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Short communication

Characterization of [^{3}H]ZM 241385 binding in wild-type and adenosine A_{2A} receptor knockout mice

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Abstract

The binding of the adenosine A_{2A} receptor antagonist [^{3}H] 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)-phenol ([^{3}H]ZM 241385) to mouse brain and spinal cord was investigated. In brain homogenates, single-site binding was observed with a B_{max} of 299±28 fmol mg^{-1} protein and a K_d of 0.75±0.08 nM. In autoradiographic studies, there was a high density of specific binding of [^{3}H]ZM 241385 in the striatum, with a very low density in the cortex and no binding elsewhere in the brain or in the spinal cord. All specific binding of [^{3}H]ZM 241385 was lost in genetically modified mice lacking the adenosine A_{2A} receptor, confirming the selectivity of this radioligand.

Keywords: Adenosine; Adenosine A_{2A} receptor; ZM 241385; Autoradiography; A_{2A} knockout; (Mouse)

1. Introduction

Adenosine is a neuromodulator in the peripheral and central nervous system, and its effects are mediated via receptors of which four classes are recognised, A_1, A_{2A}, A_{2B} and A_3 (Collis and Hourani, 1993; Fredholm et al., 2001; Ralevic and Burnstock, 1998). In the brain, adenosine A_1 receptors are highly expressed in the cortex, cerebellum and hippocampus, and adenosine A_{2A} receptors are highly expressed in the striatum, while adenosine A_{2B} and A_3 receptors are expressed only at low levels (for review, see Fredholm et al., 2001). The development of adenosine receptor knockout mice (Gimenez-Llort et al., 2002; Ledent et al., 1997) together with the currently available agonists, antagonists and radioligands, can provide new insights into adenosine receptor function.

The selective adenosine A_{2A} receptor agonist 2-[*p*-(2-carboxylethyl) phenylethylamino]-5'-*N*-ethylcarboxamido-adenosine (CGS 21680) has been widely used as a radioligand to label adenosine A_{2A} receptors in the brain (Jarvis and Williams, 1989). However, as well as labelling sites in the striatum, it has also been reported to label 'atypical' sites in the rat brain in extrastriatal structures such as the cortex and hippocampus (Cunha et al., 1996; Johansson and Fredholm, 1995; Johansson et al., 1993). A very recent study has shown that this labelling is lost in adenosine A_1 receptor knockout mice but only slightly reduced in adenosine A_{2A} receptor knockout mice, suggesting that binding may be to a form of the adenosine A_1 receptor (Lopes et al., 2004). Two selective antagonists at the adenosine A_{2A} receptor have been reported to differ in their ability to displace the extrastriatal binding of [^{3}H]CGS 21680: 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) is equipotent at displacing binding to rat brain membranes from striatum and from the hippocampus (Cunha et al., 1997), while (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) is virtually inactive at displacing the binding in the cortex (Lindstrom et al., 1996). Direct examination of the binding of [^{3}H]ZM 241385 binding to rat brain indicated specific binding to cortical membranes (Cunha et al., 1999),

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but autoradiographic studies showed that the binding in the hippocampus and the cortex was weak and failed to saturate (Alexander and Millns, 2001) or was undetectable (DeMet and Chicz-DeMet, 2002).

Although ZM 241385 is selective for the adenosine A_{2A} receptor, it is not completely specific (Fredholm and Lindstrom, 1999; Ongini et al., 1999; Poucher et al., 1995). The availability of genetically modified mice lacking the adenosine A_{2A} receptor (Ledent et al., 1997) provides an ideal model in which to validate the use of this radioligand and to check its receptor selectivity in the mouse. The binding of [^{3}H] 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol ([^{3}H]ZM 241385) to adenosine receptors in the mouse has not been reported before, but in vivo studies of locomotion suggested that, as in the rat, CGS 21680 and ZM 241385 may bind to 'atypical' sites that are not recognised by SCH 57261 and that partly mediate the locomotor depressant effects of CGS 21680 (El Yacoubi et al., 2000). However, drawing conclusions about the receptor binding of ligands from in vivo behavioural studies is difficult because of the different pharmacokinetic profiles of the drugs. We therefore directly investigated the binding of [^{3}H]ZM 241385 in the brains of wild-type and adenosine A_{2A} receptor knockout mice. Some of these results have previously been presented to the British Pharmacological Society (Kelly et al., 2003).

