

PENETRATION OF ADENOSINE ANTAGONISTS INTO MOUSE BRAIN AS DETERMINED BY *EX VIVO* BINDING

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Abstract—The penetration of the adenosine antagonists 8-cyclopentyl-1,3-dimethylxanthine (CPT), 8-cyclopentyl-1,3-dipropylxanthine (CPX), 8-(*p*-sulfophenyl)theophylline (8-PST), and 8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (XAC) into mouse brain was determined using *ex vivo* binding and locomotor studies. CPT and CPX (25 and 0.25 mg/kg, respectively) both penetrated into brain in substantial amounts: 49 and 17% of theoretical levels assuming free penetration throughout the body, 10 min after i.p. injection, respectively. Brain levels of CPT decreased rapidly, declining to undetectable levels by 30 min post-injection, whereas levels of CPX declined much more slowly. As expected, no detectable brain levels of 8-PST were found following i.p. injection of 50 mg/kg. XAC (20 mg/kg) penetrated into brain poorly: 1.6% after 10 min and 3.2% 20 min post-injection. The ability of CPT to stimulate locomotor activity paralleled the brain levels, i.e. it was similar to theophylline at short times and the effect rapidly diminished. These studies demonstrate the usefulness of *ex vivo* binding in determining CNS penetration of adenosine receptor ligands.

Caffeine, theophylline, and other alkylxanthines act as behavioral stimulants [1–3] and have been characterized biochemically as antagonists of adenosine, acting at A₁ and A₂ adenosine receptors. Xanthines have also been shown to reverse the behavioral depression elicited by adenosine agonists. The potency of xanthine derivatives in locomotor assays, both as stimulants and as antagonists of agonist-induced depression, is often not commensurate with their potency as adenosine antagonists. Although synthetic analogues of theophylline and various non-xanthine heterocycles that have 10⁴-fold or greater potency than theophylline in adenosine receptor affinity have been reported [4–6], the relationship between behavioral stimulation and adenosine receptor antagonism is unclear. To adequately characterize this relationship *in vivo*, we have measured the blood–brain barrier penetrability and half-life in the CNS of peripherally administered adenosine antagonists.

Ex vivo binding has been used to measure the ability of a variety of drugs to penetrate into the CNS following intraperitoneal injection [7–9]. Despite its ease and rapidity, this technique has yet to be used in assessing the CNS penetration of adenosine antagonists. The following report describes the time course for CNS penetration of four adenosine antagonists, 8-cyclopentyl-1,3-dimethyl-

xanthine (CPT§), 8-cyclopentyl-1,3-dipropylxanthine (CPX), 8-(*p*-sulfophenyl)theophylline (8-PST), and 8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (XAC). XAC, CPT, and CPX are A₁-selective in the rat brain by factors of 53, 130, and 460, respectively [5, 6]. CPX and XAC are of nanomolar affinity at rat and mouse A₁ receptors, and have been characterized as radioligands [10, 11]. 8-PST is of lesser potency and selectivity, but has been utilized as a peripherally-selective xanthine [12, 13].

MATERIALS AND METHODS

Compounds were obtained from the following sources: CPX, CPT, and 8-PST were from Research Biochemicals, Inc. (Natick, MA). Their structures are shown in Fig. 1. *R*-[³H]Methyl-2-phenylethyladenosine ([³H]PIA, 42.5 Ci/mmol), [³H]-8-cyclopentyl-1,3-dipropylxanthine ([³H]CPX, 109 Ci/mmol), and [*methyl*-¹⁴C] bovine serum albumin (BSA) (0.024 mCi/mg) were from NEN-Dupont (Boston, MA). XAC was synthesized as described [5]. Before injection in the mice, XAC was repurified by isocratic reverse-phase HPLC to eliminate potential minor contaminants not detected by thin-layer chromatography. Aliquots of a saturated solution of XAC (0.1 mL, 12.5/mL) in dimethylformamide were applied to a Vydac C4-protein column (1 × 25 cm), using a mobile phase of 30% acetonitrile in water, containing 0.1% trifluoroacetic acid, at a flow rate of 3 mL/min. The combined purified XAC trifluoroacetate fractions (retention time 7 min) were reduced in volume by evaporation and lyophilized to provide a 78% recovery.

