HHS Public Access

Author manuscript

Neurochem Int. Author manuscript; available in PMC 2016 April 11.

Published in final edited form as:

Neurochem Int. 1991 ; 18(2): 207–213.

[^{3}H]XAC (XANTHINE AMINE CONGENER) IS A RADIOLIGAND FOR A_2-ADENOSINE RECEPTORS IN RABBIT STRIATUM

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Abstract

The intrinsic affinity of 8-phenylxanthine analogs at striatal A_2-adenosine receptors is highly species dependent. [^{3}H]XAC (8-[2-aminoethyl[amino[carbonyl[methyl[oxyphenyl]]]]]-1,3-dipropylxan-thine), although A_1-selective in the rat brain, binds to A_2 receptors in rabbit striatal membranes with sufficiently high affinity to serve as a radioligand. In the presence of 50 nM CPX (8-cyclopentyl-1,3-dipropylxanthine), an A_1-selective antagonist added to eliminate binding to A_1 receptors, [^{3}H]XAC exhibits saturable, specific binding (70% of total) to A_2 sites with a K_d of 3.8 nM and a B_{max} of 1.23 pmol/mg protein. At 24°C, the association and dissociation rate constants were 0.13 min^{-1} nM^{-1} and 0.36 min^{-1}, respectively. Binding was performed for 1 h, with non-specific binding defined in the presence of 100 μM NECA (*N*-ethylcarboxamidoadenosine). The potency order for antagonists against 1 nM [^{3}H]XAC at rabbit A_2-receptors was XAC ≈ N^6-Me-XAC ≫ CPX = XCC > 1,3-dipropyl-8-*p*-sulfophenylxanthine > PSPT. The relative potency order for agonists was CGS ≈ NECA > APEC [= 2-(aminoethylaminocarbonyl-ethylphenylethylamino)-NECA] > PAPA-APEC > ADAC > R-PIA (N^6-phenylisopropyladenosine) > S-PIA.

The ability to characterize central A_2-adenosine receptors using an antagonist ligand that is chemically functionalized offers the possibility to design affinity labeling probes for this receptor subtype in the brain, similar to those antagonist probes already developed for A_1-receptors. The results also suggest that affinity columns containing chemically immobilized XAC may be used for isolating central A_2-adenosine receptors from rabbit striatum.

Adenosine, acting as a neuromodulator, inhibits the release of numerous stimulatory neurotransmitters, including glutamate, norepinephrine, serotonin and acetylcholine (Fredholm and Dunwiddie, 1988). Adenosine agonists acting *in vivo* via a central mechanism cause a dramatic depression of locomotor activity (Nikodijevic *et al.,* 1990). 1,3-Dialkylxan-thines act as antagonists of adenosine at A_1- and A_2- adenosine receptor subtypes (Ramkumar *et al.,* 1988). X A C (8-[4-[[[(2-aminoethyl)amino]carbonyl]-methyl]-oxy]phenyl]-1,3-dipropylxanthine), a xanthine amine congener (Jacobson *et al.,* 1986), is a potent adenosine antagonist with an A_1-selectivity ratio in the rat of 20- to 80-fold.

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[^{3}H]XAC has been utilized as a radio-ligand at central A_1-receptors, with a K_d value of 1.2 nM in rat cortex. At A_2-receptors, [^{3}H]XAC has been reported as a radioligand only in human plate-lets with a K_d-value of 12 nM (Ukena *et al.,* 1986a). At rat striatal A_2-receptors, [^{3}H]XAC (at concentrations ≤5 nM) is not a satisfactory radioligand due to relatively low affinity and to non-specific binding to the glass fiber filters (J. W. Daly, personal communication).

