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Effects of The Selective Adenosine A2 Receptor Agonist CGS 21680 on *in Vitro* Electrophysiology, cAMP Formation and Dopamine Release in Rat Hippocampus and Striatum[1]

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ABSTRACT

Evaluation of adenosine A2 receptor function in the mammalian CNS has been impeded by the lack of highly selective A2 receptor agonists. The present investigations describe the actions of a recently introduced A2 selective adenosine agonist, CGS 21680 (2-[*p*-(carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine), on various functional neural responses known to be affected by adenosine. In hippocampal slices, CGS 21680 appeared to be a weak agonist on pre- and postsynaptic measures of electrophysiological activity (putative A1 receptor mediated events) and was ineffective at stimulating the formation of cAMP (a putative $A2_b$ mediated response). 5′-*N*-ethylcarboxamidoadenosine (NECA), which is known to act at both $A2_a$ and $A2_b$ receptors, increased hippocampal cAMP levels 4-fold. In striatal slices, CGS 21680 potently stimulated the formation of cAMP with an EC_{50} of 110 nM but was ineffective at inhibiting electrically stimulated dopamine release. In contrast, adenosine and cyclohexyladenosine both inhibited the stimulus-evoked overflow of dopamine. These results agree with previous receptor binding studies suggesting that CGS 21680 is a relatively selective agonist at the high affinity adenosine $A2_a$ receptor in striatum, with little intrinsic activity at the low affinity $A2_b$ site in hippocampus.

Considerable evidence exists to suggest that adenosine acts as an inhibitory modulator of neural activity in most regions of the mammalian CNS. Receptors for adenosine have been identified initially through actions on cAMP formation and subsequently through high affinity radioligand binding. These studies have suggested the existence of at least two major adenosine receptor subtypes. The adenosine A1 receptor subtype inhibits the formation of cAMP (Londos and Wolff, 1977; Van Calker *et al.*, 1979) and corresponds to the site labeled by [^{3}H]cyclohexyladenosine (CHA; Goodman and Snyder, 1982; Lee *et al.*, 1986; Snowhill and Williams, 1986). Receptor binding studies show that the A1 binding site is widely distributed throughout the brain but is found in particularly high concentrations in hippocampus, cerebellum and striatum. At higher concentrations, adenosine stimulates the formation of cAMP *via* the adenosine A2 receptor subtype (Sattin and Rall, 1970; Londos and Wolff, 1977; Van Calker *et al.*, 1979). In contrast to the A1 receptor, high affinity binding sites with the appropriate pharmacological A2 receptor profile show a more circumscribed distribution, being found only in brain structures such as striatum, olfactory tubercle and nucleus accumbens (Yeung and Green, 1984; Jarvis *et al.*, 1989a). In addition to this high affinity, discretely localized A2 receptor ($A2_a$), there is evidence for a low affinity A2 site ($A2_b$) found throughout the brain (Yeung and Green, 1983; 1984; Bruns *et al.*, 1986). While adenosine acts at the high affinity $A2_a$ site to stimulate cAMP formation in brain slices or homogenates, the low affinity $A2_b$ receptor mediates the accumulation of cAMP only in brain slice preparations (Bruns *et al.*, 1986). This latter observation may explain why the hippocampus exhibits a paucity of high affinity A2 receptors (Yeung and Green, 1983; Jarvis, 1988; Jarvis and Williams, 1988; 1989a) yet demonstrates a robust stimulation of cAMP formation with relatively high agonist concentrations (Smellie *et al.*, 1979; Dunwiddie and Fredholm, 1984, 1989).

Electrophysiological experiments have demonstrated that iontophoretically applied adenosine can reduce cell firing rates in striatum (Kostopoulos and Phillis, 1977) and in cerebral

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ABBREVIATIONS: CGS 21680, (2-[*p*-(carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; CHA, N^6-cyclohexyladenosine; CPT, 8-cyclopentyltheophylline; DA, dopamine; EPSP, excitatory postsynaptic potential; 8-PT, 8-phenyltheophylline; G protein, guanosine triphosphate-binding protein; R-PIA, N^6-[(*R*)-1-methyl-2-phenyl-ethyl]adenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; HPLC, high-performance liquid chromatography.

(Phillis *et al.*, 1975; Stone, 1982) and cerebellar cortex (Kostopoulos *et al.*, 1975; Dunwiddie *et al.*, 1983). In brain slices adenosine reduces evoked excitatory synaptic responses in a manner that is methylxanthine reversible (Scholfield, 1978; Schubert and Mitzdorf, 1979; Dunwiddie and Hoffer, 1980; Okada and Ozawa, 1980). Potency comparisons among adenosine agonists implicate the A1 site as the receptor mediating the inhibition of synaptic transmission in the *in vitro* hippocampus (Reddington *et al.*, 1982; Dunwiddie and Fredholm, 1984, 1989). Furthermore, this inhibition of synaptic activity occurs at much lower concentrations of adenosine agonists than those required for cAMP stimulation (Smellie *et al.*, 1979; Dunwiddie and Fredholm, 1984).

The well-documented ability of adenosine to diminish the release of various neurotransmitters in the CNS, including dopamine (DA; Harms *et al.*, 1979; Michaelis *et al.*, 1979; Myers and Pugsley, 1986; O'Neill, 1986), serotonin (Harms *et al.*, 1979; Feuerstein *et al.*, 1985), norepinephrine (Harms *et al.*, 1978; Fredholm and Hedqvist, 1980; Jonzon and Fredholm, 1984), acetylcholine (Jackisch *et al.*, 1984) and glutamate (Dolphin and Archer, 1983), appears to be linked to A1 receptor activation, at least where the receptor has been identified (Dunwiddie, 1985; Fredholm and Dunwiddie, 1988). However in some cases, such as the modulation of striatal DA release, the receptor subtype has not been conclusively identified.