Male Swiss-Webster mice (20–25 g) were used in these studies. Mice were injected intraperitoneally with 100 µL of test compound. Basic stock solutions

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§ Abbreviations: CPT, 8-cyclopentyl-1,3-dimethylxanthine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; 8-PST, 8-(*p*-sulfophenyl)theophylline; XAC, 8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; ADA, adenosine deaminase; and [³H]PIA, *R*-[³H]methyl-2-phenylethyladenosine.

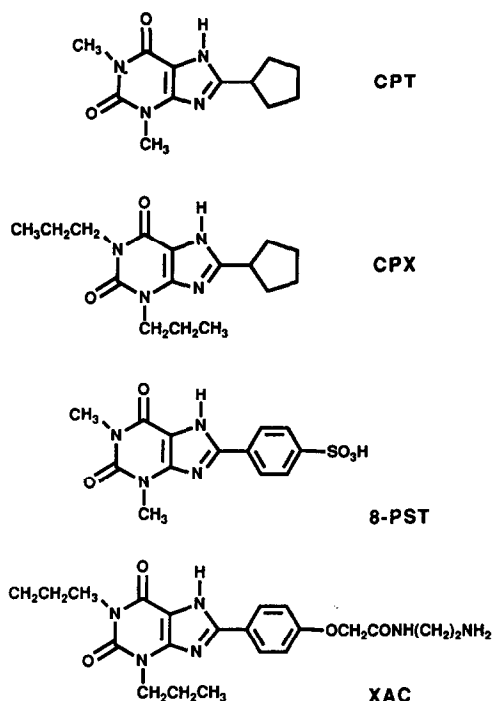


Fig. 1. Adenosine antagonists structurally related to theophylline.

of compounds were made in 0.01 N NaOH and then diluted as needed with 0.9% NaCl. After the indicated time, mice were decapitated following ether anesthesia, and the brains were removed rapidly. The cerebellum and hindbrain (pons and medulla) of each were discarded, and the remainder of the brain was weighed and homogenized in a teflon-glass homogenizer in 8 vol. of 50 mM Tris-HCl, pH 7.4. Adenosine deaminase (ADA; Sigma, Type VI from calf intestine) was added (6 U/mL of homogenate), and the homogenate was incubated at 37° for 45–60 min. Aliquots of this homogenate were used for binding studies.

Binding studies were performed by incubating a 50- μ L aliquot of homogenate with 2 nM [3 H]PIA and 50 mM Tris buffer (pH 7.4) in a total volume of 250 μ L for 60 min at 37°. Following rapid filtration over GF/B filters, the filters were washed three times with the Tris buffer, then equilibrated with scintillation counting fluid and counted. Non-specific binding was determined by co-incubation with 20 μ M 2-chloroadenosine. This amounted to less than 4% of the total [3 H]PIA bound and was routinely subtracted. K_i values for the xanthine derivatives were determined using the Cheng-Prusoff equation [14]. Standard curves were constructed by incubating brain homogenate from untreated mice with various concentrations of inhibitor using the identical conditions as above. Each determination was performed in quadruplicate using at least three different animals.

The percent brain penetration of test compounds was determined by comparing the amount of

inhibition of [3 H]PIA binding in homogenates from treated and control brain. By comparing this percent inhibition with that obtained from the standard curve, the concentration of compound entering the brain was determined. One hundred percent brain penetration was defined as the amount of compound reaching the brain in the absence of any permeability barrier, assuming that the injected dose distributed uniformly throughout the mouse. Each determination was performed in quadruplicate and data are expressed as means \pm SD from at least three different animals.

Individual adult male mice of NIH (Swiss) strain between 5 and 6 weeks of age weighing at least 25 g were studied in a Digiscan activity monitor (Omnitech Electronics Inc., Columbus, OH) with computerized data analysis (ILAM software). Animals were placed in the cage immediately after injection, and monitoring for three intervals of 10 min each was initiated after 10 min. Total distance travelled was used as a measure of locomotor activity. Xanthines were dissolved in a 20:80 (v/v) mixture of Emulphor EL-620 (GAF Chemicals Corp., Wayne, NJ) and phosphate-buffered saline and injected i.p. in a volume of 5 mL/kg body weight. Control values for vehicle-injected animals were determined for each experiment.

