A1- and A2-Selective Adenosine Antagonists: In Vivo Characterization of Cardiovascular Effects

GARY EVONIUK, KENNETH A. JACOBSON, MAH T. SHAMIM, JOHN W. DALY and RICHARD J. WURTMAN

Laboratory of Neuroendocrine Regulation, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology (R.J.W.), Cambridge, Massachusetts and Laboratories of Chemistry (G.E., M.T.S., J.W.D.), National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Accepted for publication May 15, 1987

ABSTRACT

Caffeine, a nonselective adenosine receptor antagonist, 7-methyl-1,3-dipropylxanthine, a purported A2 selective antagonist and a 1,3-dipropyl-8-phenylxanthine amine congener (XAC), an A1 selective antagonist, were evaluated for their in vivo selectivity at A1 vs. A2 adenosine receptors. Blockade of the negative chronotropic effect of bolus i.v. injections of 2-chloroadenosine, R-phenylisopropyladenosine and N-ethylcarboxamidoadenosine was utilized as an index of antagonism at A1 receptors; blockade of the hypotensive effect of the same series of adenosine agonists was used as an index of activity at A2 receptors. In addition, blockade of the potentiating effect of adenosine on the hypertensive and chronotropic effects of nicotine was studied to assess further the role of A1 and A2 adenosine receptors in this response. The potent antagonist XAC displayed considerable A1 selectivity as demonstrated by blockade of adenosine receptor-mediated bradycardia at doses 5- to 10-fold lower than those antagonizing adenosine receptor-mediated hypotension. XAC also selectively blocked potentiation by adenosine of the positive chronotropic effect of nicotine, at doses which had minimal effects on the enhancement of the hypertensive effect of nicotine. The caffeine homolog 7-methyl-1,3-dipropylxanthine exhibited A2 selectivity as demonstrated by prevention of adenosine receptor-mediated hypotension at doses which only minimally attenuated the bradycardiac effect of adenosine agonists. Caffeine displayed no selectivity for A1 vs. A2 adenosine receptors. The results indicate that selective analogs such as XAC and F-methyl-1,3-dipropylxanthine will be useful probes for investigation of receptors involved in the physiological functions of adenosine.

Adenosine acts as a physiological modulator of coronary blood flow (Berne, 1980) and possesses negative chronotropic, inotropic and dromotropic effects on the heart (Drury and Szent-Gyorgyi, 1929; Schrader et al., 1975). These actions appear to be mediated by adenosine receptors, which have been tentatively classified into A1 and A2 subtypes based on anatomical and pharmacological criteria (van Calker et al., 1979; Londos et al., 1980). Based on the relative potencies of adenosine agonists, it has been proposed that activation of A2 receptors produces coronary and systemic vasodilation, resulting in hypotension (Mustafa and Askar, 1985; Collis and Brown, 1983; Kusachi et al., 1983). In contrast, the effects of adenosine on cardiac rate and contractility are thought to be mediated by atrial A1-adenosine receptors (Collis, 1983; Haleen and Evans, 1985).

The nonselective adenosine receptor antagonist theophylline is known to possess prominent effects at the heart as well as at coronary and systemic blood vessels (Fredholm, 1984). Recently, adenosine antagonists have been synthesized which appear to be selective for either A1 or A2 receptor subtypes in vitro (Daly et al., 1986; Jacobson et al., 1985; Ukena et al., 1986a,b). The potent 1,3-dipropyl-8-phenylxanthine amine congener, XAC (fig. 1), was A1 selective at central receptors by about 40-fold (Ukena et al., 1986). Unlike many 8-phenyl substituted xanthines shown previously to be A1-selective in receptor binding studies (Bruns et al., 1983), XAC is sufficiently water-soluble to permit in vivo studies. Indeed, Fredholm et al. (1987) demonstrated a 20-fold A1-selectivity for XAC in vivo in the antagonism of the cardiovascular effects of the potent adenosine analog NECA. Recently, a series of homologs of caffeine were shown to be somewhat A2 selective in studies of receptor binding and effects on adenylyl cyclase (Daly et al., 1986; Ukena et al., 1986b): MDPX (fig. 1), 1-propargyl-3,7-

ABBREVIATIONS: XAC, xanthine amine congener; NECA, 5'N-ethylcarboxamidoadenosine; MDPX, 7-methyl-1,3-dipropylxanthine; R-PIA, R-phenylisopropyl adenosine; 2-CADO, 2-chloroadenosine; HPLC, high-performance liquid chromatography; XCC, xanthine carboxylic acid congener; ANOVA, analysis of variance.