2. Materials and methods

2.1. Animals

Adenosine A_{2A} receptor knockout mice bred on a CD-1 background (Ledent et al., 1997) and their wild-type

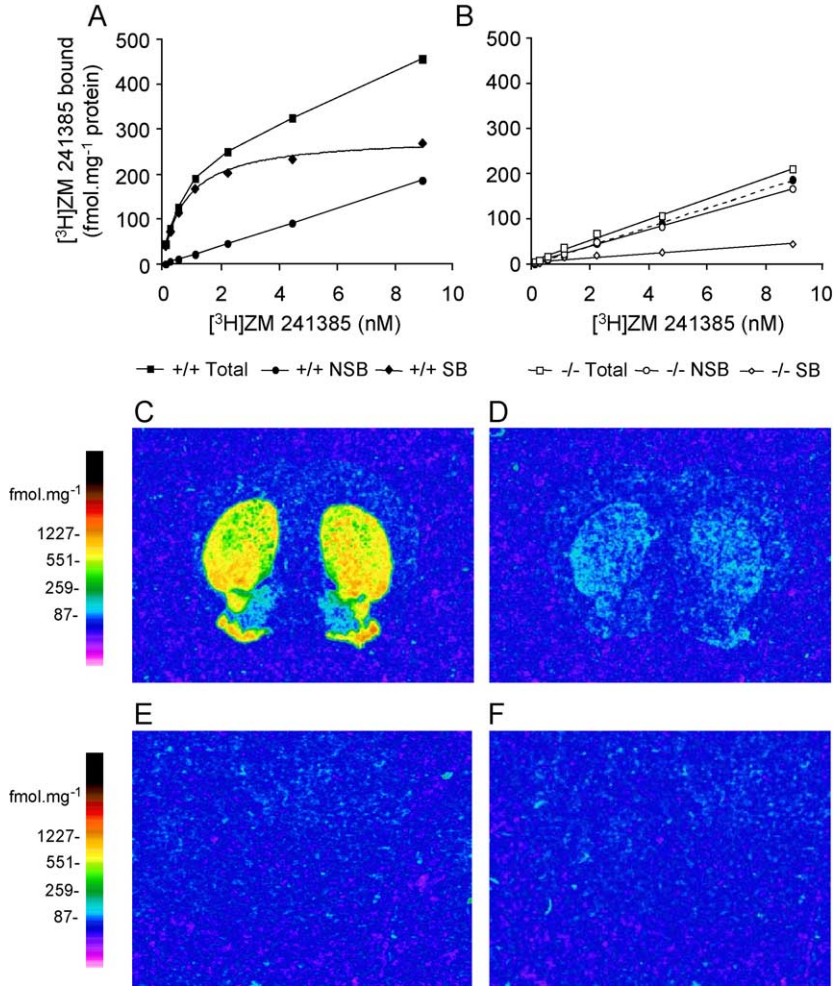

Fig. 1. Binding of [^{3}H]ZM 241385 to wild-type and adenosine A_{2A} receptor knockout mouse brains. (A) and (B) show representative saturation binding curves for the binding of [^{3}H]ZM 241385 to membranes from wild-type (+/+) and adenosine A_{2A} receptor knockout (−/−) mouse brains, respectively. Binding was carried out in triplicate at 25 °C for 30 min, nonspecific binding (NSB) was defined using 5 mM theophylline and specific binding (SB) was calculated by subtracting NSB from total binding. (C)–(F) show representative total (C and E) and nonspecific (D and F) binding of [^{3}H]ZM 241385 (2.5 nM) to coronal sections (bregma point=1.34 mm) of wild-type (C and D) or adenosine A_{2A} receptor knockout (E and F) mouse brains. Binding was carried out for 90 min at room temperature, and nonspecific binding was defined using 5 mM theophylline. The colour bars show pseudocolour interpretation of relative density of black-and-white film image calibrated in fmol mg^{-1} tissue.

