

A role for central A₃-adenosine receptors Mediation of behavioral depressant effects

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The behavioral effects of a selective A₃ adenosine receptor agonist 3-IB-MECA (*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine) in mice and the localization of radioligand binding sites in mouse brain were examined. Low levels of A₃ adenosine receptors were detected in various regions of the mouse brain (hippocampus, cortex, cerebellum, striatum), using a radioiodinated, high-affinity A₃-agonist radioligand [¹²⁵I]AB-MECA (*N*⁶-(3-iodo-4-aminobenzyl)-5'-*N*-methylcarboxamidoadenosine). Scatchard analysis in the cerebellum showed that the *K*_d value for binding to A₃ receptors was 1.39 ± 0.04 nM with a *B*_{max} of 14.8 ± 2.1 fmol/mg protein. 3-IB-MECA at 0.1 mg/kg i.p. was a locomotor depressant with > 50% reduction in activity. Although selective A₁ or A_{2a} antagonists reversed locomotor depression elicited by selective A₁ or A_{2a} agonists, respectively, the behavioral depressant effects of 3-IB-MECA were unaffected. 3-IB-MECA also caused scratching in mice, which was prevented by coadministration of the histamine antagonist cyproheptadine. The demonstration of a marked behavioral effect of A₃ receptor activation suggests that the A₃ receptor represents a potential new therapeutic target.

Adenosine receptor; Xanthine; Locomotor activity; Histamine; Radioligand binding

1. INTRODUCTION

The centrally-mediated locomotor depressant effects of selective A₁ and A_{2a} receptor agonists and their reversal by xanthine antagonists have been well documented [1–3,15]. Activation of both A₁ and A_{2a} receptors, by the A₁-selective agonist CHA (*N*⁶-cyclohexyladenosine) and the A_{2a}-selective agonist APEC (2-[(2-aminoethyl-amino)carbonyl]ethylphenylethylamino]-5'-*N*-ethylcarboxamidoadenosine), results in synergistic locomotor depression [3]. Similarly, although non-selective xanthines, such as caffeine and theophylline, cause stimulation of locomotor activity [1,5], A₁- or A_{2a}-selective xanthines, such as CPX (1,3-dipropyl-8-cyclopentylxanthine) and CSC (8-(3-chlorostyryl)caffeine), respectively, do not elevate locomotor activity except when coadministered [6]. Therefore, the functional roles of A₁ and A_{2a} receptors in the CNS appear to be interrelated. A₁ receptors occur throughout the brain, and activation of presynaptic A₁ receptors results in decreased release of many stimulatory neurotransmitters (see [7]). In contrast, A_{2a} adenosine receptors occur mainly in the striatum and are closely associated with D₂-dopamine receptors [7]. Activation of A_{2a} receptors inhibits a dopaminergic pathway in the striatum.

Recently, a third class of adenosine receptors (A₃) has been cloned from rat brain [8]. A₃ receptors appear responsible for well-documented stimulatory effects of adenosine agonists on release of inflammatory mediators from mast cells [12,20]. As with A₁ receptors, cyclic AMP formation is inhibited by A₃ receptor activation [8]. Phospholipid turnover is also stimulated by A₃ receptor activation [9]. Unlike A₁ and A_{2a} receptors, most alkylxanthines are ineffective as competitors in binding experiments at rat A₃-receptors [8,10] or as antagonists in functional assays [8,9]. The cardiovascular (hypotensive) effects of A₃ receptor stimulation have been surmised indirectly [13], through coadministration of a non-selective agonist, APNEA (1, Fig. 1), and an antagonist that blocks action only at A₁ and A_{2a} receptors.

We recently introduced a class of highly selective adenosine A₃ agonists [11], including 3-IB-MECA (*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine, 2, Fig. 1). A closely related iodinated radioligand, [¹²⁵I]AB-MECA [9], 3, was found to bind to rat brain A₃ receptors expressed in stably-transfected CHO cells with a *K*_d of 1 nM. However, this compound also binds to A₁ receptors in mouse and bovine brain. The *in vivo* effects of selective A₃ agonists have not been characterized. Since stimulation of A₁ and A_{2a} receptors produces demonstrable behavioral effects, the impact of acute administration of the A₃ agonist 3-IB-MECA on locomotor

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tor activity in mice was investigated in this study. The presence of A_3 receptors in regions of the mouse brain was also demonstrated using radioligand binding.