Received for publication November 25, 1986.

1This work was supported by grants from The International Life Sciences Institute and the Center for Brain Sciences and Metabolism Charitable Trust.

2Present address: Laboratory of Neuroscience, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892. Recipient of Fellowship Grant MH 09197-02 from the U.S. Public Health Service and a National Institute of General Medical Sciences Pharmacology Research Associate Training Fellowship.
of concentrations as low as 0.10 μg/ml (XAC) and 0.02 μg/ml (XCC).

We have now examined the selectivity of XAC and MDPX with respect to antagonism of hypotension (A2) or bradycardia (A1) induced in rats by the adenosine analogs NECA, R-PIA and 2-CADO, and with respect to antagonism by XAC of the potentiating effect of adenosine on the chronotropic and hypertensive responses to nicotine.

Methods

Animals. Male Sprague-Dawley rats (200-250 g, Charles River Breeders, Wilmington, MA) were anesthetized with pentobarbital (50 mg/kg i.p.) and a catheter was implanted in the right jugular vein for i.v. drug administration. The left carotid artery was cannulated to allow periodic withdrawal of blood samples for assay of adenosine and XAC levels and direct measurement of blood pressure and heart rate. Blood pressure was monitored with Statham (Hato Rey, PR) 23PD pressure transducers interfaced with a Grass model 7C polygraph. Heart rate was monitored via a Grass model 7P4A4 cardiometer.

Plasma XAC determinations. Arterial blood samples (0.2 ml) were withdrawn and allowed to clot on ice. Serum was separated after centrifugation and frozen for later assay. Thawed samples were deproteinated by the addition of an equal volume of 15% trichloroacetic acid followed by centrifugation and neutralization of the supernatant by addition of excess calcium carbonate (finely powdered). In some experiments animals were sacrificed by introduction of a fatal air embolism, whole brains were then excised and homogenized in 5 volumes (w/v) of 0.4 M perchloric acid and centrifuged at 20000 x g for 20 min. An aliquot was subsequently neutralized with calcium carbonate. Serum and brain samples were analyzed by HPLC (Beckman model 344) using a C8 reverse-phase column (Alltech, cartridges) with isocratic elution (85% methanol in 0.05 M triethylammonium trifluoracetate) at a flow rate of 1.0 ml/min. XAC and its carbosylic acid derivative, XCC, were detected by UV absorbance at 280 nm (retention times of 10 and 6 min, respectively), allowing quantitation of concentrations as low as 0.10 μg/ml (XAC) and 0.02 μg/ml (XCC).

Plasma adenosine determinations. Plasma adenosine levels were measured as described previously (von Borstel et al., 1986). Briefly, arterial blood samples were withdrawn into ice-cold saline containing 0.5 mM dipyridamole (Boehringer Ingelheim, Ridgefield, CT) to inhibit erythrocyte uptake of adenosine and 2.5 μM N6,N6-dimethylguanosine (P-L Biochemicals, Milwaukee, WI) as an internal standard. After centrifugation and deproteinization plasma adenosine levels were determined by HPLC.

Drug treatments. All drugs were dissolved in 0.9% saline except XAC, which was dissolved initially in a small volume (10% v/v) of 0.1 N NaOH and subsequently diluted with saline. The same vehicle injected into control animals produced no changes in blood pressure, heart rate nor their responses to adenosine or adenosine analogs. XAC, MDPX, nicotine and the adenosine analogs 2-CADO, R-PIA and NECA were administered as i.v. bolus injections. Caffeine was administered by i.p. injection. After determination of control responses to the adenosine agonists (2-CADO, R-PIA and NECA), one to two doses of a single antagonist (caffeine, XAC or MDPX) were tested in each animal. The antagonist was injected, followed by a 10-min (20 min for caffeine) equilibration period, after which responses to agonists were retested. A recovery time of at least 5 min was allowed to elapse between successive agonist injections to allow heart rate and blood pressure to return to baseline.

In another set of animals adenosine (5 mg/ml in 0.9% saline) was administered as a continuous i.v. infusion at rates of 10 to 100 μl/min using a Harvard syringe pump (Harvard Apparatus Co., Inc., South Natick, MA). After determination of control responses to nicotine and withdrawal of an arterial blood sample for determination of plasma adenosine levels, adenosine was infused for 10 min followed by withdrawal of an addition blood sample and reinfusion of nicotine. This procedure was repeated for each of three infusion rates of adenosine.

