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ZM241385 is an antagonist of the facilitatory responses produced by the A_{2A} adenosine receptor agonists CGS21680 and HENECA in the rat hippocampus

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1 In the present study, we investigated the ability of a recently introduced non-xanthine A_{2A} receptor antagonist, ZM241385 (4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3-a{1,3,5}triazin-5-yl-aminoethyl)phenol) to displace binding of the prototypical A_{2A} adenosine receptor agonist [^{3}H]CGS21680 (2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine) and to modify the facilitatory responses caused by the A_{2A} selective agonists, CGS21680 and HENECA (2-hexynl-5′-*N*-ethylcarboxamidoadenosine) in rat hippocampal preparations.

2 ZM241385 was nearly equipotent to displace [^{3}H]CGS21680 (30 nM) binding to hippocampal (K_i of 0.52 nM) and to striatal membranes (K_i of 0.35 nM), whereas HENECA was a more potent displacer of [^{3}H]CGS21680 binding to striatal (K_i of 4.5 nM) than to hippocampal membranes (K_i of 19 nM).

3 HENECA (3–30 nM) was equipotent with CGS21680 to facilitate veratridine-evoked [^{3}H]acetylcholine release from superfused hippocampal synaptosomes and ZM241385 (20 nM) inhibited the facilitatory effects of both HENECA (30 nM) and CGS21680 (30 nM); this antagonism was mimicked by CSC (250 nM).

4 In contrast, CGS21680 (10–30 nM) was more potent than HENECA (10–30 nM) to facilitate synaptic transmission in Schaffer fibres/CA1 pyramid synapses of hippocampal slices and the facilitatory effect of CGS21680 (10 nM) was blocked by ZM241385 (20 nM) whereas CSC (250 nM) caused a 40% attenuation of this CGS21680-induced facilitation.

5 These results indicate that ZM241385 is the first A_{2A} antagonist with equal potency to displace [^{3}H]CGS21680 binding to striatal and limbic regions, and with general efficiency to antagonize HENECA- or CGS21680-mediated facilitatory responses in the hippocampus.

Keywords: Adenosine receptors; hippocampus; CGS21680; ZM241385; HENECA

Introduction

It has recently been recognized that responses to adenosine are a balance between inhibitory A_1 and facilitatory A_2 receptors (e.g. Cunha *et al*., 1994a, b), and A_2 receptors are widespread in the CNS (Sebastião & Ribeiro, 1996), albeit with a lower density than A_1 receptors (e.g. Cunha *et al*., 1994b). A_2 receptors are subdivided into two classes: low-affinity A_{2B} receptors and high-affinity A_{2A} receptors. The distinction between A_{2A}- and A_{2B}-mediated responses was made possible by the introduction of the adenosine analogue 2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680) which binds with nanomolar affinity to A_{2A} receptors (Jarvis *et al*., 1989) and virtually does not bind to A_{2B} receptors at micromolar concentrations (Stehle *et al*., 1992). CGS21680 is also 180-fold selective towards A_1 receptors (Jarvis *et al*., 1989) and is unable to displace binding to A_3 receptors at micromolar concentrations (Zhou *et al*., 1992). Another adenosine analogue, 2-hexynl-5′-N-ethylcarboxamidoadenosine (HENECA) has an A_{2A}/A_1 selectivity similar to that of CGS21680 and similar A_{2A} receptor efficacy (Monopoli *et al*., 1994), but CGS21680 is still the drug currently used to pharmacologically define the A_{2A} receptor subtype.

However, the prototypical A_{2A} receptor agonist CGS21680 binds in the nanomolar range to two pharmacologically different binding sites: 'typical', i.e. striatal-like, A_{2A} receptors also present with low abundance in the cortex and hippocampus (Cunha *et al*., 1995, 1996), and an 'atypical' CGS21680 binding site which is predominant in the cortex and hippocampus (Cunha *et al*., 1996; Johansson & Fredholm, 1995). These predominant 'atypical' CGS21680 binding sites in the hippocampus are 10 fold less sensitive to two potent and selective striatal-like A_{2A} receptor antagonists (Cunha *et al*., 1996), KF17837 ((*E*)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine) which is 62-fold A_{2A}/A_1 selective (Nokada *et al*., 1994), and CSC (8-(3-chlorostyryl)caffeine) which is 520-fold A_{2A}/A_1 selective (Jacobson *et al*., 1993), and are also insensitive to a non-xanthine A_{2A} receptor antagonist (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine, SCH58261) (Lindström *et al*., 1996) which is 50-fold A_{2A}/A_1 selective and does not interfere with A_{2B} or A_3 receptors (Zocchi *et al*., 1996), raising doubts as whether these 'atypical' CGS21680 binding sites might represent A_{2A} receptors (Lindström *et al*., 1996).

