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A1- and A2-Selective Adenosine Antagonists: *In Vivo* Characterization of Cardiovascular Effects[1]

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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 analogs. 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.*, 1986a). 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 adenylate cyclase (Daly *et al.*, 1986; Ukena *et al.*, 1986b): MDPX (fig. 1), 1-propargyl-3,7-

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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.

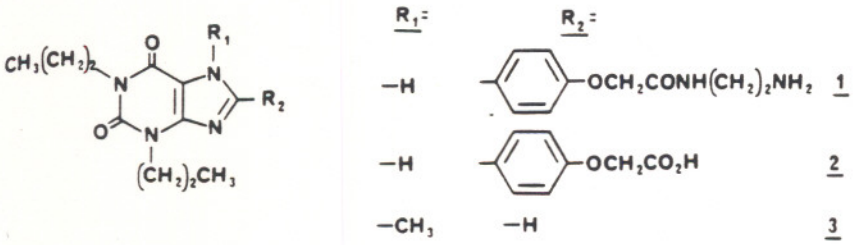

Fig. 1. Structures of XAC (1), XCC (2) and MDPX (3).

dimethylxanthine and 7-propyl-1,3-dimethylxanthine were shown to be A2 selective by 6- to 10-fold. The caffeine homologs have not been investigated for *in vivo* selectivity.

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 anesthesized 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 7P44 cardiotachometer.

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 deproteinized 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 emoblism, whole brains were then excised and homogenized in 5 volumes (w/v) of 0.4 M perchloric acid and centrifuged at 20000 × *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 C_{18} reverse-phase column (Altex Ultrasphere ODS, 4.6 mm × 25 cm) with isocratic elution (65% methanol in 0.05 M triethylammonium trifluoroacetate) at a flow rate of 1.0 ml/min. XAC and its carboxylic 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 N^2,N^2-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 μl/mg) of 0.1 N NaOH and subsequently diluted with saline. The same vehicle injected into control animals produced neither 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 base line.

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 reinjection 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-aminoethyl)-amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine), XCC (8-[4-[(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 hypotensive 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

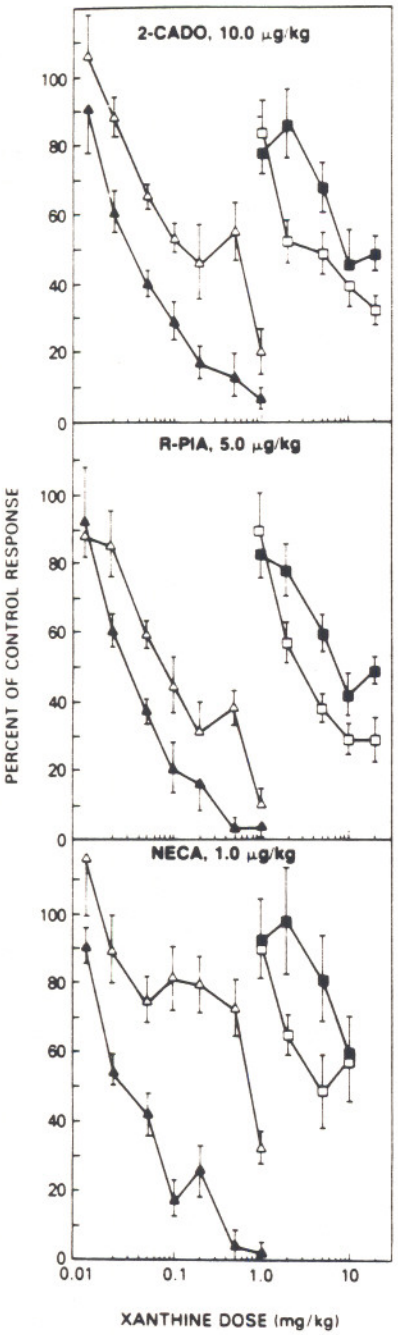

Fig. 2. Effect of XAC and MDPX doses on hypotensive and negative chronotropic responses to adenosine analogs. Blood pressure (open symbols) and heart rate responses (filled symbols) to the adenosine analogs 2-CADO, *R*-PIA and NECA were monitored in rats pretreated with varying doses of XAC (triangles) or MDPX (squares). Results are expressed as percentage of control and reported as means ± S.E.M. (Control blood pressure responses = −42, −36 and −58mm Hg; control heart rate responses = −58, −68 and −55 beats/min for 2-CADO, *R*-PIA and NECA, respectively; n ≥ 5). One-way ANOVA indicates significant ($P < .05$) effect of XAC and MDPX doses on blood pressure and heart rate responses to all three adenosine analogs except MDPX *vs.* NECA-heart rate response.