Adenosine, nicotine, caffeine and 2-CADO were obtained from Sigma Chemical Co. (St. Louis, MO). R-PIA and NECA were obtained from Research Biochemicals, Inc. (Wayland, MA). XAC (8-[4-[[[(2-aminomethyl)-aminol]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine) and XCC (8-[[carboxymethyl]oxy]phenyl]-1,3-dipropylxanthine) and MDPX were synthesized as described previously (Daly et al., 1986; Jacobson et al., 1985).

Statistics. Data are expressed as means ± S.E.M. and compared by replicated ANOVA followed by selected comparisons utilizing the Newman-Keuls test.

Results

Effect of XAC, MDPX and caffeine on cardiovascular responses to adenosine analogs. We tested the abilities of XAC, MDPX and caffeine to block the hypertensive and negative chronotropic effects of the adenosine analogs 2-CADO, R-PIA and NECA. The peak fall in diastolic blood pressure and heart rate was recorded after i.v. bolus injections (0.1 ml) of 10, 5 and 1.0 μg/kg of 2-CADO, R-PIA and NECA, respectively. Responses were tested before and after (within 30 min) administration of xanthine or vehicle. Responses after vehicle treatment were consistently within 5 to 10% of initial responses. Neither caffeine, XAC nor MDPX affected basal blood pressure or heart rate within the dose ranges tested (data not shown). XAC (fig. 2) was the most potent xanthine tested by 1 to 2 orders of magnitude. The cardiovascular effects of the three adenosine analogs were blocked substantially at doses of XAC of 1.0 mg/kg i.v. or less. XAC displayed selectivity for adenosine receptors affecting heart rate. It reduced heart rate responses to 2-CADO, R-PIA and NECA by 50% at a dose of 0.05 mg/kg or less; equivalent inhibition of hypotensive responses required doses of XAC in the 0.1 to 1.0 mg/kg range. MDPX displayed moderate selectivity for vascular adenosine receptors as evidenced by a relatively greater potency against blood pressure responses than against heart rate responses. A dose of 5 mg/kg i.v. of MDPX inhibited hypotensive responses to adenosine analogs by 50%. 10 mg/kg or more was required to inhibit bradycardia by 50%. Caffeine (fig. 3) displayed no selectivity for heart rate vs. blood pressure responses to the adenosine
agonists. Fifty percent inhibition of both responses was observed at doses of 10 to 25 mg/kg i.p.

Dose response curves were constructed for the hypotensive and bradycardic effects of 2-CADO in the absence and presence of MDPX (5 mg/kg i.v.) or XAC (0.05, 0.1 and 0.2 mg/kg i.v.). All three doses of XAC (fig. 4) inhibited the bradycardic effects of 2-CADO; responses to 2-CADO (1-100 μg/kg i.v.) were reduced by 50 to 80%. However, the effect of XAC on blood pressure was insignificant, reducing the hypotensive effect of 2-CADO by less than 15 to 20 mm Hg at any given dose. In contrast, MDPX was more potent in reducing the hypoten-
with the exception of the 25-μg/kg dose. Heart rate responses were reduced significantly (P < .05-Newman Keuls) by MDPX.

In previous studies we demonstrated that the adenosine agonists 2-CADO, R-PIA and NECA were antagonized by the selective adenosine antagonists displaying selectivity for the A1 or A2 adenosine receptor subtypes. We have demonstrated here the in vivo A1 selectivity of the 1.3 dipropyl-8-phenylxanthine amine congener XAC, and to a lesser extent the A2 selectivity of the caffeine homolog MDPX.

**Effect of XAC on potentiating by adenosine of responses to nicotine.** In previous studies we demonstrated that adenosine profoundly potentiates cardiovascular responses to nicotine (von Borstel et al., 1986). Small elevations in circulating adenosine levels that produce minimal effects on basal blood pressure and heart rate increase dramatically pressor and chronotropic responses to nicotine. We tested two doses of XAC to determine whether it displayed selectivity for cardiac vs. vascular responses to nicotine (40 μg/kg i.v.) when plasma adenosine levels were elevated by an i.v. adenosine infusion (fig. 6). XAC (0.2 and 0.5 mg/kg i.v.) had no significant effect on the hypertensive or chronotropic effects of nicotine in control animals (fig. 6, open bars). Elevation of plasma adenosine levels to 2 μM or more, produced by infusing adenosine i.v., potentiated the cardiovascular effects of nicotine, increasing by 3- to 5-fold the hypertensive and positive chronotropic effects (fig. 6, shaded bars; ANOVA P < .001). XAC (0.2 and 0.5 mg/kg i.v.) significantly reduced the chronotropic effect of nicotine when arterial adenosine levels were elevated; at adenosine concentrations of 1.8 to 2.7 μM, the chronotropic effect was reduced by 50% (Newman Keuls, P < .05). In contrast, the same XAC doses produced only minimal attenuation of the hypertensive effect of nicotine; 0.2 mg/kg XAC had no significant effect whereas 0.5 mg/kg produced less than 20 to 25% inhibition of the hypertensive effect.

