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Short communication

ZM 241385, an adenosine A_{2A} receptor antagonist, inhibits hippocampal A_1 receptor responses

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Abstract

4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3*a*}-{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) has been used as an antagonist of adenosine A_{2A} receptors, exhibiting high selectivity over adenosine A_1 receptors. We now report that ZM 241385 (10–50 nM) attenuated the inhibitory action of N^6-cyclopentyladenosine (10 nM) and $R(-)$-N^6-phenylisopropyladenosine (R-PIA, 20 nM), two selective adenosine A_1 receptor agonists, on hippocampal population spike amplitude. This effect is unlikely to be a direct antagonism of adenosine A_1 receptor since the K_i of ZM 241385 to displace [^{3}H]PIA (2 nM) binding, from hippocampal membranes ranged from 0.8 to 1.9 μM. These results question the usefulness of ZM 241385 to define adenosine A_{2A} receptors actions in functional studies.

Keywords: ZM 241385; Adenosine A_{2A} receptor; Adenosine A_1 receptor; Hippocampus

1. Introduction

Both adenosine inhibitory A_1 and facilitatory A_{2A} receptors contribute to the overall neuromodulatory effects of adenosine in the hippocampus, albeit the dominant response is an inhibitory one through adenosine A_1 receptors (Cunha et al., 1994b, 1996b). The recently synthesised non-xanthine compound ZM 241385 (4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2, 3*a*}-{1,3,5}triazin-5-yl-amino]ethyl)-phenol) displays adenosine A_{2A} receptor antagonist properties with high affinity and selectivity for A_{2A} receptors (Poucher et al., 1995; Ongini et al., 1999). Comparison of the binding characteristics of ZM 241385 in cortical and striatal membranes indicate a 500–1000 fold selectivity for adenosine A_{2A} over A_1 receptors and no significant affinity for adenosine A_3 receptors (Poucher et al., 1995). In addition, several functional studies reveal that low nanomolar concentrations of ZM 241385 effectively counteract the effects resulting from adenosine A_{2A} receptors activation (reviewed by Ongini and Fredholm, 1996), namely in the rat hippocampus (Cunha et al., 1997).

However, we now provide evidence that ZM 241385 attenuates adenosine A_1 receptor inhibition of neuronal excitability in the rat hippocampus.

2. Methods

Electrophysiological recordings were performed in 400 μm thick hippocampal slices from male Wistar rats (5–6 weeks old), as previously described (Cunha et al., 1996b).

Synaptosomes from the hippocampal CA1 area were obtained by sucrose/Percoll differential centrifugation (Cunha et al., 1994c) after separation of the CA1 area (Cunha et al., 1994b). Membranes were prepared as previously described (Cunha et al., 1996c) either from the whole hippocampus, from slices of the isolated CA1 area or from the synaptosomal fraction obtained as described before. Displacement binding curves of [^{3}H]$R(-)$-N^6-phenylisopropyladenosine (PIA, 2 nM) binding by ZM 241385 (10 different concentrations ranging from 0.1 nM to 10 μM) were performed by incubation for 2 h at 37°C in 50 mM Tris and 2 mM $MgCl_2$ in a final volume of 300 μl, as previously described (Cunha et al., 1996c). Non-

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specific binding was evaluated in the presence of 2 μM of {4-[(2-aminoethyl)amino]carbonylmethyloxyphenyl}-xanthine (XAC) and represented nearly 25% of total binding. Membrane protein was determined according to Peterson (1977).

XAC, PIA and N^6-cyclopentyladenosine (CPA) were from Research Biochemicals International. ZM 241385 was from Tocris and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 58261) was generously provided by Dr. E.Ongini (Schering-Plough, Milan). [^{3}H]PIA (63Ci/mmol) was from DuPont-NEN and adenosine deaminase [from calf intestine, 200 U/mg protein — 2 mg/10 ml, solution in 50% glycerol (v/v), 10 mM potassium phosphate, pH approximately 6] from Boehringer Mannheim. All drugs were diluted daily into the appropriate media from 5 mM stock solutions made up in dimethylsulphoxide stored at −20° C, except adenosine, adenosine deaminase and [^{3}H]PIA which were prepared directly into the incubation solution each day and XAC which stock solution was prepared in 0.1 M NaOH.