RESULTS

Scatchard analysis of [3 H]PIA binding to membranes from crude homogenates treated with 6 U/mL of ADA revealed two affinities: a high-affinity site with a K_d of 1.5 ± 0.2 nM ($N = 3$) and a B_{\max} of 398 ± 29 fmol/mg protein, as well as a site of much lower affinity. The high affinity observed for *R*-PIA at A_1 -adenosine receptors compares favorably with values previously published [6].

Omission of ADA resulted in a higher K_d (4.5 nM) and lower B_{\max} (250 fmol/mg), consistent with the assumption that endogenous adenosine, present even in the membrane preparation, partially occupies the receptor. To demonstrate that this difference was not an artifact produced by the presence of ADA (for example, [3 H]PIA binding hypothetically to the enzyme, which may be immobilized in the cell membrane), we treated membranes from PC12 cells, that do not contain A_1 -adenosine receptors, with ADA (1.5 mg of membranes in 0.5 mL buffer treated with 6 U of enzyme for 60 min at 37°) and found no resulting increase in binding. These results further substantiate the idea that the added ADA is removing endogenous adenosine and not providing an additional binding site for the radioligand.

Inhibition of [3 H]PIA binding to brain homogenates by xanthine derivatives occurred with K_i values consistent with A_1 -adenosine receptor affinities (8-PST, 2.6 μ M; CPT, 28 nM; CPX, 4.1 nM; XAC, 8.2 nM). An example of a competition curve for CPT is shown in Fig. 2. The detection limits for *ex vivo* binding are related to these K_i values. Since brain tissue was diluted a total of 40-fold by homogenization and dilution for binding studies, a concentration of compound 40 times its K_i value would be readily detectable in this *ex vivo* assay. The detection limits of this method depend on the

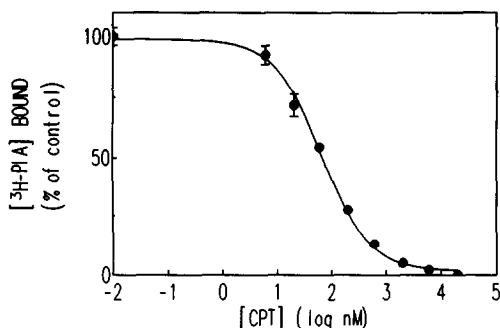


Fig. 2. Competition curve for CPT inhibition of [^3H]PIA binding to homogenate of mouse brain. Homogenate was treated with ADA (6 U/mL), and then incubated with 2 nM [^3H]PIA in 50 mM Tris-HCl buffer (pH 7.4) for 60 min at 37°. Data points represent averages of duplicate values and the line is a non-linear regression analysis of the data. In the absence of CPT, specific binding amounted to about 5500 cpm (100% of control).

brain penetrability of the compound being measured: a highly permeable compound will have a detection limit of around four times its K_i value, at which concentration a 10% inhibition in binding would be observed. In this connection, under control conditions, total [^3H]PIA binding amounted to around 5500 cpm per 50- μL aliquot tested, with about 150 cpm of this being non-specific binding. Since the maximal inhibition of binding caused by injection of 25 mg/kg of CPT (93% inhibition) still left about 350 cpm of specific binding, sufficient counts remained to allow for reasonably accurate estimates of drug concentration.

To demonstrate that the xanthines detected in brain homogenate of treated animals were primarily from brain rather than from the brain vasculature, the following experiments were undertaken to measure the approximate contribution of brain vasculature to total brain volume. Mice were injected i.v. (tail vein) with [^{14}C]BSA (0.1 $\mu\text{Ci}/\text{animal}$) and after 15 min, radioactivity levels in serum and in forebrain were determined. At this time, 100 μL of serum contained $11,041 \pm 832$ cpm (average \pm range, $N = 2$), whereas the entire forebrain (approximate mass 0.32 g) contained only 411 ± 48 cpm (average \pm range, $N = 2$), indicating that the vascular space is extremely small (roughly 1%) compared to brain volume. Therefore, the levels of xanthines detected by the *ex vivo* binding procedure ($>1\%$ of theoretical) represent primarily those from brain rather than from vasculature.