In addition to these presynaptic effects, adenosine can act directly on postsynaptic receptors to cause hyperpolarization through the activation of a potassium channel that is coupled to a pertussis toxin-sensitive guanosine triphosphate-binding protein (G protein; Trussell and Jackson, 1985, 1987; O'Reagan and Phillis, 1987). This phenomenon probably underlies the adenosine-mediated inhibition of repetitive spiking induced by antidromic activation of pyramidal neurons in low calcium medium in the hippocampus (Haas and Jefferys, 1984; Lee *et al.* 1984; Dunwiddie and Fredholm, 1989). These postsynaptic effects of adenosine also appear to be mediated by A1 receptors (Dunwiddie and Fredholm, 1989) and, like the synaptic modulatory effect of adenosine, occur independently of effects upon cAMP levels (Trussell and Jackson, 1985, 1987; Dunwiddie and Fredholm, 1989).

From the above discussion it is clear that most functional responses to adenosine in the CNS can be ascribed to the A1 receptor. Although there is indirect evidence that A2 receptor stimulation may be involved in locomotor behavior (Green *et al.*, 1982) and discriminative stimulus properties of purinergic drugs (Spealman and Coffin, 1988), the only well-documented A2-specific response is that of enhanced cAMP accumulation.

Resolution of the relative contributions of adenosine A2 receptors to functional responses in brain has been in part impeded by the lack of A2-selective agonists (Williams, 1987). A recently introduced adenosine A2 agonist, 2-[*p*-(carboxyethyl)-phenylethylamino]-5′*N*-ethylcarboxamidoadenosine (CGS 21680), demonstrates approximately 140-fold selectivity for the A2 *versus* the A1 receptor (Jarvis *et al.*, 1989b; Jarvis and Williams, 1989) and has low nanomolar affinity (K_d = 7–20 nM) for the $A2_a$ binding site in striatum. This compound also demonstrates a high degree of activity at nanomolar concentrations in cardiovascular bioassays for A2 receptor activation (Hutchison *et al.*, 1989). In the present experiments we have examined the actions of CGS 21680 on electrophysiological responses and cAMP formation in rat hippocampal slices and cAMP formation and DA release in striatal slices.

Methods

Animals

Male Sprague-Dawley rats (Sasco Animal Laboratories, Omaha, NE) weighing 150–250 g were used for all experiments. They were housed in groups of four to six under a 12-hr light-dark cycle with food and water available *ad libitum*. For electrophysiology and biochemistry subjects were decapitated, and the hippocampus or striatum was dissected free of surrounding tissue.

Electrophysiology

Coronal sections taken from the middle portion of the hippocampi were prepared as described previously (Dunwiddie and Lynch, 1978; Mueller *et al.*, 1981). Slices were cut at 400 μm on a Sorvall tissue chopper and immediately placed in ice-cold medium consisting of (in mM) 124 NaCl; 4.9 KCl, 1.2 KH_2PO_4, 2.4 $MgSO_4$, 2.5 $CaCl_2$, 25.6 $NaHCO_3$ and 10 glucose (pH 7.5) that had been saturated with 95% O_2-5% CO_2. Slices were transferred within 5 min to a recording chamber maintained at 33 ± 1°C. Slices were not superfused with medium during a 40–60-min recovery in which the level of the medium was maintained level with or just below the upper surface of the slice. For experiments involving antidromic bursting, the slice medium was exchanged with one containing 4.0 mM $MgSO_4$ and 0.24 mM $CaCl_2$ at least 30 min before recording.

Electrophysiological recordings were made with 2–3 MΩ glass microelectrodes filled with 5 M NaCl, placed in either stratum radiatum or stratum pyramidale of the CA1 region under visual guidance. Twisted nichrome wire stimulation electrodes were placed in the stratum radiatum near the border of CA1-CA2 (synaptic response) or on the alveus near the CA1-subicular border (antidromic stimulation). Monophasic 0.1-msec pulses of 6–30 V were delivered to the synaptic pathway at 1-min intervals, and the voltage was adjusted so as to evoke a field EPSP of ~1 mV amplitude. For antidromic bursting, stimulation voltages of 10–50 V were used, with the intensity set to elicit at least three well-defined secondary spikes.

Because the supply of CGS 21680 was limited all dose-response curves were determined using a static bath procedure. In this procedure responses were recorded from slices maintained at the gas-liquid interface while suspended on nylon netting in a 2-ml slice well. After a stable base line had been recorded 25 μl of a concentrated drug solution (80 times desired final concentration, in water) were added to the 2-ml slice well using a calibrated microliter syringe mounted in a microelectrode holder. Control injections of 25 μl distilled water into the slice well had no effect on the responses. Occasionally theophylline was added to the slice well, subsequent to agonist administration, to reverse agonist effects. Although washout of CGS 21680 was not possible using this static bath procedure, preliminary experiments using superfused CGS 21680 demonstrated comparable drug effects that were reversible with extended washing.

