS 21680, although this lower concentration of DPCPX still considerably surmounts its K_D value measured in binding studies (Lohse *et al*., 1987). Moreover, the modulation of NMDA receptor channels by A_1 receptors is highly unlikely, since two structurally unrelated A_1 receptor agonists CCPA and **S**(−)-ENBA were both ineffective (see above). Taken together, our data suggest that the inhibitory action of adenosine on NMDA receptor channel conductance in medium spiny neurones of the rat striatum is mediated indeed by adenosine A_{2A} receptors. However, the possibility that the functional correlate of a DPCPX-sensitive high-affinity binding site for CGS 21680 (Cunha *et al*., 1996) modulates the NMDA receptor conductance cannot be excluded. This binding site is sensitive to DPCPX and, thereby, does not conform to any of the hitherto characterized adenosine receptor-types.

The endogenous agonist adenosine was inhibitory only when the nucleoside uptake was blocked by the transport inhibitor NBTG (Paterson *et al*., 1977). Such uptake sites are present in the striatum (Geiger & Nagy, 1990) and taking into account their high transport rates (Banay-Schwartz *et al*., 1980) may heavily interfere with agonist efficacy. By contrast, ATP remained inactive even in the presence of NBTG, excluding the possibility that under our experimental conditions a considerable quantity of ATP is enzymatically converted into adenosine.

The question arises by which mechanism do A_{2A} receptors inhibit NMDA receptor channels? The CGS 21680-induced inhibition of NMDA-currents appears to be due to a direct effect on medium-spiny neurones rather than to an indirect effect via neighbouring presynaptic structures (e.g. on corticostriatal afferents, Malenka & Kocsis, 1988). A localization of A_{2A} receptors on the medium spiny neurones themselves was suggested by the persistence of the CGS 21680 effect in the presence of TTX or a Ca^{2+}-free medium. While TTX did not alter the response to CGS 21680, the omission of Ca^{2+} from the bath medium increased it. This may be due to a still unresolved interaction with the transduction mechanism of A_{2A} receptors, since both manipulations are supposed to interrupt action potential-mediated synaptic inputs with similar efficiency.

Inactivation of G proteins with intracellular GDP-β-S (Sternweis & Pang, 1990), abolished the inhibitory effect of CGS 21680, indicating G protein coupling of the A_{2A} receptor. A_2 receptors are defined by their ability to stimulate adenylate cyclase (Van Calker *et al*., 1979) and are therefore thought to be coupled to the G_s type of G proteins (Fredholm *et al*., 1994). Hence, the most likely transduction mechanism of A_{2A} receptors is the activation of the adenylate cyclase/protein kinase A (PKA) system. Almost all neurotransmitter receptors examined to date have been shown either to be regulated by protein phosphorylation or to contain consensus sequences for phosphorylation by protein kinases (Huganir & Greengard, 1987; Swope *et al*., 1992). Such mechanisms have been demonstrated previously both the nicotinic acetylcholine receptor channels (Huganir & Greengard, 1987; Nörenberg *et al*., 1995) and EAA receptor channels (Swope *et al*., 1992; Raymond *et al*., 1993; Raman *et al*., 1996). However, it is not known whether there are G proteins other than G_s that interact with A_{2A} receptors or whether the G_s activated can influence effectors other than adenylate cyclase (Fredholm *et al*., 1994).

P1 purinoceptor agonists did not inhibit the function of NMDA-channels in about 20% of striatal neurones. Otherwise the CGS 21680-sensitive and -insensitive cells had similar membrane potentials, input resistances and soma diameters. These findings, together with the relative abundance of medium spiny neurones in the striatum (90–95%) suggest that all recordings were made from this cell population. High levels of A_{2A} binding were detected only in a subset of GABAergic medium spiny output neurones also containing enkephalins (Schiffmann *et al*., 1991; Schiffmann & Vanderhaeghen, 1993). This subset of cells is estimated to contribute 40–50% to the whole medium-spiny neuronal population, whereas less striatal output cells (25–40%) belong to a second class of GABAergic neurones positive also for substance P (Penny *et al*., 1986).

A differential sensitivity of medium spiny neurones to A_{2A} receptor agonists may be of functional significance, since the two groups of neurones participate in divergent efferent pathways. A direct route is formed by the substance P positive cells which connect the striatum with the basal ganglia output nuclei (internal segment of globus pallidus and substantia nigra pars reticularis). Enkephalin positive cells, which project to the external segment of the globus pallidus (GPe), are part of an indirect loop. This loop consists additionally of GPe neurones projecting to the subthalamic nucleus (STN) and of fibres connecting the STN with the output nuclei (Penny *et al*., 1986). Activity in the direct pathway facilitates, whereas activity in the indirect pathway depresses locomotor behaviour. In conclusion, agonists at striatal A_{2A} receptors may selectively interfere with EAA neurotransmission onto enkephalin-containing medium spiny neurones and may, thereby, alleviate locomotor depression e.g. in Parkinson's disease.

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