We now report that a recently introduced non-xanthine A_{2A} receptor antagonist, ZM241385 (4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3-a{1,3,5}triazin-5-yl-aminoethyl)phenol) which is 500 to 1000 fold A_{2A}/A_1 selective and does not interfere with A_{2B} or A_3 receptors (Poucher *et al*., 1995), is a potent displacer of 'typical' and 'atypical' CGS21680 binding sites, and antagonizes both CGS21680- and HENECA-induced facilitatory responses in hippocampal preparations.

Methods

Drugs and solutions

2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680) and 8-(3-chlorostyryl)caffeine (CSC) were from RBI. [methyl-^{3}H]choline (specific activity 76.0–86.3 Ci/mmol) was obtained from Amersham and [^{3}H]CGS21680 (specific activity 38.3–42.2 Ci/mmol) was from

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DuPont NEN. 4-(2-[7-amino-2-(2-furyl{1,2,4}-trizolo{2,3a}{1,3,5}triazin-5-yl-aminoethyl)phenol (ZM241385) was a gift from ZENECA and 2-hexynyl-5′-N-ethylcarboxamidoadenosine (HENECA) was kindly provided by Professor Gloria Cristalli (University of Camerino, Italy). Hemicholinium-3, 2-chloroadenosine and adenosine deaminase (type VI) were from Sigma. All other reagents were of the highest purity available.

CSC, CGS21680, HENECA and ZM241385 were made up into 5 mM stocks in DMSO. Aqueous dilution of these stock solutions were made daily. When testing the effect of CSC, care was taken to protect the solutions and perfusion tubes from light.

[^{3}H]CGS21680 binding to hippocampal and striatal membranes

[^{3}H]CGS21680 binding studies were performed as previously described using freshly prepared rat hippocampal or striatal membranes (Cunha *et al*., 1996). Briefly, competition curves of [^{3}H]CGS21680 were performed by incubation of 30 nM [^{3}H]CGS21680 for 4 h at room temperature (23–25°C) with 150–340 μg of membrane protein in a final volume of 300 μl in a solution containing 50 mM Tris and 10 mM $MgCl_2$ pH 7.4 and 5 u ml^{-1} adenosine deaminase, and 12 different concentrations (ranging from 0 to 10 μM) of the tested competitors, ZM241385 or HENECA. Specific binding was determined by subtraction of the non-specific binding which was measured in the presence of 100 μM 2-chloroadenosine. The binding reactions were stopped by vacuum filtration through Whatman GF/C glass fiber filters, followed by washing of the filters and reaction tubes with 15 ml of a solution containing 50 mM Tris and 10 mM $MgCl_2$, pH 7.4, kept at 4°C. The filters were then placed in scintillation vials and 5 ml of scintillation liquid (Scintran Cocktail T, BDH) were added. Radioactivity bound to the filters was determined after 12 h with an efficiency of 55–60% for 4 min. All binding assays were performed in triplicate using 12×15 polyproplene tubes. Membrane protein was determined according to Peterson (1977).

The averaged data were fitted by non-linear regression using the Raphson–Newton method, performed with the GraphPAD InPlot Software package to determine the IC_{50} values, converted into K_i values using values of K_D for CGS21680 of 62 nM (hippocampus) and 12 nM (striatum) (see Cunha *et al*., 1996). An F-test ($P > 0.05$) was used to determine whether the competition curves were fitted best by one or two independent binding sites. The confidence intervals (95%) of the K_i values were determined from the log values.

The pharmacological dissection of [^{3}H]CGS21680 binding to 'typical' and 'atypical' CGS21680 binding sites was performed as previously described (Cunha *et al*., 1996). Briefly, displacement of [^{3}H]CGS21680 binding to hippocampal membranes by ZM241385 was performed in the presence of CSC (200 nM) to isolate 'atypical' CGS21680 binding sites or in the presence of DPCPX (100 nM) to isolate 'typical' CGS21680 binding sites.