To characterize the postsynaptic effects of adenosine and CGS 21680, we used an extracellularly recorded response, low calcium-induced antidromic bursting (Dunwiddie and Fredholm, 1989). Antidromic bursting reflects the repetitive cell firing that occurs in slices maintained in low calcium medium when the axons of hippocampal CA1 pyramidal neurons are activated antidromically. This response is highly sensitive to adenosine (Lee *et al.*, 1984; Schubert and Lee, 1986) and has been shown to be purely postsynaptic. The absolute, as well as relative, potencies of N^6-[(*R*)-1-methyl-2-phenyl-ethyl] adenosine (R-PIA), its *S*-isomer (Lee *et al.*, 1984) and the affinity of the A1 selective antagonist 8-cyclopentyltheophylline (CPT; Dunwiddie and Fredholm, 1989) for the receptor that mediates this response suggest that depression of antidromic bursting is mediated *via* an A1 receptor. Dose-response curves for CGS 21680 were analyzed by EC_{50} values determined by using least-squares lines fitted to the data points and converted to a Hill plot (Tallarida and Jacob, 1979). Biochemical data (see below) were analyzed in the same manner.

Biochemistry

Cyclic AMP levels. Coronal hippocampal and striatal slices (400 μm nominal thickness) were made with a tissue chopper and incubated in a metabolic shaker at 34°C for 60 min in 10–15 ml Krebs' buffer (in mM: 118 NaCl, 4.7 KCl, 11.1 glucose, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.0 NaH_2PO_4, 1.3 $CaCl_2$, 0.11 ascorbic acid and 0.004 Na_2EDTA) saturated with 95% O_2-5% CO_2 (pH 7.4). The slices were then rinsed with 15 ml fresh buffer and transferred to glass superfusion chambers (1.6 ml volume, containing two platinum electrodes). The slices were superfused with aerated Krebs' buffer, maintained at 34°C, for 15 min at 1.0 ml/min. They were then superfused for an additional 15 min (30 min for hippocampal slices) with buffer alone (controls) or with buffer containing various concentrations of CGS 21680 (0.001–10 μM) or 10 μM 5′-*N*-ethylcarboxamidoadenosine (NECA). In an additional experiment, slices of hippocampus were incubated with various concentrations of CGS 21680 with the selective A1 receptor antagonist CPT (Bruns *et al.*, 1986; Dunwiddie and Fredholm, 1989) added to reduce A1 effects on cAMP formation. After superfusion the slices were transferred to microcentrifuge tubes, and 100 μl of 10% trichloroacetic acid were added to each tube. The samples were immediately sonicated for 10–20 sec and then placed in a boiling water bath for 5 min. The solubilized cAMP was separated from the precipitated protein by centrifugation (13,600 × *g*; 8 min) and the supernatant extracted four times with 5 vol water-saturated diethyl ether. cAMP in the final aqueous layer was measured by radioimmunoassay (Biomedical Technologies, Stoughton, MA). The protein in each slice was measured after first solubilizing the precipitated protein with 1 N NaOH (Bradford, 1976).

The data were analyzed with the repeated-measures ANOVA with Newman Keuls *post hoc* tests and with the independent groups Student's *t* test.

Endogenous dopamine release. Striatal slices were prepared and incubated as described above. After placing the slices into the superfusion chambers they were superfused at 1.0 ml/min for 60 min to allow the rate of spontaneous DA release to stabilize. The Krebs' buffer for all DA release experiments contained 10 μM nomifensine to inhibit the synaptic DA transporter and obtain detectable levels of DA. After the 60-min superfusion, 17 1-ml samples associated with each of two electrical stimulation periods were collected on ice. Two prestimulus samples were collected, then stimulation applied (unipolar square-wave stimulations, 1 Hz for 1 min, 2-ms pulse duration, 16–25 mA current flow) and nine more consecutive samples collected. Six additional samples were collected at 12, 15, 18, 21, 24 and 27 min after stimulation. After collection of the last sample from the first stimulation, slices were superfused with buffer containing adenosine (50 μM), adenosine (50 μM) plus 8-PT (10 μM), N^6-cyclohexyladenosine (CHA; 100 nM), CGS 21680 (100 nM or 1 μM) or buffer with no drug added (controls). Thirty minutes after beginning drug superfusion, the stimulation and sample collection procedure was repeated. After sample collection, slices were removed from the chambers and weighed. Superfusate samples were stored at −70°C until assay (1–14 days).

Dopamine levels were determined using high-performance liquid chromatography (HPLC) coupled with electrochemical detection as described in detail previously (Gerhardt *et al.*, 1989). Briefly, 10-μg ascorbate oxidase was added to each sample, and 50 μl of the solution were injected directly into the HPLC system. The HPLC system consisted of a Beckman model 116 pump, Beckman model 501 autoinjector, Keystone Hypersil ODS 3 μm particle (100 mm × 4.6 mm) reverse phase column and an ESA, Bedford, MA, 5100A Coulochem electrochemical detector with a model 5011 dual-detector analytical cell (detector 1 set at +0.01 V; detector 2 set at +0.32 V). Chromatograms were recorded from detector 2 using a dual-pen strip chart recorder. The eluent was 0.07 M citrate/0.1 M acetate buffer containing 50 mg/l Na_2-EDTA, 130–150 mg/l octanesulfonic acid sodium salt, 650 mg/l NaCl and 8% methanol. The flow rate was 2.0 ml/min, and all separations were performed at room temperature. Complete separation and re-equilibration of the system required 7 min with a nominal detection limit of 1.0 pg/injection.