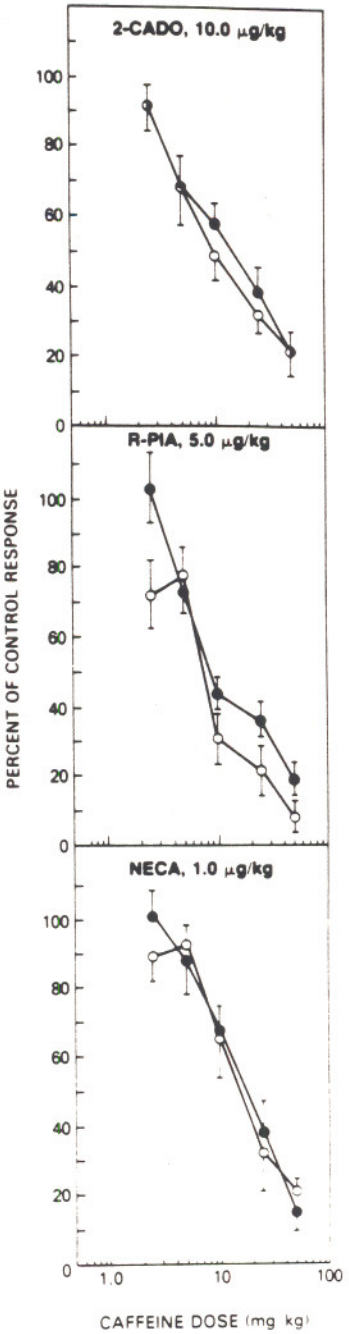

Fig. 3. Effect of caffeine doses on hypotensive and negative chronotropic responses to adenosine analogs. Blood pressure (open symbols) and heart rate responses (filled symbols) to the the adenosine analogs 2-CADO, *R*-PIA and NECA were monitored in rats pretreated with varying doses of caffeine. Results are expressed as percentage of control and reported as means ± S.E.M. (see fig. 1 for control responses; $n \geq 6$). One-way ANOVA indicates significant ($P < .001$) effect of caffeine dose on blood pressure and heart rate responses to all three adenosine analogs.

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 bradycardiac 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 bradycardiac 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-

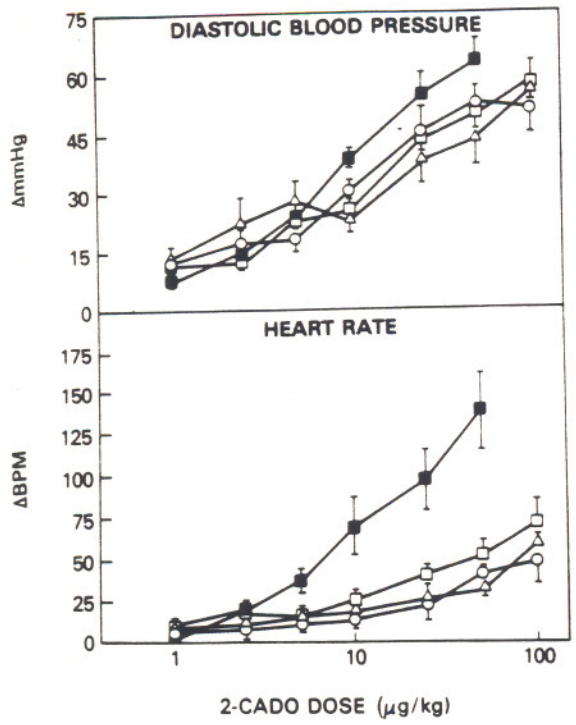

Fig. 4. Effect of XAC doses on blood pressure and heart rate dose-response to 2-CADO. Hypotensive and bradycardiac responses to varying 2-CADO doses were monitored in rats pretreated with vehicle (■) or XAC (□, 0.05; △, 0.10; ○, 0.20 mg/kg i.v.). Results are expressed as means ± S.E.M. ($n \geq 6$). Two-way ANOVA indicates significant ($P < .001$) effect of 2-CADO on blood pressure, heart rate and significant ($P < .05$) effect of XAC dose on heart rate. BPM, beats/min.