[^{3}H]Acetylcholine release from hippocampal synaptosomes

The release of [^{3}H]acetylcholine ([^{3}H]ACh) from a rat hippocampal synaptosomal fraction (see Cunha *et al*., 1994c) was performed as previously described (Cunha *et al*., 1995). Briefly, the synaptosomes were equilibrated at 37°C for 5 min in Krebs solution of the following composition (mM): NaCl 115, KCl 3, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, $MgSO_4$ 1.2, glucose 10, and adenosine deaminase 2 U ml^{-1}. From this time onwards, all solutions applied to the synaptosomes were kept at 37°C and gassed with 95% O_2 and 5% CO_2. After the equilibration period, the synaptosomes were loaded with [^{3}H]choline (10 μCi ml^{-1}, 0.125 μM) for 5 min, centrifuged and washed twice with 1 ml of Krebs solution containing hemicholinium-3 (10 μM), which was present up to the end of the experiment to prevent the reuptake of choline. Synaptosomes were then resuspended in 5 ml of Krebs solution and layered over Whatman GF/C filters into eight parallel 90 μl perfusion chambers (adapted from Swinny filter holders, Millipore) with the aid of a roller pump (flow rate: 0.6 ml min^{-1}, which was kept constant through the experiment). The chamber volume plus dead volume was approximately 0.6 ml. A series of eight parallel perfusion chambers was used to enable both control and test conditions to be performed in duplicate from the same batch of synaptosomes. After a 5 min washout period, the effluent was collected in 1 min fractions for scintillation counting analysis (Cunha *et al*., 1994c). The synaptosomes were stimulated with veratridine (10 μM) for 2 min at 4 and 16 min after starting sample collection (S_1 and S_2). When testing the effect of CGS21680, CGS21680 was added to the perfusion medium 7 min before S_2 and remained in the bath up to the end of the release period. The effect of CGS21680 on the release of acetylcholine was expressed by alterations of the S_2/S_1 ratio. When we evaluated the modifications of the effect of CGS21680 by ZM241385, ZM241385 was applied from the beginning of the perfusion period and hence was present during S_1 and S_2. When present during S_1 and S_2 ZM241385 did not significantly alter ($P > 0.05$) the S_2/S_1 ratio compared to the S_2/S_1 ratio obtained in its absence. At the end of the experiments, the filters were removed from the superfusion chambers and analysed by scintillation counting for determination of tritium retained by the synaptosomes (4 min, error < 2%). Radioactivity was expressed in terms of desintegrations per second per mg of protein (Bq mg^{-1}) in each chamber. The fractional release was expressed in terms of the percentage of total radioactivity present in the preparation at the beginning of the collection of each sample. The amount of radioactivity (expressed as fractional release) released by each pulse of veratridine (S_1 and S_2) was calculated by integration of the area of the peak upon subtraction of the estimated basal tritium release from the total release of tritium obtained upon veratridine stimulation, and is a good measure of [^{3}H]ACh release (Cunha *et al*., 1994c).

The values are presented as mean ± s.e.means. To test the significance of the effect of CGS21680, a paired Student's *t* test was used. When making comparisons of the effect of CGS21680 in the absence and in the presence of ZM241385, a one-way variance analysis (ANOVA) was used, followed by a Dunnett's test. *P* values of 0.05 or less were considered to represent significant differences.

Electrophysiological recording of synaptic transmission in hippocampal slices

Rat hippocampal slices (400 μm thick) were prepared as previously described (Cunha *et al*., 1994b) and allowed to recover for 1 h at room temperature in artificial cerebrospinal fluid (CSF) of the following composition (mM): NaCl 124, KCl 3, KH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10, pH 7.4, gassed with a 95% O_2 and 5% CO_2 mixture. One slice was transferred to a 1 ml recording chamber for submerged slices and continuously superfused with gassed CSF solution, kept at 30.5°C, at a flow rate of 3 ml min^{-1}. Drugs were added to this superfusion solution.

Electrophysiological recordings of field excitatory postsynaptic potentials (fEPSP) were obtained as previously described (Cunha *et al*., 1994b). Monopolar stimulation (rectangular pulses of 0.1 ms applied once every 10 s) was delivered through an electrode placed on the Schaffer fibres, in the stratum radiatum near the CA3/CA1 border. Orthodromically-evoked fEPSPs were recorded through an extracellular microelectrode (4 M NaCl, 2–5 MΩ resistance) placed in the stratum radiatum of the CA1 area. The intensity of the stimulus (160–230 μA) was adjusted to evoke the largest fEPSP without population spike contamination. The individual responses were displayed on a Tektronix (2430A) oscilloscope and the averages of eight consecutive responses were digitally recorded. fEPSP responses were quantified as the initial slope of the averaged fEPSPs. Perfusion of a slice

with the tested drugs was started after a stable response was recorded.