The values are shown as mean ± S.E.M. of *n* (number of experiments), except the K_i values that are presented as mean (95% confidence interval). The significance of differences was evaluated by the paired Student's *t*-test. *P* values < 0.05 were considered significant.

3. Results

The selective adenosine A_1 receptor agonist, CPA (10 nM), inhibited by 57 ± 7% ($n = 10$, $P < 0.05$) the amplitude of population spikes in CA1 area, an effect prevented by the selective adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (Alzheimer et al., 1991), therefore mediated by adenosine A_1 receptor activation. Upon two successive applications in the same hippocampal slice, CPA elicited a similar inhibition of population spike amplitude (the ratio between CPA inhibitory effect elicited by the second and first application was 1.05 ± 0.05, $n = 2$).

As illustrated in Fig. 1A, the adenosine A_{2A} receptor antagonist, ZM 241385 (50 nM), decreased the inhibitory response of CPA (10 nM). The application of ZM 241385 (50 nM) caused a mild excitatory effect (11 ± 4%, $n = 5$, $P < 0.05$) on basal neuronal excitability. This ability of ZM 241385 to attenuate adenosine A_1 receptor mediated inhibition of neuronal excitability effect was concentration dependent (Fig. 1B). To exclude any direct drug interaction, we also tested the effect of ZM 241385 on the inhibitory response caused by another selective adenosine A_1 receptor agonist, R-PIA. R-PIA (20 nM) caused 74 ± 2% ($n = 3$) inhibition of population spike amplitude. Again, ZM 241385 (50 nM) caused a 54 ± 3% ($n = 3$) attenuation of R-PIA inhibitory effect. This unexpected ability of nanomolar concentration of ZM 241385 to attenuate A_1 receptor mediated effects prompted us to re-assess, in the hippocampus, the reported high selectivity of ZM 241385 over adenosine A_1 receptors. Thus, we performed displacement binding curves of [^{3}H]PIA (2 nM), an adenosine A_1 receptor agonist, by increasing concentrations of ZM 241385 in whole hippocampal membranes (Fig. 2). These experiments revealed a K_i of 0.8 μM [95% confidence interval (CI): 0.5–1.3 μM, $n = 3$]. Since the electrophysiological studies were conducted specifically on CA1 synapses, we repeated the binding experiments with membranes prepared exclusively from CA1 hippocampal area. Again, the K_i for the displacement of [^{3}H]PIA by ZM 241385 was 1.9 μM (95% CI: 1.1–3.2 μM, $n = 3$). Since the inhibitory effect of adenosine A_1 receptor activation on neuronal excitability has mainly a presynaptic nature (reviewed by Thompson et al., 1993), we performed the same binding protocol with membranes of CA1 nerve terminals. Again, low nanomolar concentrations of ZM 241385 were