2. EXPERIMENTAL

2.1. Agents

3-IB-MECA and CSC were synthesized as described [6,11]. CPA and CPX were obtained from Research Biochemicals International (Natick, MA). The preparation and characterization of [125 I]-labeled AB-MECA (N^6 -(4-aminobenzyl)-5'- N -methylcarboxamidoadenosine) will be described elsewhere [9].

2.2. Locomotor activity

Adult male mice (NIH Swiss strain, 25–30 g) were housed in groups of 10 animals per cage with a light-dark cycle of 12:12 h. The animals were given free access to standard pellet food and water and were acclimatized to laboratory conditions for 24 h prior to testing. Each animal was used only once in the activity monitor.

Locomotor activity of individual animals was studied in an open field using a Digiscan activity monitor (Omnitech Electronics Inc., Columbus, OH) equipped with an IBM-compatible computer. The computer-tabulated measurements represent multivariate locomotor analysis with specific measures, such as simultaneous measurements of ambulatory, rearing, stereotypical, and rotational behaviors. Data was collected in the morning, for three consecutive intervals of 10 min each, and analyzed as a group. Statistical analysis was performed using Student's *t*-test. The results are reported as mean $\pm$ S.E.M. for each point. All drugs were dissolved in a vehicle consisting of a 20:80 v/v mixture of Alkamuls EL-620 (Rhône-Poulenc, Cranbury, NJ) and phosphate-buffered saline, except for CSC, which was dissolved initially in DMSO and diluted in at least 20 volumes of vehicle. Drugs were administered i.p. in a volume corresponding to 5 ml/kg body weight. Where applicable, the antagonist was injected 10 min before the agonist. After injection of agonist, the mouse was placed in the activity monitor for 5 min before data collection was begun.

2.3. Membrane preparation

Twenty white male mice (NIH Swiss strain, 25–30 g) were killed by cervical fracture. Brains were rapidly removed, placed in ice-cold 50 mM Tris-HCl buffer (pH 7.4), and cortex, cerebellum, hippocampus, brain stem and striatum were dissected. Tissue was homogenized in 20 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a Polytron (setting no. 6) for 10 s. The homogenates were centrifuged at $35,000 \times g$ for 15 min at 4°C. The pellets then were resuspended in fresh volume of the same buffer, homogenized with the Polytron and re-centrifuged. The final pellet was stored at -70°C before use in the receptor binding assay. For the binding assay membranes were diluted at a protein concentration of 1–3 mg/ml. Protein concentrations were determined by the BCA protein assay reagents (Pierce Chemical Co., Rockford, IL) using bovine albumin as a standard.

2.4. Radioligand binding

[125 I]AB-MECA binding to A_3 adenosine receptor in mouse brain membranes was performed in 50 mM Tris, 10 mM MgCl_2 , 1 mM EDTA buffer (pH 7.4) containing 100 μl membrane suspension with adenosine deaminase (3 units/ml) added. Where applicable, the A_1 component of binding was eliminated by the addition of 100 nM CPX to the medium. The final concentration of [125 I]AB-MECA ranged from 0.1 to 4 nM, while the final volume of the preparation was 0.5 ml. Incubations were carried out in duplicate for 90 min at 25°C. Nonspecific binding was defined in the presence of 40 μM *R*-PIA (N^6 -*R*-phenylisopropyladenosine) and constituted approximately 30% of the total binding. Binding reactions were terminated by filtration through Whatman GF/B filters using a Brandel M24R cell harvester (Brandel Gaithersburg, MD). Filters were washed three times with 3 ml ice-cold buffer and placed in vials. Radioactivity was determined in a Beckman 5500B gamma-counter.

2.5. Analysis of data

Linear fitting of Scatchard plots and the saturation experiments, analyzed by nonlinear regression using computer program GraphPad InPlot (Version 4.0, San Diego, CA), gave similar results for determination of K_d and B_{max} values. Each experimental result is reported as mean $\pm$ S.E.M. from three or four experiments.