When testing the ability of an antagonist (ZM241385 or CSC) to modify the effect of an agonist (CGS21680 or HENECA), the effect of the agonist was tested first in the absence of the antagonist; the agonist was then washed out, and the antagonist was perfused for at least 45 min before perfusing the agonist in the presence of the antagonist. The two substances were then washed out and the effect of the agonist in the absence of the antagonist was usually tested again.

Results

Effect of A_{2A} ligands on [^{3}H]CGS21680 binding to membranes

We compared the relative potency of ZM241385, an A_{2A} antagonist (Poucher *et al*., 1995), and of HENECA, an A_{2A} agonist (Monopoli *et al*., 1994), to displace [^{3}H]CGS21680 binding to hippocampal membranes, where 'atypical' CGS21680 binding sites predominate (Cunha *et al*., 1996) and to striatal membranes where CGS21680 binding sites are assumed to represent 'typical' binding to adenosine A_{2A} receptors (Cunha *et al*., 1996; Sebastião & Ribeiro, 1996). In membranes from the hippocampus and striatum, the displacement curves of [^{3}H]CGS21680 (30 nM) binding by ZM241385 (10^{-11} to 10^{-5} M) and HENECA (10^{-11} to 10^{-5} M)

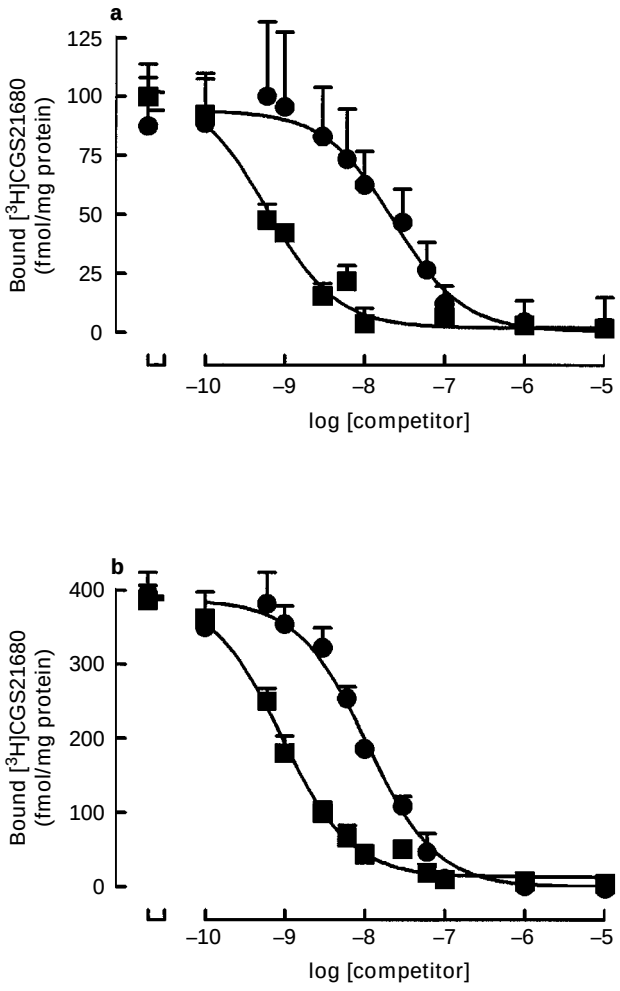

Figure 1 Inhibition of [^{3}H]CGS21680 (30 nM) binding to whole membranes of the hippocampus (a) and striatum (b) by the adenosine A_{2A} receptor antagonist, ZM241385 (■), and by the A_{2A} agonist, HENECA (●). The ordinates represent the specific binding of [^{3}H]CGS21680 obtained upon subtraction of the non-specific binding, determined in the presence of 100 μM 2-chloroadenosine, from the total binding. The specific binding of [^{3}H]CGS 21680 corresponded to about 39% of total binding in the hippocampus and 82% in the striatum. Curves were generated from the average binding parameters obtained upon fitting by nonlinear regression assuming a single binding site model. Results are the mean ± s.e.mean of four experiments performed in duplicate.