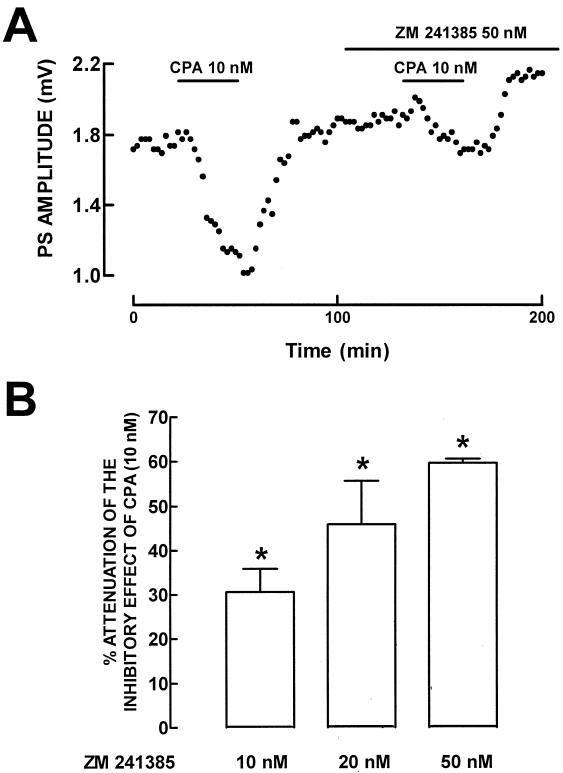

Fig. 1. Attenuation by ZM 241385 of the adenosine A_1 receptor agonist, CPA, inhibition of neuronal excitability in hippocampal slices. In (A), the time course of a single representative experiment is shown. After obtaining a stable population spike (PS) response, CPA (10 nM) was added to the superfusion solution and then washed out. To test the effect of ZM 241385 (50 nM) the drug was added at least 30 min before the second application of CPA. The horizontal bars represent the period of application of each drug to the superfusion solution. In (B), the ordinates represent the average percentage attenuation of the population spike amplitude caused by the concentration of ZM 241385 indicated under each bar. Each value was calculated as the percentage decrease of the effect of CPA (10 nM) in the presence of ZM 241385 in relation to the inhibitory effect of CPA alone in each experiment. The values are mean ± S.E.M of three to four experiments. $^*P < 0.05$ (paired Student *t* test).

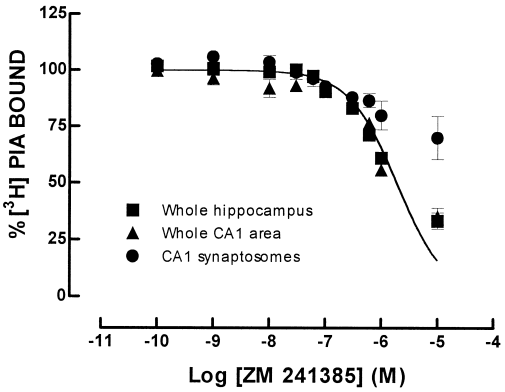

Fig. 2. Displacement binding curves of the adenosine A_1 receptor agonist [^{3}H]PIA (2 nM), by ZM 241385 (0.1 nM to 10 μM) in the indicated hippocampal membranes. The ordinates represent the percentage binding of [^{3}H]PIA compared to control (i.e., absence of ZM 241385). The specific binding, obtained upon subtraction of non-specific binding determined in the presence of 2 μM XAC from the total binding, corresponded to about 75% of total binding, except in membranes from CA1 synaptosomes where the specific binding was 35% of total binding due to the low amount of tissue available. Curves were generated from the average binding parameters obtained upon fitting by non-linear regression assuming a single binding site model for whole hippocampus and CA1 area, which are superimposed. For CA1 synaptosomes no curve is shown since, due to the reasons explained above, no fitting was made. No individual experiment have data points that were best fitted by two independent binding sites (F-test, $P > 0.05$). The results are means ± S.E.M from three experiments performed in triplicate.

ineffective to displace [^{3}H]PIA binding and 10 μM ZM 241385 caused a maximum displacement of 30 ± 4% (n = 3) (Fig. 2).

We then investigated if SCH 58261, a different non-xanthine adenosine A_{2A} receptor antagonist, also attenuated adenosine A_1 receptor-mediated responses. SCH 58261 only partially displaces the binding of the adenosine A_{2A} receptor agonist 2-[4-(2-*p*-carboxy-ethyl)phenylamino]5′-*N*-ethylcarboxamidoadenosine (CGS 21680) to hippocampal membranes (Lindström et al., 1996), precisely the CGS 21680 binding corresponding to striatal-like adenosine A_{2A} receptors (Cunha et al., 1999). CPA (10 nM) caused a 47%–51% and a 44%–45% inhibition of population spike amplitude in the absence and in the presence of SCH 58261 (50 nM), respectively ($n = 2$). By itself, SCH 58261 (50 nM) decreased by 9%–13% population spike amplitude ($n = 2$).