Retention times of standards were used to identify peaks, and peak heights were used to calculate absolute amounts of DA (expressed in picograms per milligram wet weight tissue per minute). Spontaneous release was determined by averaging the values from the two samples preceding each stimulation (designated as SP1 for stimulus 1 and SP2 for stimulus 2). Stimulation-evoked overflow was calculated by subtracting spontaneous release from total release in the remaining samples and summing all positive values (designated S1 and S2, respectively).

Drugs. All drugs were dissolved in deionized water in concentrations 80–100 times greater than final desired concentrations. CPT and 8-PT were dissolved in dimethyl sulfoxide that was added to all superfusion buffers in these experiments (final concentration dimethyl sulfoxide 0.0036%).

CGS 21680 was synthesized at CIBA-GEIGY (Summit, NJ) and generously donated by Dr. Michael Williams. NECA, CPT and 8-PT

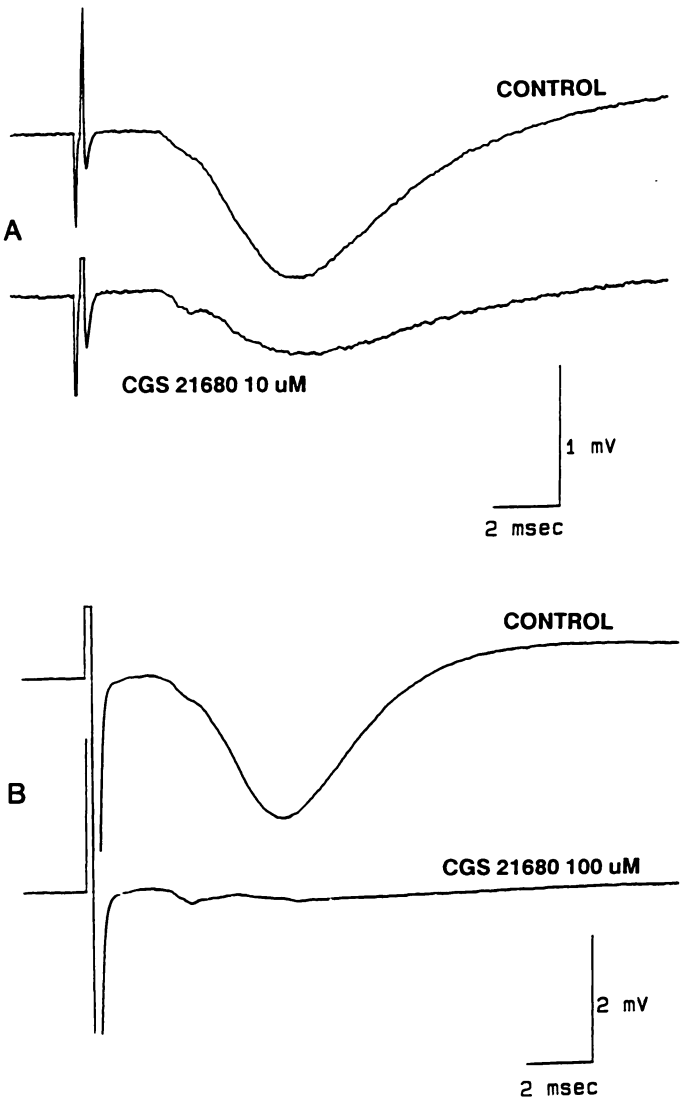

Fig. 1. Effects of CGS 21680 on field EPSP response. These waveforms represent digital averages of five field EPSP responses in two separate hippocampal slices before CGS 21680 (control) and 15 min after CGS 21680 administration. A, inhibition of field EPSP with 10 μM CGS 21680; B, inhibition of field EPSP with 100 μM CGS 21680. Peak inhibitions of these field EPSP responses were used to construct a dose-response curve (fig. 2). Although washout of this response is not shown because CGS 21680 was applied using a static bath procedure (see "Methods"), recovery of response after prolonged washouts in superfusion experiments was commonly observed (data not shown).

were purchased from Research Biochemicals (Wayland, MA). Nomifensine maleate was a gift from Hoechst-Roussel Pharmaceuticals (Somerville, NJ). Adenosine and CHA were purchased from Calbiochem (LaJolla, CA).

Results

Electrophysiology. The prominent inhibitory effect of adenosine and related compounds on evoked field EPSPs is almost certainly due to a presynaptic action at adenosine A1 receptors (Dunwiddie, 1985; Dunwiddie and Haas, 1985; Proctor and Dunwiddie, 1987). This inhibitory response was also seen with the putative adenosine A2 receptor agonist CGS 21680 (fig. 1). However, this compound was nearly equipotent compared with adenosine at inhibiting the field EPSP response; the EC_{50} for inhibition of the field EPSP by adenosine is 29 μm (26–33 μM, 95% confidence intervals; see table 1, Dunwiddie and Fredholm, 1984), whereas the EC_{50} for CGS 21680 was determined to be 11 μM (7–15 μM, 95% confidence intervals). These effects are not consistent with the low nanomolar affinity of CGS 21680 for the A2 receptor but correspond well with its micromolar affinity for the A1 receptor (Hutchison *et al.*, 1989), which mediates this response. At 100 μM CGS 21680 completely suppressed the field EPSP response (fig. 1B; fig. 2).

In experiments where agonists were perfused, the latency to response onset and washout were considerably slower for CGS 21680 than for adenosine. This has been observed previously using other adenosine agonists (Dunwiddie *et al.*, 1983) and is characteristic of lipophilic adenosine analogs that are poor substrates for enzymatic degradation or uptake. In some experiments the adenosine receptor antagonist theophylline (250 μM; Sattin and Rall, 1970) was added to demonstrate the adenosine receptor mediation of the CGS 21680 effect. In every case theophylline antagonized the CGS 21680-induced inhibition of the field EPSP (data not shown).