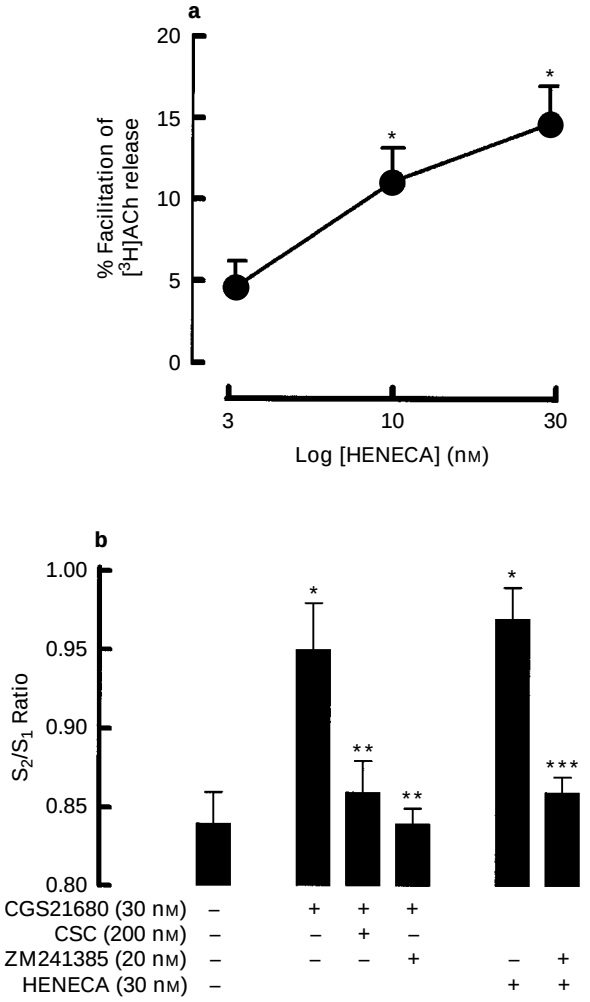

Figure 2 Concentration-dependent facilitation by HENECA of the release of [^{3}H]ACh from rat hippocampal synaptosomes (a) and efficacy of the A_{2A} antagonists ZM241385 and CSC to antagonize A_{2A} receptor-mediated facilitation of [^{3}H]ACh release (b). The synaptosomes (115 ± 5 μg protein per chamber) were superfused in the presence of 2 U ml^{-1} adenosine deaminase and stimulated twice (S_1 and S_2) with veratridine (10 μM). The evoked tritium release was Ca^{2+}-dependent (74%) and mainly constituted by [^{3}H]ACh (for details see Cunha *et al*., 1994c), and was assumed to represent exocytotic ACh release. HENECA or CGS21680 were added 7 min before S_2 and remained in the bath up to the end of the experiment. The facilitatory effects of HENECA or CGS21680 were calculated from the alteration of the S_2/S_1 ratio compared to the S_2/S_1 ratio in the absence of these A_{2A} agonists. In (b), the left column (control) corresponds to the absence of added drugs. ZM241385 (20 nM) or CSC (200 nM) were present at the beginning of superfusion (i.e. they were present during S_1 and S_2) and did not modify the S_2/S_1 ratio compared with control. As facilitatory effects of A_{2A} agonists were not observed in all hippocampal synaptosomal batches (see Cunha *et al*., 1995), the effect of the A_{2A} agonist in the absence and in the presence of the tested A_{2A} antagonists was always compared in the same batch of synaptosomes in parallel experiments. The results are mean ± s.e.mean of two to four experiments in (a) and (b), except the control which is $n = 52$. $^{*}P < 0.05$ compared to control, $^{**}P < 0.05$ compared to the effect of CGS21680 (30 nM) in the absence of any other drug (second column from the left in b). $^{***}P < 0.05$ compared to the effect of HENECA in the absence of any other drug (second column from the right in b).