3. RESULTS

The high affinity radioligand, [125 I]AB-MECA [12], bound specifically to both A_1 and A_3 adenosine receptors in membranes prepared from regions of NIH Swiss mouse brains. Specific binding represented ca. 70% of total binding, with B_{max} values (expressed as fmol specifically bound/mg protein in parentheses): hippocampus (215 ± 17), cortex (159 ± 15), cerebellum (120 ± 5), and striatum (123 ± 23). Scatchard analysis indicated K_d values ranging from 1.9 to 2.8 nM. The A_{2a} selective antagonist 8-(3-chlorostyryl)caffeine (CSC) failed to displace specific binding of [125 I]AB-MECA. The specific binding consisted of two components, since the A_1 selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (CPX) completely displaced the most but not all of the binding with high affinity (sigmoidal competition curves had K_i values ranging from 2 to 8 nM). Thus, the majority of the specific binding of [125 I]AB-MECA was to high-affinity A_1 receptors. The residual binding, which was not displaced by CPX at concentrations as high as 1 μM , represented binding to A_3 adenosine receptors [9]. Low levels of A_3 adenosine receptors (18% of total specific binding using 0.4 nM [125 I]AB-MECA in the presence of 100 nM CPX) were detected in the cerebellum and striatum, with even lower levels in the hippocampus and cortex (7–9% of total specific binding). A Scatchard analysis showed that in the presence of 100 nM CPX the K_d for binding of [125 I]AB-MECA to cerebellar A_3 receptors was 1.39 ± 0.04 nM with a B_{max} of 14.8 ± 2.1 fmol/mg protein ($n = 3$) (Fig. 2).

A potent (K_i 1.1 ± 0.3 nM at rat brain A_3 receptors) and selective (50-fold less potent in binding to either A_1 or A_{2a} rat brain receptors) A_3 agonist [11], 3-IB-MECA (2, Fig. 1), was selected for in vivo studies. The locomotor effects in mice of 3-IB-MECA alone or in combina-

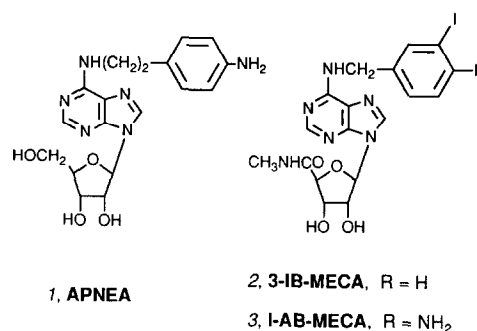


Fig. 1. Structures of adenosine agonists that have been used to characterize A_3 receptors.

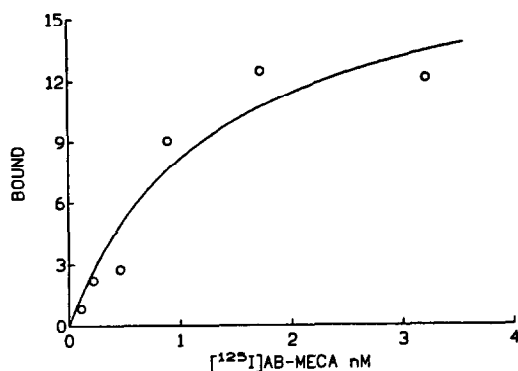


Fig. 2. A representative saturation curve for binding at A_3 receptors in mouse cerebellar membranes using [125 I]AB-MECA in the presence of 100 nM CPX. Conditions are described in section 2 (specific binding shown). The K_d value for binding to A_3 receptors was 1.39 ± 0.04 nM with a B_{max} of 14.8 ± 2.1 fmol/mg protein.

tion with potent and selective A_1 and A_{2a} receptor antagonists were examined. 3-IB-MECA administered i.p. in a dose range of 0.01 to 0.3 mg/kg was found to be locomotor depressant (total distance travelled) with a threshold dose of 0.01 mg/kg and maximal depression reached at ~ 0.1 mg/kg (Fig. 3). Unlike depression elicited by selective A_1 and A_{2a} agonists, the depression did not exceed 90% at the higher doses, but reached a plateau at ca. 60% below control level (Fig. 3). Vertical activity, stereotypy counts, and rotational movement were depressed in a dose dependent manner following administration of 3-IB-MECA, however average distance per move and average speed were not changed significantly.

Administration of 3-IB-MECA also caused rapid scratching behavior, whose frequency appeared to increase with the dose of the A_3 agonist. Since activation of A_3 receptors causes release of histamine in cultured mast cells, it was proposed that the scratching could be related to histamine. Coadministration of a histamine H_1 -antagonist, cyproheptadine at 10 mg/kg i.p. eliminated this behavior, while at 1 mg/kg dose of cyproheptadine the effect was only partial.

The highly A_1 -selective antagonist CPX (0.25 mg/kg) completely reversed locomotor depression elicited by the potent A_1 agonist CPA at its determined ED_{50} value of 100 μ g/kg i.p. (Fig. 4). CPX did not diminish the depressant effects of either the A_{2a} -selective agonist APEC or 3-IB-MECA at doses chosen to cause comparable ca. 50% reduction in locomotor activity.