**Effect of XAC dose on plasma concentrations.** Plasma concentrations of XAC and its metabolite, XCC (a xanthine carboxylic acid congener), were monitored in rats receiving various i.v. doses of the drug (table 1). Measurable quantities of XAC were observed in rats receiving injections of 0.05 mg/kg or more. The plasma half-life of XAC appeared to be on the order of 30 min. Its volume of distribution, 0.18 to 0.20 liters/kg, corresponded to the volume of extracellular water (plasma plus interstitial fluid). Brain levels of XAC remained undetectable after injection of as much as 0.5 to 1.0 mg/kg i.v.

**Discussion**

A recent goal of pharmacologic investigation of adenosine and the methylxanthines has been the development of potent and bioavailable adenosine antagonists displaying selectivity for the A1 or A2 adenosine receptor subtypes. We have demonstrated here the in vivo A1 selectivity of the 1.3 dipropyl-8-phenylxanthine amine congener XAC, and to a lesser extent the A2 selectivity of the caffeine homolog MDPX.

Antagonism of cardiac and systemic vascular responses to the adenosine agonists 2-CADO, R-PIA and NECA was utilized as the initial test for selectivity at cardiovascular adenosine receptors. The doses of agonists utilized produced nearly equivalent decrements in blood pressure (42, 36 and 58 mm Hg, respectively) and heart rate (58, 68 and 55 bpm). Thus, whereas...
COIlCMtr8tiona of XAC end XCC their physiological effects at the doses utilized. here indicate a

tencies in blocking both the bradycardiac and hypotensive

trations R-PIA is considered a partially selective A1 agonist,

(P < .05) effect of XAC on heart rate response. . P < .05 compared to

in lJitro

previous

Data .. expressed as IM81$ % S.E.~. (n ~ 4). P < .05 (~y AHOVA) for

at same

vehicle

effect 01 time. dose on plasma )CAC COlICIN ob atiolls.

Effect of dose .nd time .Iter injection on pl88118 nd brain

TABLE 1

Effect of dose and time after injection on plasma and brain

contifications of XAC and XCC

Data are expressed as means ± S.E.M. (n ≥ 4). P < .05 (two-way ANOVA) for
effect of time, dose on plasma XAC concentrations.

<table>
<thead>
<tr>
<th>Conc.</th>
<th>10 min after injection</th>
<th>45 min after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Brain</td>
</tr>
<tr>
<td>mg/kg</td>
<td>µg/ml</td>
<td>µg/ml</td>
</tr>
<tr>
<td>0.05</td>
<td>0.45 ± 0.07</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>0.1</td>
<td>0.93 ± 0.11</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>0.5</td>
<td>1.50 ± 0.31 0.078 ± 0.016 0.84 ± 0.14 0.078 ± 0.028 &lt;.10</td>
<td>0.79 ± 0.32</td>
</tr>
<tr>
<td>1.0</td>
<td>2.65 ± 0.45 0.103 ± 0.028 0.95 ± 0.03 1.114 ± 0.016 &lt;.10</td>
<td>1.0 ± 0.21</td>
</tr>
</tbody>
</table>

NECA is extremely potent at A2 receptors and at low concentrations R-PIA is considered a partially selective A1 agonist, their physiological effects at the doses utilized here indicate a mixture of both A1 (negative chronotropic) and A2 (vasodilating) actions.

The adenosine antagonist caffeine displayed indetical potencies in blocking both the bradycardiac and hypotensive effects of the three adenosine analogs, in agreement with previous in vitro results demonstrating its lack of selectivity for A1 vs. A2 receptors (Daly et al., 1986). In contrast, the functionalized congener XAC was at least 10-fold more potent in vivo than any other antagonist tested and displayed a 10-fold greater than those required to produce similar blockade of A1 receptor-mediated bradycardia.