Intraperitoneal administration of 25 mg/kg CPT resulted in a rapid accumulation of CPT in brain that reached maximal levels by 7 min after injection (Fig. 3). At this time, the concentration of CPT in brain was 49 μM which represents 49% of theoretical penetration. One hundred percent penetration is defined here as the concentration of compound expected in brain without any diffusion barrier. This level of CPT was maintained in brain for about 4 min, after which it declined markedly. By 19 min

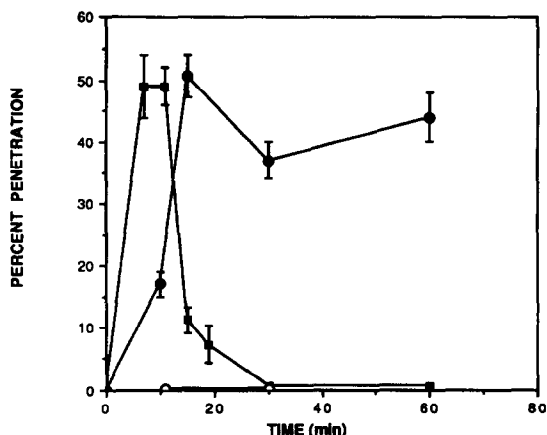


Fig. 3. Time course for penetration of xanthines in the mouse brain. Mice were injected i.p. with: (■) CPT, 25 mg/kg; (●) CPX, 0.25 mg/kg; and (○) 8-PST, 50 mg/kg. After the indicated time, their forebrains were removed, homogenized in 8 vol. of Tris-HCl, pH 7.4, and, after treatment with ADA (6 U/mL) used directly for [^3H]PIA binding. Concentrations of xanthines in brain were determined by comparing [^3H]PIA binding to that from a standard curve. One hundred percent brain penetration is defined as the amount of compound reaching the brain in the absence of any permeability barrier assuming that the injected dose distributes uniformly throughout the mouse. Each determination is the mean \pm SD from at least three different animals.

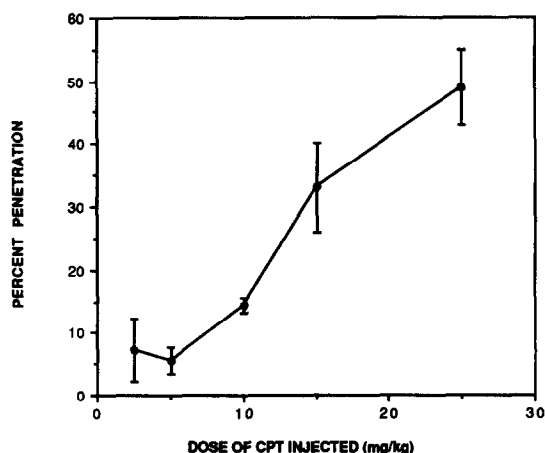


Fig. 4. Dose-dependency for CPT penetration into brain relative to each injected dose. The indicated doses of CPT were injected i.p., and *ex vivo* binding was performed 10 min after injection. The error bars represent average \pm range of duplicate animals.

following injection, it had declined to 7.3 μM , or 7.3% theoretical penetration assuming uniform tissue distribution of the injected dose. As shown in Fig. 4, the percent penetration of CPT was dose dependent.

Levels of CPT in the plasma were also measured

at various time points following i.p. injection of 25 mg/kg doses. These levels decreased from 50.8 μM at 7 min following injection, to 45.9 μM , 19.3 μM and 10.5 μM at 11, 19 and 30 min following injection of this compound. This rapid decrease in serum levels parallels the rapid decline in brain and may reflect either elimination or metabolic breakdown of this compound.