The xanthine antagonist CSC is highly A_{2a} -selective in binding assays, functional adenylate cyclase assays, and in vivo with respect to locomotor activity [6]. A dose of CSC of 1 mg/kg caused a small, statistically insignificant reversal of the 3-IB-MECA-mediated locomotor depression (Fig. 4). Previously it was shown that the same dose of CSC caused a complete reversal of the behavioral depression elicited by APEC in the same experimental model [6].

4. DISCUSSION

A_3 receptors were found to be present in the mouse brain, with highest density in the cerebellum and striatum. Thus, the striatum, which is critical to the locomotor depressant effects of A_{2a} agonists [21], contains A_3 receptors at a density at least 10-fold lower than A_{2a} receptors. Since previously reported levels of mRNA coding for A_3 receptors in rat brain indicated the greatest density in hippocampus and cerebellum [14], there is either a species difference between rat and mouse, or the level of message is not entirely predictive of the level of receptor density. A_1 receptors are localized in high density in the hippocampus and appear to be involved in the cerebroprotective effects of adenosine [16]. A_3 receptors were also detected in the hippocampus but at densities 1–2 orders of magnitude lower than A_1 receptors.

The non-reversal of locomotor depression by the A_1 - and A_{2a} -adenosine antagonists is consistent with in vitro observations with rat A_3 -receptors, i.e. the inability of xanthines to antagonize at this site [8,10]. Both CPX and CSC are known to act centrally [6]. The lack of a selective A_3 antagonist, to act peripherally or centrally, is a disadvantage in this study. Previous studies of locomotor depression by A_1 - and A_{2a} -adenosine agonists [3] have utilized both centrally-active xanthines and other xanthines, such as 8-sulphophenyltheophylline, that do not penetrate the blood brain barrier, to demonstrate the central nature of the behavioral effects.

There is considerable interest in the therapeutic potential of selective A_1 -adenosine agonists and antagonists to treat neurological and other CNS disorders. The cognition-enhancing [16,17], cerebroprotective [18], and anti-convulsant [19] effects of selective A_1 -adenosine agents have frequently been demonstrated. A_{2a} -antagonists may be useful in treating Parkinson's disease [7]. The demonstration of a marked behavioral effect of A_3 receptor activation suggests that the A_3 receptor may also represent a unique therapeutic target.

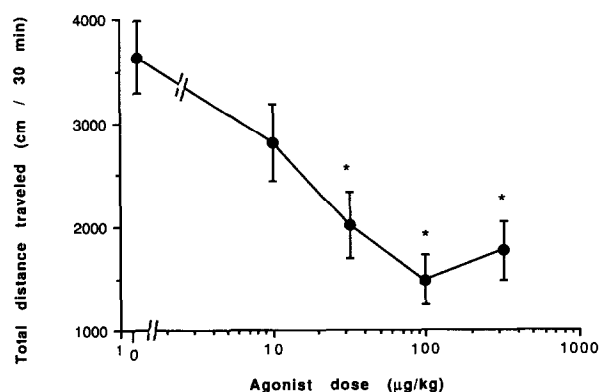


Fig. 3. Locomotor activity in male NIH Swiss mice. Effect of the A_3 -selective adenosine agonist 3-IB-MECA. * P value is < 0.05 vs. vehicle control ($n = 6-19$).

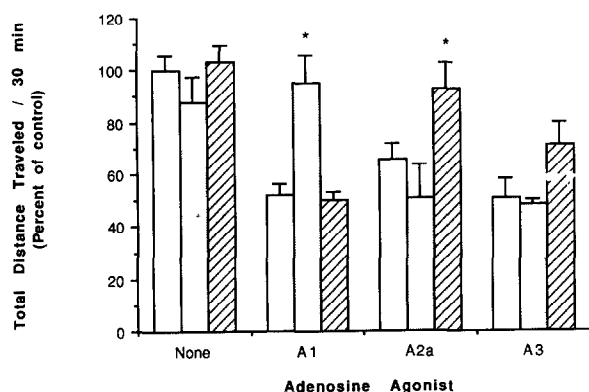


Fig. 4. Locomotor activity in mice following injection of an A_1 - (CPA, 100 μ g/kg), A_{2a} - (APEC, 16 μ g/kg), or A_3 - (3-IB-MECA, 100 μ g/kg) selective agonist and the effects of coadministration of selective antagonists ($n = 6-19$). Locomotor activity as a percent of control is shown for no antagonist (unshaded bars) or for coadministration of selective xanthine antagonists: CPX (shaded bars, A_1 , 0.25 mg/kg) or CSC (hatched bars, A_{2a} , 1.0 mg/kg). * P value < 0.05 vs. adenosine agonist alone.

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