A large species-dependent variation in the potencies of certain substituted purine derivatives at A_1 and at A_2 receptors has been noted. For example, a given 8-phenylxanthine or N^6-phenyladenosine derivative may have affinities for central A_1-receptors ranging over several orders of magnitude depending on species (Ukena *et al.,* 1986b). Typically, the species-dependent order of potency for 8-phenylxanthines at A_1-receptors is: calf > rat > guinea pig, human. At A_2-receptors, the K_i-values for 1,3-diethyl-8-phenyl-xanthine (DPX) were shown to be at least an order of magnitude less in rabbit or human than in rat or bovine brain (Stone *et al.,* 1988). Thus, the subtype selectivity of a given xanthine derivative is highly dependent on the species.

We have shown that XAC, like DPX, is of much greater affinity at A_2-receptors in the rabbit than in the rat. This enhanced affinity allows the measurement of specific binding of [^{3}H]XAC to rabbit striatal A_2-receptors.

EXPERIMENTAL PROCEDURES

Materials

XAC, ADAC (N^6-[4[[[4-[[[[(2-aminoethyl)amino]carbonyl] methyl]anilino]carbonyl]methyl]phenyl]adenosine), NECA (*N*-ethylcarboxamidoadenosine), 8-*p*-sulfophenyl-xanthines (1,3-dimethyl and 1,3-dipropyl, abbreviated PSPT and DPSPX, respectively) and CPX (8-cyclopentyl-1,3-dipropylxanthine) were obtained from Research Bio-chemicals, Inc. (Natick, Mass.). N^6-Cyclopentyladenosine was a gift from Dr Ray Olsson. [^{3}H]XAC was obtained from Dr D. Ahern of Dupont NEN (Boston, Mass.). 2-Thio-CPX (8-cyclopentyl-1,3-dipropyl-2-thioxanthine), N,N^{α}-Me_2-XAC (8-[2-dimethylaminoethyl[amino[carbonyl[methyl[oxy-phenyl]]]]]-1,3-dipropyl-xanthine), and the corresponding monomethyl XAC analog were synthesized by methods reported (Jacobsen *et al.,* 1989a). *m*- and *p*-DITC-XAC isomers (1,3-dipropyl-8-[4-[[[[[2-[[[(isothiocyanatophenyl) amino] thiocarbonyl] amino] ethyl] amino] carbonyl] methyl] oxy]phenyl]xanthine) were synthesized as described (Jacobson *et al.,* 1989b). APEC (2-[4-[2-[2-aminoethylamino-carbonyl]ethyl]phenyl] - ethylamino] - 5′ - *N* - ethylcarbox - amidoadenosine) and its N^{α}-*p*-aminophenylacetyl derivative, PAPA-APEC, were prepared as described (Jacobson *et al.,* 1990). PD115,199 (8 - [2-dimethylaminoethyl[*N*-methyl-amino[sulfonyl[phenyl]]]]-1,3-dipropylxanthine) was the generous gift of Dr J. Bristol. Parke-Davis Warner Lambert. Ann Arbor Mich.

Preparation of striatal membranes

Rabbit striatal tissue (100 150 mg per brain) was isolated by dissection from whole rabbit brains, obtained frozen from Pel-Freeze Biologicals Co. (Rogers, Arkansas). Striatal

membranes were homogenized in 20 vol of ice-cold 50 mM Tris, adjusted to pH 7.4 with hydrochloric acid, using a polytron (Kinematica, Gmbh, Luzerne, Switzerland) at a setting of 2–3 for 10 s. The membrane suspension was then centrifuged at 37,000 *g* for 20 min at 4°C. The pellet was resuspended in the above buffer solution, and the membranes were again homogenized and centrifuged. Finally the pellet was suspended in buffer (100 mg wet weight per ml) and stored frozen at −70°C until use. Protein was determined using the BCA protein assay reagents (Pierce Chemical Co., Rockford, Ill.), which is based on the complex with cuprous ions and bicinchoninic acid (Smith *et al.,* 1985).