littermate controls were obtained from a breeding colony maintained at The University of Surrey (Prentice et al., 2002). Adult male mice aged at least 12 weeks were used in these experiments. All studies were carried out in accordance with protocols approved by the University of Surrey ethics committee and by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

2.2. Binding of [^{3}H]ZM 241385 to brain homogenates

Three brains (minus cerebella) were used for each saturation binding experiment and prepared by repeated homogenisation and centrifugation (Alexander et al., 1994). Saturation binding was carried out in triplicate as described for the rat brain by Alexander and Millns (2001). Briefly, the homogenate was used at 10 mg wet weight ml^{-1}, [^{3}H]ZM 241385 was used in a concentration range of 0.25–16 nM, and the incubation was carried out at 25 °C for 30 min. Nonspecific binding was determined in the presence of 5 mM theophylline. Analysis of the binding data was carried out using GraphPad Prism (Intuitive Software for Science, San Diego).

2.3. Autoradiographic binding of [^{3}H]ZM 241385

Brain and spinal cord sections were prepared, and autoradiographic binding was carried out using the general procedures previously described in our laboratory (Kitchen et al., 1997). Coronal or sagittal sections throughout the brain and sections throughout the spinal cord were cut 20-μm thick at 300-μm intervals, mounted on gelatin-coated slides and dried. [^{3}H]ZM 241385 binding was carried out as described for rat brain by Alexander and Millns (2001). Briefly, the sections were incubated with 2.5 nM [^{3}H]ZM 241385 in buffer [50 mM Tris HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 U ml^{-1} adenosine deaminase, pH 7.4] for 90 min at room temperature. Nonspecific binding was determined in the presence of 5 mM theophylline. At the end of the incubation, the sections were washed three times by immersion for 5 min in ice-cold 170 mM Tris HCl (pH 7.4) and then once by immersion for 5 min in ice-cold reverse osmosis water before being dried in a cold air stream. The sections were then apposed for 16 weeks to [^{3}H]Hyperfilm (Amersham, batch RPN 535B) together with [^{3}H] microscales (Amersham) to allow densitometric analysis.

Quantitative analysis of the brain sections was carried out, as previously described (Kitchen et al., 1997), using an MCID image analyser (Imaging Research, Canada), and brain structures were identified using an atlas (Franklin and Paxinos, 1997).

2.4. Chemicals

[^{3}H]ZM 241385 (specific activity 0.629 TBq mmol^{-1}) was a generous gift from Tocris Cookson, Bristol, UK. All other reagents were of analytical grade and were obtained from Sigma, UK or Fischer Scientific, UK.

3. Results

3.1. Binding of [^{3}H]ZM 241385 to brain homogenates

Saturable specific binding was observed in the homogenate binding studies in tissues from wild-type mice (Fig. 1A), with a B_{max} of 299±28 fmol mg^{-1} protein and a K_d of 0.75±0.08 nM (n=5). Analysis indicated a single-site model, and a two-site model could not be fitted. Specific binding at K_d was 90–95%. Only a low level of non-saturable binding, 5±1 fmol mg^{-1} protein at K_d (n=4), was observed in adenosine A_{2A} receptor knockout brain homogenates, similar to the nonsaturable component of the binding in the tissue from wild-type mice (Fig. 1B).

3.2. Autoradiographic binding of [^{3}H]ZM 241385

High-density specific binding of [^{3}H]ZM 241385 to coronal brain sections from wild-type mice was confined to the caudate putamen, nucleus accumbens, globus pallidus and olfactory tubercle (Table 1), a distribution confirmed by the binding observed in sagittal sections (data not shown). In these areas, specific binding was > 85% (Table 1). A very low level of binding was detectable in the cortex, but in all other regions of the brain, the specific binding was below the limits of detection (<3.0 fmol mg^{-1}), as it was in all areas of the adenosine A_{2A} receptor knockout brain (Fig. 1C–F). No specific binding was detected in the spinal cord at any level in wild-type or adenosine A_{2A} receptor knockout mice. Specific binding in heterozygote brain was approximately