In addition, XAC selectively antagonized adenosine-mediated potentiation of the positive chronotropic effect of nicotine. Previous studies have demonstrated that a slow i.v. adenosine infusion which, by itself, produces minimal hypotension and bradycardia profoundly enhances the hypertensive and positive chronotropic effects of nicotine (von Borstel et al., 1986). The persistence of this effect after decentralization of sympathetic ganglia suggests that the locus of interaction between adenosine and nicotine lies within or distal to the sympathetic ganglia in which nicotine acts. In the present study XAC (0.2-0.5 mg/kg i.v.) blunted the potentiating effect of adenosine on cardiac rate, but had only a minimal effect on enhancement of the hypertensive effect of nicotine. Two non-selective antagonists, caffeine and 8-(p-sulfophenyl)-theophylline have been shown previously to block adenosine-mediated potentiation of both the tachycardiac and hypertensive effects of nicotine (von Borstel et al., 1986; Evoniuk et al., 1987). Thus, selective access of xanthine antagonists to adenosine receptors mediating potentiation of the cardiac effects of nicotine is an unlikely basis for the selectivity exhibited by XAC. Instead, it appears that either ganglionic adenosine receptors are the site of the nicotine-adenosine interaction, their subtypes mirroring those found at the end organs (i.e., A1 for cardiac and A2 for vascular smooth muscle) or alternatively, that pre- or postsynaptic adenosine receptors within the tissues are the site of the potentiating effect of adenosine on nicotine.

Additional studies provided data on the pharmacokinetic fate of XAC. Brain XAC levels remained undetectable after injection of as much as 1.0 mg/kg i.v. This inability to cross freely the blood brain barrier is likely a result of the presence of a charged ammonium group at physiological pH. The volume of distribution (0.18-0.20 liters/kg) corresponded to the volume of extracellular water, as expected for a relatively polar, watersoluble drug. The disappearance of XAC from plasma occurred with a half-time on the order of 30 min, somewhat faster than was indicated by the preliminary data reported by Fredholm et al. (1987). This discrepancy could be due to different routes of administration used in the two studies (i.v. vs. i.p.) or might reflect differences in animal strains. A predicted metabolite XCC was detected, but at levels roughly 5% of the level of XAC. The presumed route for the formation of this metabolite is by enzymatic hydrolysis of XAC, which also liberates an equivalent of ethylene diamine (see fig. 1). Taken together these data indicate that, in vivo, XAC acts as a remarkably potent, A1-selective adenosine receptor antagonist with high water solubility and subsequent bioavailability.

The present studies also demonstrate a degree of in vivo selectivity for the caffeine analog MDPX. Previously this compound has demonstrated 5- to 6-fold selectivity for A2 vs. A1 receptors in vitro (Daly et al., 1986; Ukena et al., 1986b). Within the dose range of 2.5 to 5.0 mg/kg, MDPX antagonized the hypotensive effects of adenosine analogs by 40 to 50% or more, while decreasing their chronotropic effects by 25% or less. MDPX (5 mg/kg) also antagonized significantly the hypertensive effect of several doses of 2-CADO while causing minimal blockade of the bradycardiac effect. Preliminary studies (G. Evoniuk, unpublished results) have indicated that other caf-
Feine homologs displaying in vitro A2 selectivity such as 1-propargyl-3,7-dimetanthine and 7-propyl-1,3-dimetanthine do not exhibit significant in vivo selectivity under the same conditions. Thus, although not displaying the same degree of selectivity for A1 vs. A2 receptors as XAC, MDPX nevertheless does exhibit a greater degree of in vivo selectivity for A2 receptors than any adenosine antagonist investigated to date.

In summary, the functionalized congener XAC was demonstrated to be a potent and selective A1 adenosine receptor antagonist as assessed by its ability in vivo to prevent the bradycardiac effects of several adenosine analogs. In addition, XAC selectively antagonized the potentiating effect of adenosine on the positive chronotropic effect of nicotine, while having little effect on potentiation of the hypertensive effect of nicotine. The caffeine homolog MDPX displayed moderate A2 selectivity in vivo under the same conditions. These drugs may provide a rational basis for the development of therapeutically useful adenosine receptor antagonists.

References
BRUNA, R. F., DALY, J. W. AND SNYDER, S. H.: Adenosine receptors in brain membranes: Binding of 