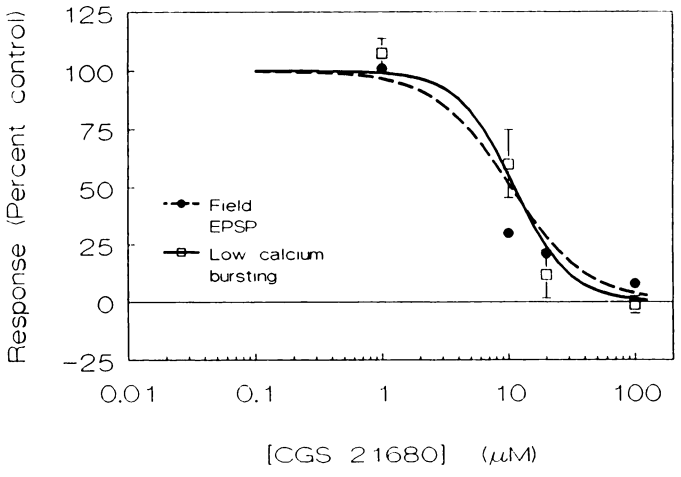

Fig. 2. Dose-response curves for inhibition of field EPSPs and inhibition of low calcium bursting by CGS 21680 in hippocampus. Each point represents mean (±S.E.M.) of five hippocampal slices. Maximal response was assumed to be 100% for computer analysis of dose-response curves. Curves shown were fit using the equation:

$$R = \frac{1}{1 + (EC_{50}/A)^H}$$

where R is fractional response, A is agonist concentration and EC_{50} and H correspond to EC_{50} value and Hill slope derived from a Hill plot. EC_{50} for inhibition of both the field EPSP and low calcium bursting was determined to be 11 μM.

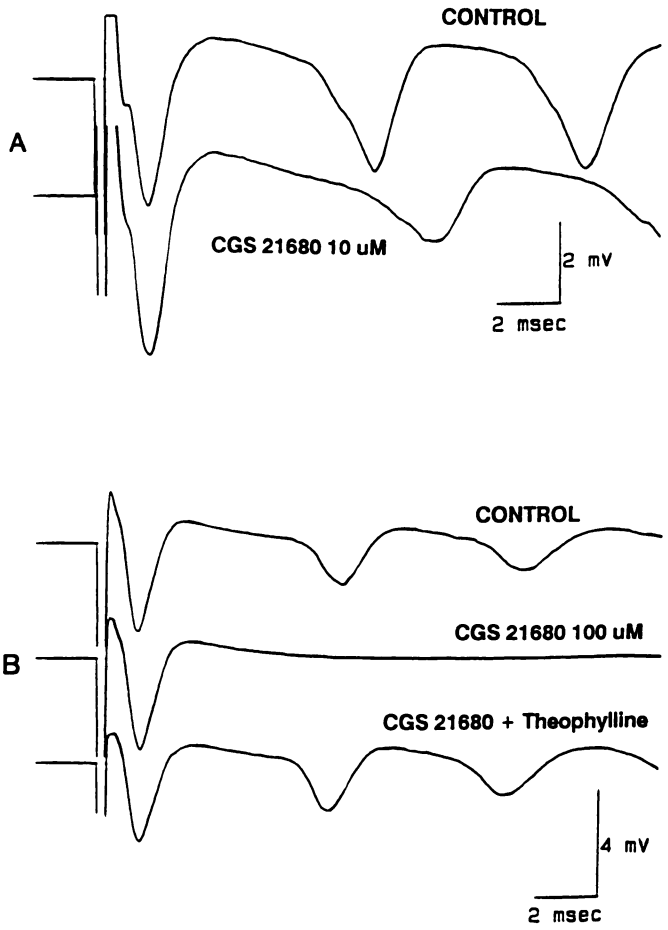

Fig. 3. Effects of CGS 21680 on low calcium bursting in two separate hippocampal slices. Each waveform represents average of five individually evoked responses. Magnitude of these responses was determined by measuring amplitude of second spike of an antidromically activated afterdischarge. *A*, low calcium bursting before (control) and 15 min after addition of 10 μM CGS 21680 to static bath. Note decrease in second spike and shift in spike latency in presence of CGS 21680. B, complete inhibition of low calcium bursting in 100 μM CGS 21680. When 250 μM theophylline was added to the chamber containing 100 μM CGS 21680, inhibition of this response was completely antagonized. Note in both A and B that the initial antidromic spike is unaffected by CGS 21680, as is the case for adenosine (Dunwiddie and Fredholm, 1989).

In a second series of experiments we evaluated the effects of CGS 21680 on low-calcium bursting, a response that is observed after antidromic stimulation in medium containing lowered levels of extracellular calcium and elevated magnesium. Previous reports have shown that adenosine can inhibit these low calcium afterdischarge bursts but not the primary antidromic response (Haas and Jefferys, 1984; Lee *et al.*, 1984). CGS 21680 also had no effect on the first evoked spike but reliably reduced subsequent spiking (fig. 3) with an EC_{50} of 11 μM (6–20 μM, 95% confidence intervals; fig. 2). At 100 μM CGS 21680 completely inhibited the burst response, and this effect was also theophylline reversible (fig. 3B).

cAMP levels. In hippocampal slices, CGS 21680 at concentrations up to 10 μM failed to significantly promote the formation of cAMP (table 1). In a separate experiment, the A1 selective adenosine receptor antagonist CPT was added along with CGS 21680 to reduce the possibility that A1-mediated inhibition of cAMP accumulation may have obscured an increase in cAMP levels enacted through the A2 receptor. The

TABLE 1

Effects of CGS 21680 and NECA on hippocampal and striatal cAMP formation

Values are means ± S.E.