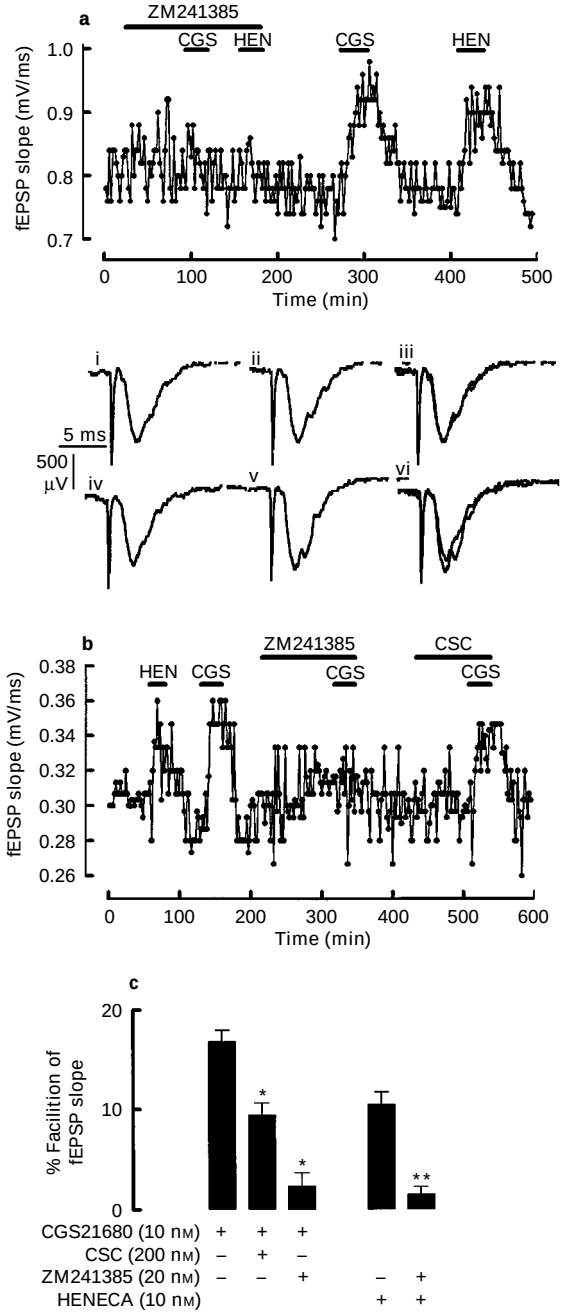

Figure 3 Facilitatory effects of the A_{2A} agonists HENECA and CGS21680 on field excitatory postsynaptic potentials (fEPSP) slope and different efficiency of the A_{2A} antagonists, ZM241385 and CSC, to prevent the A_{2A} receptor-mediated facilitation of synaptic transmission. (a) and (b) shown time courses of the slope of averages of eight consecutive fEPSPs recorded from the CA1 area of the hippocampus in typical experiments. In (a), the hippocampal slice was perfused with CGS21680 (CGS, 10 nM) and HENECA (HEN, 10 nM) first in the presence of ZM241385 (20 nM) and then in the absence of this A_{2A} antagonist, as indicated by the bars above in (a). The fEPSP recordings were derived from data presented in (a) and correspond to (upper row) 84 min (i, ZM241385), 106 min (ii, ZM241385 + CGS21680) and these two fEPSPs superimposed (iii), and (lower row) 262 min (iv, control), 296 min (v, CGS21680) and these two fEPSPs superimposed (vi). (b) Hippocampal slice was first perfused with HENECA (10 nM) and then with CGS21680 (10 nM) in the absence and in the presence of either ZM241385 (20 nM) or CSC (200 nM) as indicated by the bars above in (b). In (c), the ordinates represent the average percentage modification of the fEPSP slope caused by either CGS21680 (10 nM) alone (left column) or HENECA (10 nM) alone (second column from the right), and by CGS21680 (10 nM) or HENECA (10 nM) in the presence of drugs indicated below each column. As exemplified in (a) and (b), in each experiment, the A_{2A} antagonists were applied to the preparations 40–50 min before testing the effect of the A_{2A} agonists. The effect of the A_{2A} agonist in the absence and in the presence of the tested A_{2A} antagonists was always compared in the same slice. It was previously observed that three successive applications of CGS21680 (10 nM) separated by a 45 min period produce similar facilitation of synaptic transmission. $^{*}P<0.05$ compared to the facilitatory effect of CGS21680 alone. $^{**}P<0.05$ compared to the facilitatory effect of HENECA alone. The results in (c) are mean ± s.e.mean of two to four experiments.

were monophasic (Figure 1). As shown in Figure 1, HENECA was less potent to displace [^{3}H]CGS21680 binding in hippocampal membranes ($K_i = 19$ nM; 95% confidence interval: 6–32 nM; $n = 3$) than in striatal membranes ($K_i = 4.5$ nM; 3.3–5.7 nM; $n = 3$). In contrast, the K_i of ZM241385 was similar in hippocampal and striatal membranes, being 0.52 nM (95% confidence interval: 0.04–1.50 nM, $n = 3$) in the hippocampal membranes and 0.35 nM (0.21–0.50 nM, $n = 3$) in striatal membranes (Figure 1).