4. Discussion

The major finding in the present work is that low nanomolar concentrations of ZM 241385, a selective adenosine A_{2A} receptor antagonist, attenuated adenosine A_1 receptor-mediated responses in the hippocampus. We have excluded a direct antagonism of adenosine A_1 receptors since the K_i of displacement of the binding of the adenosine A_1 receptor agonist, [^{3}H]PIA, was between 0.8–1.9 μM either in whole hippocampal, whole CA1 area or in CA1 area synaptosomal membranes. These K_i values are similar to those previously obtained in cerebral cortical membranes (Poucher et al., 1995) and contrasts with the subnanomolar K_i of ZM 241385 to displace the binding of the adenosine A_{2A} receptor agonists in the hippocampus (Cunha et al., 1997). Therefore, according to binding criteria ZM 241385 behaved as a selective adenosine A_{2A} versus A_1 receptor ligand in the hippocampus. A direct molecular interaction between CPA and ZM 241385 was also excluded by the observation that ZM 241385 also attenuated the effect of another adenosine A_1 receptor agonist, R-PIA. Furthermore, it was recently reported that the inhibitory effect of ATP on neuronal excitability, caused by activation of A_1 receptors, disappears in the presence of ZM 241385 (O'Kane and Stone, 1999).

A hypothesis that remains to be explored relies on a possible interaction between adenosine A_1/A_{2A} receptors. In order to explain these results, it has to be postulated that adenosine A_{2A} receptors would be tonically enhancing adenosine A_1 receptor inhibitory responses on neuronal excitability, an effect which would disappear upon adenosine A_{2A} receptor blockade with ZM 241385. However, this does not agree with several studies showing that activation of adenosine A_{2A} receptors causes a desensitisation-like effect on adenosine A_1 receptor (Cunha et al., 1994a; Dixon et al., 1997; O'Kane and Stone, 1998; Lopes et al., 1999), an effect blocked rather than mimicked by ZM 241385. Alternatively one could conceive an intense tonic adenosine A_{2A} receptor-mediated facilitation of γ-aminobutyric acid (GABA) release, which when blocked could raise the threshold for inhibition by adenosine A_1 receptor agonists. Activation of adenosine A_{2A} receptors facilitates the evoked GABA release from hippocampal synaptosomes (Cunha et al., 1996a) but the facilitatory effect of adenosine A_{2A} receptor agonists on neuronal excitability is not changed by blockade of GABAergic transmission (Cunha and Ribeiro, unpublished observations) suggesting that facilitation of GABA release elicited by adenosine A_{2A} receptor activation is not directly related to the control of CA1 hippocampal neuronal excitability.

Interestingly, another adenosine A_{2A} receptor antagonist, SCH 58261, did not attenuate adenosine A_1 receptor-mediated responses in the hippocampus. This further reinforces the previous proposal of the existence of pharmacologically different adenosine A_{2A} receptors in the hippocampus (e.g., Cunha et al., 1996c): low amounts of striatal-like adenosine A_{2A} receptors, which are recognised by SCH 58261 and ZM241385, and a predominant 'atypical' adenosine A_{2A} receptor, which is recognised by ZM 241385 but not by SCH 58261 (Cunha et al., 1997, 1999).

Independently of the mechanism involved, the present results raise doubts about how innocuously ZM 241385 acts towards adenosine A_1 receptor mediated effects. In particular great care must be taken on the interpretation of the effects of ZM 241385 resulting from activation of the

facilitatory adenosine A_{2A} receptors without proper control of the functional selectivity of ZM 241385 over inhibitory adenosine A_1 receptors.

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