Agonist Concentration	0 μM	0.1 μM	1 μM	10 μM
		(pmol cAMP/mg protein)		
Hippocampus[a]				
Control	4.2 ± 0.3			
NECA				17.8 ± 4.3**
CGS 21680		4.6 ± 0.8	4.9 ± 0.7	4.9 ± 0.4
CGS 21680 + 360 nM CPT		5.2 ± 1.1	3.9 ± 0.1	3.9 ± 0.3
Striatum[b]				
Control	7.1 ± 0.5			
NECA				22.5 ± 2.5**
CGS 21680		9.5 ± 0.4*	13.0 ± 0.8*	12.2 ± 0.4*

[a] n = 4 slices/concentration from two animals.
[b] n = 3 slices/concentration from three animals.
* $P < .05$, compared with control. Repeated-measures ANOVA with Newman-Keuls *post hoc* analysis.
** $P < .001$ compared with control. Student's independent groups t test.

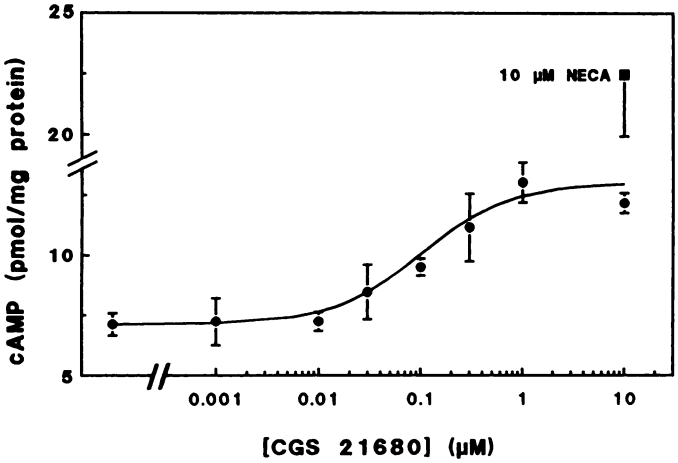

Fig. 4. Dose-response curves for stimulation of cAMP formation in striatal slices by CGS 21680 and NECA. See "Methods" for details. Mean values ± S.E.M. for three experiments (one slice per concentration per experiment) are shown. Note that 10 μM NECA resulted in a greater stimulation of cAMP formation than 10 μM CGS 21680. Curve corresponds to a least-squares fit with basal level of cAMP assumed to be 7.1 pmol/mg protein (table 1) and maximal level to be 12.7 pmol/mg protein. Hill slope was constrained to 1, and EC_{50} was determined to be 110 nM.

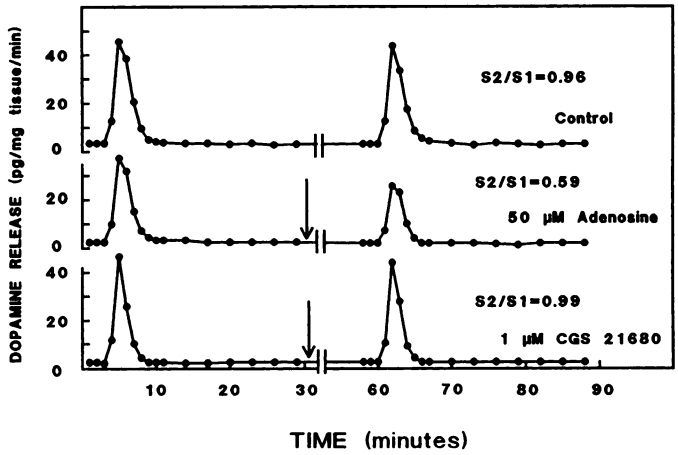

Fig. 5. Time course of adenosine and CGS 21680 effects on release of endogenous DA from striatal slices. Nomifensine (10 μM) was present throughout entire experiment. Electrical stimulation (1.0 Hz) was applied at 3 and 60 min. Adenosine (50 μM) or CGS 21680 (1.0 μM) was added to superfusion buffer at 30 min (arrows) and continued throughout experiment. Ratios of amount of DA released after electrical stimulation period 2 (S2; after drug perfusion) *versus* stimulation period 1 (S1; before drug perfusion) are shown at right. Mean values for three experiments (two slices per experiment) are shown.

addition of CPT was also without effect on cAMP levels at a concentration that was determined to shift the A1 receptor dose-response curve 10-fold (360 nM) while having negligible effects on the A2 dose-response curve. NECA however, at concentrations previously shown to elicit near-maximal increases in cAMP accumulation (Dunwiddie and Fredholm, 1984), produced a significant 4-fold increase in hippocampal cAMP content (table 1).