In contrast to the rapid accumulation and rapid decline of CPT levels in brain, i.p. administration of CPX (0.25 mg/kg) resulted in a somewhat slower brain penetration, reaching maximal levels by 15 min. At this time, the brain concentration of CPX was 344 nM, representing 50.6% penetration. This compound cleared the brain much more slowly than CPT (see Fig. 3), and significant levels (252 nM) remained in brain 30 and 60 min following injection.

We next measured the brain penetration of 8-PST, a compound that should not penetrate the blood-brain barrier because of its high charge [15]. Injection of 50 mg/kg of 8-PST resulted in no detectable accumulation in brain either 10 or 30 min post-injection. This injected dose would have resulted in a brain concentration of 200 μM had it been 100% permeable into brain. Since the K_i of this compound was 2.6 μM , we conclude that less than 4.8% of it penetrated into brain.

To account for the possibility that the CPX observed in the brain extracts was from brain and not some other peripheral compartment expressing A_1 receptors, [^3H]CPX (1 μCi) was administered (i.p.) into each of six animals. Three of these mice were co-injected with 50 mg/mL of the peripheral antagonist 8-PST. After 15 min, the animals were killed, and brains were removed, homogenized in Tris buffer, and processed for scintillation counting. The 8-PST failed to displace any of the observed radioactivity from the brain extract, demonstrating that the CPX had indeed penetrated into brain rather than simply bound to A_1 receptors in some other compartment. By this time, $1.1 \pm 0.1\%$ of this injected dose had accumulated in the brain: since brain is approximately 1.3% of the weight of the whole animal, this represents approximately 85% of theoretical penetration. The difference between this value and the lower value (50.6%) determined by *ex vivo* binding may be due to tracer amounts used in this experiment compared to the larger doses used for the *ex vivo* binding.

XAC (xanthine amine congener) is mainly but not entirely charged at physiological pH, by virtue of the primary amino group. Injection of 20 mg/kg XAC resulted in 1.6% ($N = 2$, range 1.4 to 1.8, corresponding to 600 nM) penetration 10 min following i.p. injection, and 3.2% ($N = 2$, range 2.7 to 3.7, corresponding to 1.2 μM) penetration 20 min following i.p. injection.

We carried out locomotor studies in mice using a computerized monitoring apparatus, based on previous studies of the locomotor depressant effects of adenosine agonists [12, 16]. Doses of CPT or theophylline (both 10 mg/kg) were injected intraperitoneally, and locomotor activity as indicated by total distance travelled was measured in 10-min intervals, beginning 10 min post-injection. From the *ex-vivo* binding data, this injected dose of CPT

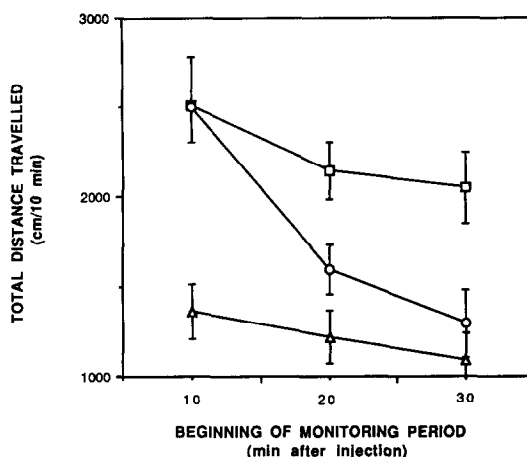


Fig. 5. Time course of locomotor activity. Mice were treated with 10 mg/kg CPT (○) or 10 mg/kg theophylline (□) compared to vehicle control (△). The total distance travelled (cm/30 min) in an Omnitech Activity analyser (dimensions of plexiglass cage: 40 × 40 cm, 30 cm high) was calculated using ILAM software. Animals were placed in the cage immediately after injection, and monitoring for three intervals of 10 min each was initiated after 10 min. Error bars shown represent the standard error of the mean of six to eight animals.

resulted in brain concentrations of 5.5 and 1 μM , 10 and 20 min respectively, following injection. Both xanthines were found to stimulate locomotor activity (Fig. 5). In the first 10-min interval measured, the degree of stimulation relative to vehicle control by theophylline (42%) or CPT (47%) was nearly identical. At longer times, the stimulation by theophylline persisted, but the effect of CPT disappeared, and at 35-min post-injection the level of locomotor activity was similar to that of the control mice. Thus, CPT stimulates locomotor behavior, but only within the first 20 min post-injection. These findings are consistent with the pharmacokinetics of this compound (Fig. 3) which show that brain concentrations decline rapidly by 20 min, and suggest that the locomotor activity is centrally mediated. In contrast to CPT, injection of 0.25 mg/kg of CPX failed to elicit a statistically significant elevation in locomotor activity ($N = 6$, data not shown), despite the previous findings that CPX penetrates rapidly into brain.