In hippocampal membranes, the K_i of ZM241385 to displace [^{3}H]CGS21680 binding (30 nM) in the presence of CSC (200 nM), which pharmacologically isolates 'atypical' CGS21680 binding sites (Cunha *et al*., 1996), was 0.58 nM (0.06–1.86 nM, $n = 4$), confirming that ZM241385 was a potent displacer of [^{3}H]CGS21680 binding to 'atypical' CGS21680 binding sites. In the presence of DPCPX (100 nM), which pharmacologically isolates 'typical' CGS21680 binding sites (Cunha *et al*., 1996), the K_i of ZM241385 to displace [^{3}H]CGS21680 binding (30 nM) to hippocampal membranes was 1.08 nM (0.16–3.12 nM, $n = 4$), confirming that ZM241385 was a nearly equipotent displacer of [^{3}H]CGS21680 binding to 'typical' and 'atypical' CGS21680 binding sites.

Effect of A_{2A} ligands on [^{3}H]acetylcholine release from synaptosomes

To investigate if ZM241385 could antagonize A_{2A}-mediated facilitatory responses in hippocampal preparations, we tested the ability of ZM241385 to inhibit A_{2A}-induced enhancement of veratridine-induced [^{3}H]acetylcholine ([^{3}H]ACh) release from hippocampal synaptosomes, an effect mediated by a 'typical' or in other words a 'striatal-like' A_{2A} receptor (Cunha *et al*., 1995). HENECA (10–30 nM) facilitated [^{3}H]ACh release (Figure 2a) with a concentration–response curve similar to that previously obtained for CGS21680 (see Cunha *et al*., 1995). Within the concentration range tested, 30 nM HENECA produced the greatest facilitation of [^{3}H]ACh release (15 ± 2%, $n = 5$) and ZM241385, in a concentration (20 nM) nearly 25-fold greater than its K_i as an inhibitor of [^{3}H]CGS21680 binding to hippocampal membranes, blocked this facilitatory effect of HENECA ($n = 3$) (Figure 2b). As previously described (Cunha *et al*., 1995), CGS21680 (30 nM) enhanced [^{3}H]ACh release by 14 ± 3% ($n = 4$) and, as shown in Figure 2b, ZM241385 (20 nM) also blocked the facilitatory effect of CGS21680 (30 nM, $n = 4$) an effect mimicked by 200 nM CSC (Cunha *et al*., 1995).

Effect of A_{2A} ligands on synaptic transmission in hippocampal slices

To further test the ability of ZM241385 to antagonize the CGS21680-mediated facilitatory responses in hippocampal preparations, we also investigated whether ZM241385 could inhibit the CGS21680-induced facilitation of synaptic transmission in Schaffer fiber/CA1 pyramid synapses of hippocampal slices. This facilitatory effect of CGS21680 involves the activation of a 3,7-dimethyl-1-propalgylxanthine-sensitive A_2 receptor (Sebastião & Ribeiro, 1992). CSC (2.5 μM) also attenuates the facilitatory effect of 30 nM CGS21680 (de Mendonca & Ribeiro, 1994) but this concentration of CSC does not allow to discriminate between 'typical' and 'atypical' CGS21680 binding sites (see Cunha *et al*., 1996). Thus, we now compared the efficiency of a fully effective concentration of

ZM241385 (20 nM) on hippocampal A_{2A} receptors (see above) and of a lower concentration of CSC (200 nM), which is fully effective on 'typical' hippocampal A_{2A} receptors (Cunha *et al*., 1995) and discriminates 'atypical' limbic CGS21680 binding sites (Cunha *et al*., 1996), to prevent the facilitation of synaptic transmission caused by CGS21680 (10 nM).

CGS21680 (10–30 nM) enhanced synaptic transmission in 11 of the 17 tested slices. In the slices where an effect of CGS21680 was detected, it enhanced synaptic transmission by $17\pm1\%$ ($n=8$) in a 10 nM concentration and by $21\pm2\%$ ($n=4$), although at the highest concentration the effect of CGS21680 was not always fully reverted upon washout. HENECA (10 nM and 30 nM) also facilitated synaptic transmission by $10\pm1\%$ ($n=4$) in a 30 nM concentration and $7\pm1\%$ ($n=3$), respectively. Thus, within the same concentration range, HENECA was less efficient ($P<0.05$) to facilitate synaptic transmission than CGS21680 (Figure 3).