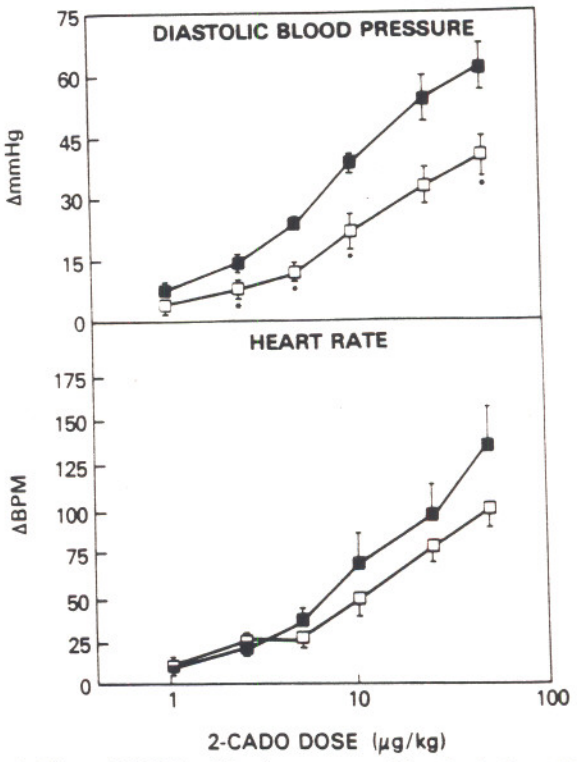

Fig. 5. Effect of MDPX on blood pressure and heart rate dose-response to 2-CADO. Hypotensive and bradycardiac responses to varying 2-CADO doses were monitored in rats pretreated with MDPX (5 mg/kg, □) or vehicle (■). Results are expressed as means ± S.E.M. ($n \geq 5$). Two-way ANOVA indicates significant ($P < .001$) effect of 2-CADO on blood pressure, heart rate and significant ($P < .05$) effect of MDPX on blood pressure. * $P < .05$ compared to vehicle at same 2-CADO dose (Newman-Keuls). BPM, beats/min.

sive effects of 2-CADO than in antagonizing effects on heart rate (fig. 5). Blood pressure responses to all doses of 2-CADO were reduced significantly ($P < .05$-Newman Keuls) by MDPX with the exception of the 25-μg/kg dose. Heart rate responses to 2-CADO were not reduced significantly at any dose of MDPX, although a trend toward reduction was seen at higher doses (10–50 μg/kg).

Effect of XAC on potentiation 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

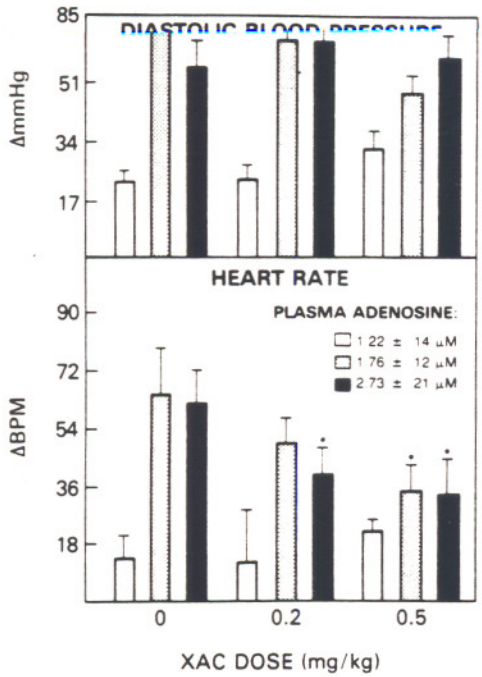

Fig. 6. Effect of XAC on potentiation by adenosine of hypertensive and positive chronotropic effects of nicotine. Pressor and chronotropic responses to nicotine (40 μg/kg i.v.) were monitored in rats at various plasma adenosine concentrations pretreated with indicated dose of XAC. Results are expressed as means ± S.E.M. ($n \geq 6$). Two-way ANOVA indicates significant ($P < .001$) effect of plasma adenosine concentration on blood pressure and heart rate responses to nicotine and significant ($P < .05$) effect of XAC on heart rate response. * $P < .05$ compared to vehicle at same adenosine concentration (Newman-Keuls). BPM, beats/min.

TABLE 1

Effect of dose and time after injection on plasma and brain concentrations of XAC and XCC

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

	Conc.				
	10 min after injection		45 min after injection		
	Plasma		Plasma		Brain
Dose	XAC	XCC	XAC	XCC	XAC
mg/kg	μg/ml		μg/ml		
0.05	0.45 ± 0.07		0.28 ± 0.03		
0.1	0.93 ± 0.11		0.46 ± 0.05		
0.2	0.78 ± 0.21		0.79 ± 0.32		
0.5	1.50 ± 0.31	0.078 ± 0.016	0.84 ± 0.14	0.078 ± 0.028	<.10
1.0	2.65 ± 0.45	0.103 ± 0.028	0.95 ± 0.03	0.114 ± 0.016	<.10

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 indentical 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 5- to 10-fold greater potency in blocking vascular responses to adenosine agonists required doses 3- to 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 nonselective 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, water-soluble 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 hypotensive 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 a 1-propargyl-3,7-dimethylxanthine and 7-propyl-1,3-dimethylxanthine 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.

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