DISCUSSION

These studies demonstrate quantitatively that the A_1 -selective adenosine analogues CPT and CPX penetrate into brain, and have considerably different pharmacokinetics. 8-PST, a polar compound by virtue of an aryl sulfonate group that is permanently charged at physiological pH, was found not to penetrate into brain. XAC, which bears an amino group, was found to enter the mouse brain to a small degree, consistent with its reported proconvulsant effects at very high intravenous doses [17], which are presumably centrally-mediated. The brain

Table 1. Brain penetration of adenosine antagonists

Compound	Dose (mg/kg)	Max. brain concentration (μ M)	Detection limit (nM)	% Penetration
CPT	25	49 \pm 5*	272	49
CPX	0.25	0.34 \pm 0.02	32	50.6
8-PST	50	<9.6	9600	<4.8
XAC	20	1.2 \pm 0.1	80	3.2

Compounds were injected i.p. at the indicated doses, and the mice were killed at various times to measure *ex vivo* binding. Maximum brain concentrations occurred at varying times as shown in Fig. 3. The detection limit refers to the concentration of compound in brain that would cause 10 \pm 5% inhibition of binding. Percent penetration is calculated by dividing the maximum brain concentration by the theoretical brain concentration of compound in the absence of any blood-brain barrier.

* Mean \pm SD, N = 3-4.

penetrability of these compounds is summarized in Table 1. Given the recent interest in the behavioral effects of methylxanthines, such studies are especially important. CPT and CPX have been reported to be centrally active in the reversal of adenosine agonist induced locomotor depression [12, 18]. [11 C]CPT has been proposed as an *in vivo* receptor imaging ligand for central adenosine receptors.* 8-PST has been utilized as a peripherally selective adenosine antagonist by Evoniuk *et al.* [19], Seale *et al.* [16], von Lubitz and Marangos [13], and others, based on indirect evidence [1] for exclusion from the brain.

Concentrations of simple xanthines in the CNS following peripheral administration have been measured. Stahle *et al.* [20] used microdialysis to measure the concentration of theophylline in rat brain following subcutaneous administration. They found that a dose of 20-24 mg of theophylline resulted in a brain-free extracellular concentration of 60-90 μ M. The injected doses and the resulting concentrations of xanthine in the brain were comparable to those with CPT administration in the present study. It is interesting to note, however, that both theophylline in the study by Stahle *et al.* [20] and CPX in the present study remained elevated in brain for much longer times than CPT. Similarly, Kaplan *et al.* [21] found that the levels of caffeine in the plasma and brains of mice after parenteral administration were maintained up to several hours post-injection (a 20 mg/kg dose had half-lives of 1.25 and 0.93 hr in the brain and plasma, respectively). In the current studies, the rapid decrease in CPT concentration in brain was paralleled by decreasing CPT concentrations in serum, and may be caused by either elimination or metabolism of this compound.

Although a correlation between the time course of locomotor stimulation by CPT and its measured levels in the brain was observed, no such correlation was observed for CPX. The reason for the absence of locomotor stimulation by CPX is not clear: the

ex vivo binding studies clearly indicate that this compound readily penetrates into brain. It is interesting to note that Bruns *et al.* [18] reported an absence of locomotor stimulation by CPT at doses up to 30 mg/kg. Possibly this might be related to the rapid decrease in CPT from the CNS. This absence of correlation, however, demonstrates the difficulty in interpreting adenosine receptor-mediated locomotor stimulation. Because of its relatively high K_i , *ex vivo* binding of theophylline could not be reliably measured, so no such correlations could be made for theophylline.

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