Table 1
Quantitative autoradiography of the binding of [^{3}H]ZM 241385 to brain regions in the wild-type CD-1 mouse

	Specific binding of [^{3}H]ZM 241385 (fmol mg^{-1})	Specific binding %
Bregma=1.34 mm		
Olfactory tubercle	955±87	94.8±0.8
Caudate putamen	526±70	89.0±1.3
Nucleus accumbens		
Core	370±61	89.1±2.9
Shell	261±25	87.3±2.5
Somatosensory cortex		
Superficial	3±1	8.1±2.5
Deep	2±1	3.9±1.0
Bregma=−0.34 mm		
Globus pallidus	170±44	84.7±3.2

The specific binding of 2.5 nM [^{3}H]ZM 241385 (n=5, mean±S.E.M.) to brain regions in wild-type CD-1 mice. Nonspecific binding was defined using 5 mM theophylline. Measures at the regions indicated were taken at the bregma coordinates taken from the atlas of Franklin and Paxinos (1997). Regional determinations were made from both left and right sides of the sections, which were cut 300 μm apart.

50% of that in wild-type brain (data not shown), and there was no binding detected in heterozygote spinal cord.

4. Discussion

Specific binding of [^{3}H]ZM 241385 to the mouse brain was localised to the striatum, with the highest levels being found in the olfactory tubercle and slightly lower levels in the caudate putamen, nucleus accumbens and globus pallidus. Very low levels of binding, at least 100-fold lower than in the striatum, were detectable in the cortex, but binding was undetectable in other brain areas and in the spinal cord. The low levels of binding of the agonist radioligand [^{3}H]CGS 21680 to cortical areas detectable in autoradiographic studies has recently been shown to be lost in A_1 knockout mice but not in A_{2A} knockout mice (Lopes et al., 2004). This suggests that the 'atypical' receptors to which [^{3}H]CGS 21680 has been reported to bind in extrastriatal areas (Cunha et al., 1996; Johansson and Fredholm, 1995; Johansson et al., 1993; Kirk and Richardson, 1995) may result from a combination of a very low level of adenosine A_{2A} receptors together with a form of the adenosine A_1 receptor. In support of the presence of a low level of adenosine A_{2A} receptors in extrastriatal regions, mRNA for the adenosine A_{2A} receptor has been detected in the hippocampus and cortex (Dixon et al., 1996), and Western blots have identified adenosine A_{2A} receptors in nerve terminal membranes from cerebral cortex of wild-type but not of adenosine A_{2A} receptor knockout mice (Lopes et al., 2004). In our studies, the low levels of specific binding of [^{3}H]ZM 241385 detected in the cortex were completely lost in the adenosine A_{2A} receptor knockout mice, suggesting that this ligand, in contrast to [^{3}H]CGS 216870, only labels the adenosine A_{2A} receptor.

The K_d value for [^{3}H]ZM 241385 from the homogenate binding studies of 0.75 nM compares well with that found in rat brain [0.84 nM; (Alexander and Millns, 2001), 1.1 nM; (DeMet and Chicz-DeMet, 2002)] and with the pA_2 values of around 9, originally reported from its in vitro pharmacology in the guinea pig Langendorff heart preparation (Poucher et al., 1995). The autoradiographic density of binding of [^{3}H]ZM 241385 in the caudate putamen (526±70 fmol mg^{-1}) and nucleus accumbens (370±61 fmol mg^{-1} core, 261±25 fmol mg^{-1} shell) reported here for the mouse is similar to that reported for the rat, where it was around 450 and 370 fmol mg^{-1}, respectively, while the density in the olfactory tubercle (955±87 fmol mg^{-1}) was much higher than in the rat, where it was only around 60 fmol mg^{-1} (DeMet and Chicz-DeMet, 2002). Although the concentration of radioligand used in the rat studies was 1.5 nM rather the 2.5 nM used here, this cannot explain the regional discrepancies, which most probably reflect a species difference.

Overall, our results show that [^{3}H]ZM 241385 is a selective high-affinity radioligand for the investigation of adenosine A_{2A} receptors, as in the adenosine A_{2A} knockout mouse brain all specific binding was lost, both in the homogenate studies and the autoradiography.

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