[^{3}H]XAC binding

The binding of [^{3}H]XAC to rabbit striatal membranes was measured in a total volume of 1 ml, each in a 13 × 100 mm glass tube. The unlabeled competing ligand or NECA (at a final concentration of 100 μM, for determination of non-specific binding) was dissolved in 25 μl of DMSO. To this solution was added 50 μl of 200 mM $MgCl_2$, 50 μl of 1 μM CPX to eliminate binding to A_1-adenosine receptors, 725 μl of 50 mM Tris*, at pH 7.4 at room temperature, and 100 μl of radioligand to produce a final concentration of 1 nM. Finally 100 μl of a striatal tissue suspension (final concentration of approx. 200 μg protein per ml), containing 3 IU/ml adenosine deaminase, Type VI from calf intestinal mucosa (Sigma, St Louis, Mo.), was added. The mixture was incubated with shaking for 60 min at 24°C. All assays were done in triplicate. Bound and free radioligand were separated by addition of 4 ml of ice-cold 50 nM Tris, pH 7.4, followed by rapid filtration using a Brandel Cell Harvester (Brandel, Gaithersburg, Md). The contents of each tube were filtered through a Whatman GF/B filter that had been soaked in a flat pan in buffer containing 0.3% polyethylene imine for 60 min. The filters were washed twice with 4 ml of ice-cold 50 mM Tris, pH 7.4. Each filter disk was added to 4 ml of scintillation fluid, vortexed and counted after 6 h.

Data analysis

IC_{50} values were computer-generated from competition binding data, using a non-linear regression formula on the GraphPAD program (Institute for Scientific Information), were converted to K_i values using a K_d value for [^{3}H]XAC of 3.8 nM and the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

RESULTS

[^{3}H]XAC was tested as a radioligand at A_2-aden-osine receptors in rabbit striatal membranes. Since XAC is not A_2-selective, even in the rabbit, it was necessary to add a sufficient quantity of an A_1-selective antagonist to selectivity block the A_1 component. CPX (8-cyclopentyl-1,3-dipropylxanthine) was selected for this purpose and added to the incubation medium. CPX at a concentration of 50 nM was found to occupy 90% of the A_1-receptors, but only 6.5% of the A_2-receptors in rabbit striatum. A curve representing competition for [^{3}H]XAC binding sites in the rabbit striatum (data not shown) fit a 2-site

*The addition of 0.01% CHAPS (3-[(cholamidopropyl)di-methylammonio]-1-propanesulfonate) to the Tris buffer solution was later found to enhance specific [^{3}H]XAC binding.

model when analyzed by a computer-assisted curve-fitting program (Scatfit). The K_i-value for CPX at the high affinity (A_1) site was found to be 2.9±2.2 nM and at the low affinity (A_2) site 250±144 nM. The high affinity component represented 46% of the combined, specific [^{3}H]XAC binding.

A representative saturation isotherm for specific [^{3}H]XAC binding, in the presence of 50 nM CPX is shown in Fig. 1. The Scatchard plot of the data (Fig. 2) is linear indicating a homogeneous population of non-cooperative binding sites with a K_d value of 3.80±0.15 nM and a binding capacity (B_{max}) of 1.23±0.17 pmol/mg protein. The amount of specific binding was approx. 70% of total binding.

Association and dissociation kinetics for [^{3}H]XAC were rapid and monophasic at 24°C (Fig. 3). The association rate constant, k_a and the dissociation rate constant, k_b, for [^{3}H]XAC calculated from the least squares plot were 0.13 min^{-1} nM^{-1} and 0.36 min^{-1}, respectivey, resulting in a K_d estimate of 2.77 nM. This value is in good agreement with the K_d of 3.8 nM determined from saturation experiments.