The base-line level of cAMP in untreated superfused rat striatal slices was 7.1 ± 0.5 pmol/mg protein. NECA (10 μM) significantly increased striatal cAMP levels 3-fold (table 1; fig. 4). CGS 21680 significantly increased striatal cAMP levels in a dose-dependent manner (EC_{50} 110 nM), but in contrast to NECA, a maximally effective concentration of CGS 21680 (1 μM) produced only a 2-fold increase in cAMP levels (fig. 4). These data were obtained from slices that were superfused with buffer for 15 min and then exposed to drugs for 15 min. Similar results were obtained when slices were superfused for 60 min and exposed to drug for 30 min (per the protocol used in DA release, data not shown). In addition, the presence or absence of nomifensine (10 μM) in the superfusion buffer had no effect on cAMP levels (data not shown).

Endogenous dopamine release. The unstimulated basal level of DA release from the striatal slices was 2.9 ± 0.33 pg DA/mg tissue/min (n = 3 rats, four slices per rat). Addition of CGS 21680, adenosine or CHA to the superfusion buffer had no effect on basal release (F = 0.82, dF = 3,6, P = .5294).

The time course for the stimulated release of endogenous DA from superfused rat striatal slices is shown in figure 5. Stimulation-evoked overflow of endogenous DA from the striatal slices was 96 ± 7.3 pg DA/mg tissue (n = 3 rats, four slices per rat), and typically the second response to stimulation was nearly identical to the first (S2/S1 ratio = 0.96 ± 0.01). CGS 21680 (1 μM) had no effect on stimulation-evoked overflow of DA, whereas adenosine (50 μM) reduced DA overflow by ~40%. The effect of adenosine was completely prevented by co-perfusion with 10 μM 8-PT (fig. 6). Cyclohexyladenosine (0.1 μM), a selective A1 adenosine receptor agonist, reduced the release of DA in a manner similar to that of adenosine (fig. 6).

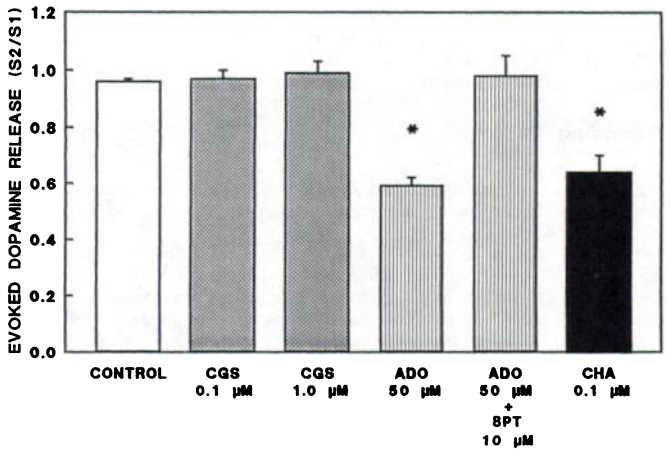

Fig. 6. Effects of various adenosine agonists on electrically stimulated dopamine release in striatal slices (same protocol as in fig. 5). Adenosine (ADO) and A1 receptor selective agonist CHA significantly reduced stimulation-evoked overflow of DA, whereas CGS 21680 did not. Mean values for three experiments at each drug concentration (two slices per experiment) are shown. An ANOVA was performed on ratios SP2/SP1 and S2/S1 with drug present during S2 as a single within factor. *Posthoc* comparisons were made using Newman-Keuls analysis. *, $P < .05$ *versus* control 8-phenyltheophylline (8-PT).

Discussion

The novel adenosine A2 receptor agonist, CGS 21680, has been shown to be selective for the A2 receptor in physiological and radioligand binding assays (Hutchison *et al.*, 1989; Jarvis *et al.*, 1989b; Jarvis and Williams, 1989). The pharmacological profile of binding sites for [^{3}H]CGS 21680 demonstrates a high degree of correlation ($r = 0.98$; Jarvis *et al.*, in press) with that for [^{3}H]NECA binding in the presence of 50 nM N^6-cyclopentyladenosine, which is added to mask 98% of A1 receptors (Bruns *et al.*, 1986). These observations thus suggest that CGS 21680 represents a highly A2-selective adenosine agonist. Moreover, the lack of significant [^{3}H]CGS 21680 binding in areas such as the hippocampus suggests that it is relatively selective for the $A2_a$ receptor subtype.

Consistent with its low affinity for A1 receptors, CGS 21680 was a relatively weak agonist at receptors mediating electrophysiological responses in the hippocampal slice. The EC_{50} value of 11 μM for CGS 21680 at inhibiting the field EPSP is similar to that for adenosine (29 μM, Dunwiddie and Fredholm, 1984) and is far below the affinity of CGS 21680 for A2 receptors in striatal tissue (7–20 nM; Hutchison *et al.*, 1989; Jarvis *et al.*, 1989b; Jarvis and Williams, 1989). Our results therefore agree well with binding experiments that yield IC_{50} values of ~3 μM at the A1 receptor subtype (Hutchinson *et al.*, 1989; Jarvis *et al.*, 1989b). This represents strong evidence that at higher concentrations CGS 21680 not only binds to A1 receptors in hippocampus but is an agonist at this receptor as well. In addition, this evidence reinforces the contention that adenosine's inhibition of synaptic transmission in the hippocampus is mediated by the A1 receptor subtype (Reddington *et al.*, 1982; Dunwiddie and Fredholm, 1984).