As shown in Figure 3a, ZM241385 (20 nM) prevented the facilitatory effects of both CGS21680 (10 nM) and of HENECA (10 nM). Figure 3b shows the time course of a typical experiment illustrating the ability of ZM241385 to prevent, whereas CSC (200 nM) only attenuated, but did not block, the facilitatory effect of CGS21680 (10 nM). Neither ZM241385 (20 nM) nor CSC (200 nM) caused by themselves any apparent modification of fEPSP slope.

Discussion

The present results show that ZM241385 is a potent displacer of [^{3}H]CGS21680 binding to hippocampal membranes and also behaves as an efficient antagonist of CGS21680-induced facilitatory responses in hippocampal preparations. Thus, ZM241385 was nearly equipotent to displace [^{3}H]CGS21680 binding to A_{2A} receptors in striatal membranes and to 'atypical' [^{3}H]CGS21680 binding sites which predominate in hippocampal membranes. As anticipated, ZM241385 was as efficient as the striatal A_{2A} receptor antagonist CSC to prevent CGS21680-induced facilitation of [^{3}H]ACh release from hippocampal synaptosomes, an effect mediated by 'typical, striatal-like' A_{2A} receptors. Furthermore, even when CSC was found to have low efficacy to inhibit CGS21680-mediated facilitatory effects, such as in the CGS21680-induced facilitation of synaptic transmission, ZM241385 effectively prevented the facilitatory effect of CGS21680 on synaptic transmission.

The present results also extend previous suggestions (Cunha, 1997; Sebastião & Ribeiro, 1996) that there are 'typical' and 'atypical' CGS21680 binding sites, both of which share A_{2A} receptor characteristics. Thus, we now observed that the 'atypical' CGS21680 binding site which predominates in the limbic cortex is not only recognized with nanomolar affinity by CGS21680 but also by another selective A_{2A} agonist, HENECA, and by a potent and selective A_{2A} antagonist, ZM241385. That pharmacologically different facilitatory receptors activated by A_{2A} agonists are present in hippocampal preparations was suggested by the different efficacy of CGS21680 and HENECA to facilitate [^{3}H]ACh release and synaptic transmission in hippocampal preparations: HENECA and CGS21680 are nearly equipotent to facilitate [^{3}H]ACh release, an effect mediated by striatal-like A_{2A} receptors (Cunha *et al*., 1995; Jin & Fredholm, 1997), whereas CGS21680 had greater efficacy than HENECA to facilitate synaptic transmission in Schaffer fiber/CA1 pyramid synapses, an effect where 'atypical' receptors activated by A_{2A} agonists may be involved. Furthermore, the observation that ZM241385 antagonizes CGS21680-induced facilitatory responses in the hippocampus, even when CSC shows little potency, suggests that 'atypical' CGS21680 binding sites might represent a different form of A_{2A} receptors. It is interesting to note that membrane studies have shown the existence of immunological isoforms of A_{2A} receptors (Palmer *et al*., 1992), and different SDS–PAGE spots with a pharmacological displacement profile compatible with A_{2A} receptors have been identified (Nanoff *et al*., 1991; Piersen *et al*., 1994), which might correspond to different processing forms of the single A_{2A} receptor gene, which yields different transcripts (Chu *et al*., 1996; Peterfreund *et al*., 1996). Binding of agonists and antagonists to A_{2A} receptors could involve different receptor amino acid residues (e.g. Jiang *et al*., 1996), so it is possible that different glycosylation patterns and particularly different proteolytic processing of the long carboxy terminal of A_{2A} receptors (Nanoff *et al*., 1991; Piersen *et al*., 1994) might differentially affect agonist and antagonist binding and/or favour coupling of the pleiotropic A_{2A} receptor (e.g. Gubitz *et al*., 1996) to a particular G protein, which eventually results in pharmacologically distinct A_{2A} receptors. However, it remains to be explored whether limbic 'atypical' CGS21680 binding sites might correspond to particular interactions between different established adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) with a peculiar pharmacology, which functionally result in a facilitation similar to that produced by A_{2A} receptor activation or whether differences in drug distribution in the vicinity of the receptors due to differences in lipidic environment of the receptors may occur.

But, irrespective of the type of receptor(s) mediating the facilitatory responses to A_{2A} adenosine receptor agonists, the present work shows that in contrast to previously described A_{2A} antagonist (e.g. CSC), ZM241385 is an efficient antagonist of both CGS21680- and HENECA-induced facilitatory responses in the rat hippocampus.

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