Some known agonists and antagonists at adenosine receptors competitively displaced specific [^{3}H]XAC binding to rabbit striatal membranes as shown in Table 1. The rank order of potency expected for binding to rabbit A_2-adenosine receptors was observed, i.e. for antagonists (Fig. 4) XAC ≈ N^6-Me - XAC ≫ CPX ≈ XCC > 1,3-dipropyl-8-*p*-sulfo-phenylxanthine > PSPT, and for agonists CGS-21680 ≈ NECA > APEC > PAPA-APEC > ADAC > R-PIA > S-PIA. The curves for antagonists were monophasic with slope factors approximately equal to 1. The agonist inhibition curves also fit mono-phasic analysis with correlation coefficients in the range of 0.9–1.0. The K_i values obtained for agonists were higher than those reported in rat brain using [^{3}H]NECA as a radioligand, similar to the findings of Bruns *et al.* (1987) using another tritiated antagonist, PD115,199 at rat brain A_2-adenosine receptors.

XAC itself was a very potent inhibitor of [^{3}H]XAC binding with a K_i value of 7.5 nM. This value is in good agreement with the K_d value derived from saturation experiments with [^{3}H]XAC. Certain derivatives of XAC (structures shown in Table 2), particularly compounds 1–3, were also very potent, with K_i values in the range of 10^{-8} M at rabbit A_2-adenosine receptors. *p*-DITC-XAC, 3, has been shown to be an irreversible inhibitor of A_1-adenosine receptors of bovine brain. we are currently examining whether there exists irreversible binding of 3 and 5 at rabbit A_2-receptors based on the high affinity.

The enhancement of affinity of xanthines at rabbit vs rat A_2-adenosine receptors is indicated by ratios of K_i values in Table 1. Previously, Stone *et al.* (1988) comparing inhibition of [^{3}H]NECA binding at striatal A_2-receptors in rat vs rabbit found ratios of K_is of 10.2 and 8.1 for 8-phenyltheophylline and 1,3-diethyl-8-phenylxanthine, respectively. The ratio of K_i-values for XAC (an 8-phenylxanthine analog) at rat and at rabbit brain A_2-receptors was 8.4. This was in contrast to the ratio for CPX (an 8-cycloalkyl analog) of 1.9. The irreversibly-binding adenosine antagonists, *meta*-and *para*-isomers of DITC-XAC, showed an even greater enhancement of affinity at rabbit brain A_2-receptors, with ratios of 22 and 46, respectively. Even 8-phenylxanthines such as XCC, 9, and DPSPX, 10, containing

negatively charged groups, showed enhancement of affinity similar to XAC, which contains a positively charged amino group. Thus, it seems that the species-dependent enhancement of affinity at A_2-adenosine receptors in rabbit is most pronounced for 8-phenylxanthines. The enhancement appears to be dependent on the presence of 1,3-dipropyl groups, as well as on the nature of the 8-phenyl substitutent. Compound 11 (ratio of 0.5) is identical to compound 10 (ratio of 40) except for methyl vs propyl groups at the 1- and 3-positions. 8-*p*-Sulfophenylcaffeine, similar in structure to PSPT, yet nearly inactive at rat adenosine receptors (Shamim *et al.,* 1989), failed to displace [^{3}H]XAC from rabbit A_2-adenosine receptors.

Curiously, the gain in potency observed at the rat A_2-receptor upon *N*-methylation of XAC, e.g. analogs 1 and 2, was not observed in the rabbit striatum. The K_i-value for another dimethylamino xanthine derivative, PD115,199, vs [^{3}H]XAC binding was found to be 3.28±0.86 nM.

DISCUSSION

It would be desirable to have a readily available adenosine antagonist radioligand for A_2-adenosine receptors in the striatum, the brain region in which the greatest amount of A_2-receptor sites are found, to complement studies that are being carried out with adenosine agonists. Comparative studies of agonist and antagonist binding have already been performed at central A_1-adenosine receptors.