Antidromic activation of pyramidal neurons in slices maintained in low calcium medium results in repetitive synchronous bursting of these cells. This response is clearly postsynaptic since stimulus-secretion coupling is prevented by the unavailability of calcium to the presynaptic nerve terminal. These low calcium-induced afterdischarges have also been shown to be very sensitive to adenosine (Lee *et al.*, 1984; Dunwiddie and Fredholm, 1989). CGS 21680 reduced the amplitude of the secondary bursts after this antidromic pyramidal cell activation with an EC_{50} value of 11 μM, approximately the same potency as adenosine (Dunwiddie and Fredholm, 1989). As before, the low potency of CGS 21680 implies an action through A1 rather than A2 receptors and is consistent with previous investigations that have implicated the A1 receptor in the postsynaptic effects of adenosine compounds (Dunwiddie and Fredholm, 1989). It therefore appears that the A1 receptor is responsible for the inhibition of electrophysiological activity, both pre- and postsynaptically in the hippocampus, and that the effects of high concentrations of CGS 21680 on these responses are mediated *via* these A1 receptors.

At concentrations of $\leq$10 μM CGS 21680 had no effects on cAMP accumulation in hippocampal slices, even in the presence of the A1 selective antagonist CPT, which was added to block any competing A1 receptor inhibition of adenylate cyclase. In contrast to the ineffectiveness of CGS 21680 in stimulating cAMP formation, NECA (10 μM) caused a significant 4-fold increase in cAMP levels in hippocampal slices. The fact that [^{3}H]NECA can label both high and low affinity A2 binding sites (Bruns *et al.*, 1986), whereas [^{3}H]CGS 21680 binds to only the $A2_a$ sites (which are absent in the hippocampus) suggests that NECA is acting at $A2_b$ sites in the hippocampus to stimulate cAMP accumulation and agrees with the lack of an observed effect of CGS 21680 on cAMP formation in this structure. In contrast to hippocampus CGS 21680 stimulated the formation of cAMP in striatum at nanomolar concentrations. However, the maximal increase in cAMP levels induced by CGS 21680 in striatal slices was smaller than the response to NECA (see fig. 4). One explanation for this result is that CGS 21680 can only increase cAMP levels through interaction with the $A2_a$ receptor in striatum, whereas NECA may act *via* both $A2_a$ and $A2_b$ receptors. A similar effect of greater maximal stimulation of cAMP accumulation with NECA *versus* other adenosine agonists has also been seen in pituitary homogenates (Anand-Srivastava, 1988). An alternative explanation is that CGS 21680 may be a partial agonist at striatal $A2_a$ receptors. This possibility was not eliminated by the present experiments because concentrations of CGS 21680 >10 μM were not examined. At these concentrations one would expect a loss of selectivity, such that inhibitory actions at the A1 receptor may offset effects on the $A2_a$ or $A2_b$ receptor; just as the A2 actions of R-PIA can interfere with the A1-mediated inhibitory effects on cAMP accumulation at higher concentrations (Dunwiddie and Fredholm, 1989). Based upon these results we propose that CGS 21680 is a specific agonist at the high affinity striatal A2 receptor (the $A2_a$ site), but has little or no effect on the low affinity $A2_b$ sites in hippocampus or striatum. This is consistent with data from [^{3}H]CGS 21680 binding showing that this compound interacts with only one high affinity A2 binding site in striatal tissue and negligibly in hippocampal tissue (Jarvis *et al.*, 1989b). In contrast, [^{3}H]NECA can label both the high and low affinity A2 sites (Bruns *et al.*, 1986), and NECA can increase cAMP levels in hippocampus as well as striatum.

The ability of adenosine to inhibit the release of DA *in vitro* and *in vivo* is well-known (Harms *et al.*, 1979; Michaelis, 1979; Meyers and Pugsley, 1986; O'Neill, 1986), and some investigations have implicated the A1 receptor in the inhibition of DA turnover and release in striatum (Myers and Pugsley, 1986; Wood *et al.*, 1989). However, no direct study of DA release has

made a clear distinction between A1 or A2 adenosine receptor involvement in this phenomenon, and in some cases it appears as if neither receptor is involved (Ebstein and Daly, 1982). The absence of a CGS 21680 effect on stimulated DA release from striatal slices, even at a concentration that resulted in a maximal increase in the accumulation of cAMP (1 μM), and the marked inhibition of DA release by the specific A1 agonist CHA (fig. 6) suggest that this effect is mediated through adenosine A1 receptors. Also, the demonstration of a relatively robust stimulation of cAMP without alterations in DA overflow is consistent with the idea that these two responses to adenosine agonists are independent. Thus, elevated cAMP accumulation does not appear to be necessary for the adenosine-induced inhibition of DA release in striatal slices, and indeed these responses appear to be mediated by different adenosine receptor subtypes. This conclusion is supported by the observation that NECA enhances forskolin-stimulated cAMP formation without affecting catecholamine secretion in adrenal chromaffin cells (Chern *et al.*, 1988).

Assignment of the various known adenosine-mediated effects on neuronal function to receptor subtypes has suffered from a lack of specific agonists to differentiate the primary role of the A1 and A2 receptors. This investigation demonstrates that adenosine's ability to diminish neuronal transmission in the hippocampus and to reduce DA release in striatum are likely mediated through the A1 receptor. The present findings also provide further support of a functional nature for the subdivision of the A2 receptor class into the $A2_a$ and $A2_b$ subtypes and suggest that CGS 21680 is a highly selective agonist at the $A2_a$ receptor. Also, due to the distinct functional localization of the A2 receptor subtypes, these data support the suggestion that $A2_a$ and $A2_b$ receptors are separate entities and not merely different affinity states of the same receptor site (Bruns *et al.*, 1986). A further understanding of the functional significance of the $A2_a$ receptor subtype and its importance in neuronal function will hopefully be aided through further investigation with specific agonists of this kind.

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