Several agonist-derived, tritiated and iodinated radioligands have been used to characterize the binding properties and molecular structure of A_2-adenosine receptors of striatum. A binding assay based on [^{3}H]NECA, a non-selective agonist, requires the addition of a selective A_1-ligand (N^6-cyclopentyladenosine) to the medium to selectively occupy the A_1-sites (Bruns *et al.,* 1986). CGS21680 (Hutchison *et al.,* 1989, 1990; Jarvis *et al.,* 1989) and an iodinatable functionalized congener related to it, PAPA-APEC (Barrington *et al.,* 1989; Jacobson *et al.,* 1989c), are high affinity agonist radioligands that are selective for A_2-receptors. There are only two reports of adenosine antagonists, namely XAC and PD115,199, as radioligands at A_2-adenosine receptors (Ukena *et al.,* 1986a; Bruns *et al.,* 1987). Both XAC and PD115,199 bind to A_2-receptors under the proper circumstances, but neither ligand is A_2-selective.

We now show that [^{3}H]XAC binds to A_2-adenosine receptors in the rabbit brain, unlike in the rat brain, where it is A_1-selective and unsatisfactory as an A_2-radioligand. Displacement of binding by other adenosine ligands occurs with a rank order of potency expected for A_2-adenosine receptors. The rabbit striatal A_2-receptor labeled by [^{3}H]XAC appears to be a high affinity (A_{2a}) type, consistent with characterization of the corresponding brain region in rat using [^{3}H]NECA. This is indicated by the relatively high affinity for substituted adenosine agonists, such as NECA and CGS21680. K_i values derived for displacement of [^{3}H]XAC by adenosine agonists are somewhat higher than previously determined K_i-values using agonist radioligands (Bruns *et al.,* 1986; Jarvis *et al.,* 1989). This is to be expected, since we are using agonists to compete against an antagonist radioligand. These apparent K_i values may represent a combination of high and low affinity states for agonist.

In order to accomplish affinity labeling of central A_2-adenosine receptors with an antagonist ligand, we desired to identify a functionalized congener (having a chemically derivatizable pendant chain) which binds to the receptor. Derivatives of XAC have been used as spectroscopic and chemical affinity probes at A_1-adenosine receptors (Stiles and Jacobson, 1988; Jacobson *et al.,* 1987, 1989b). An agarose affinity column containing an immobilized form of XAC was used successfully to concentrate A_1-adenosine receptors from bovine brain (Olah *et al.,* 1989) and purify to homogeneity (Nakata, 1989) the rat brain A_1-adenosine receptor. Based on our results, it is expected that a similar XAC affinity column might be used to isolate from rabbit brain the A_2-adenosine receptor, which has not yet been isolated from any species. The results of Stone *et al.* (1988) indicate that the human striatal A_2-adenosine receptor resembles the rabbit receptor in having a much enhanced affinity for 8-phenyl substituted xanthines. Therefore, antagonist molecular probes developed for rabbit A_2-adenosine receptors will also likely be of use in human tissue.

The ability to characterize central A_2-adenosine receptors using an antagonist ligand that is chemically functionalized offers the possibility to design affinity labeling probes for this receptor subtype in the brain, similar to those antagonist probes already developed for A_1-adenosine receptors. The results suggest that affinity columns containing chemically immobilized XAC may be used for isolating rabbit central A_2-adenosine receptors.

Acknowledgments

We are most grateful to Research Bio-chemicals Incorporated for financial support.

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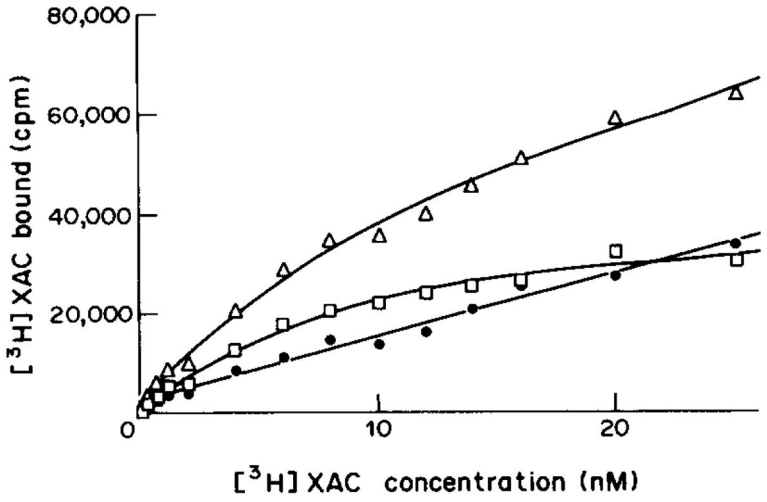


Fig. 1.

Saturation of [^{3}H]XAC binding to rabbit striatal A_2-adenosine receptors. Specific (□), non-specific (○) and total (△) binding were determined for 60 min at 24°C. Values are means of a typical experiment done in triplicate. The calculated K_d-value of 3.8 nM represents the mean of 3 separate experiments.

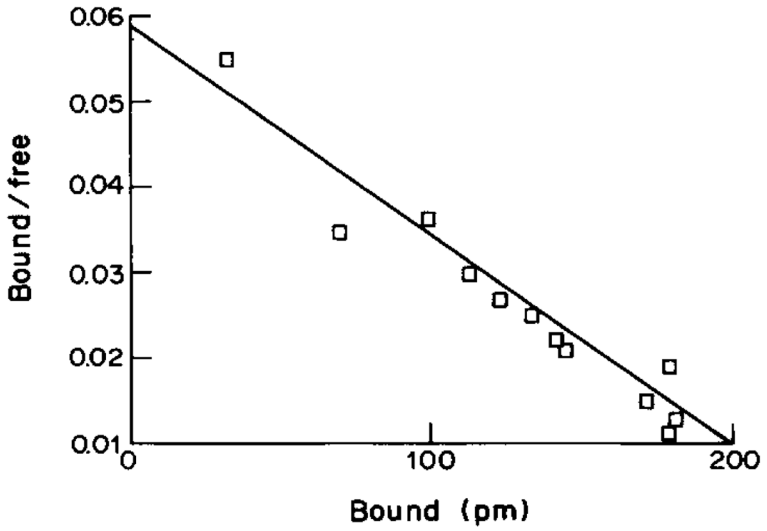


Fig. 2.

Scatchard plot for the binding of [^{3}H]XAC to rabbit striatal A_2-adenosine receptors (data same as for Fig. 1). A K_d-value of 3.8 nM and a B_{max} value of 1.23 pmol/mg protein were determined.

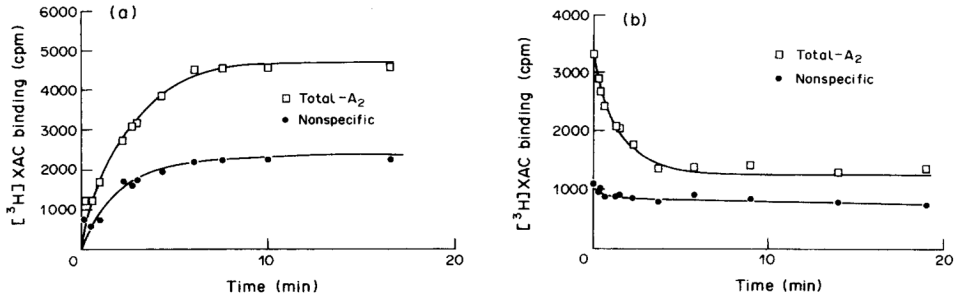


Fig. 3.

Association (a) and dissociation (b) curves for [^{3}H]XAC binding to rabbit striatal A_2-adenosine receptors. Total binding at A_2-receptors (□) and non-specific binding (○) are shown. Concentration of the radioligand was 0.7 nM, in the presence of 50 nM CPX. Dissociation was initiated after 75 min by the addition of 100 μM N^6-cyclopentyladenosine. Data were fit to exponential curves by weighted non-linear least squares curvefitting. The experiments were carried out twice with essentially identical results. Association and dissociation constants of 0.13 min^{-1} nM^{-1} and 0.36 min^{-1}, respectively, were calculated from linear transformation of the data. The linear regression coefficients for association and dissociation plots were 0.09 and 0.824, respectively.

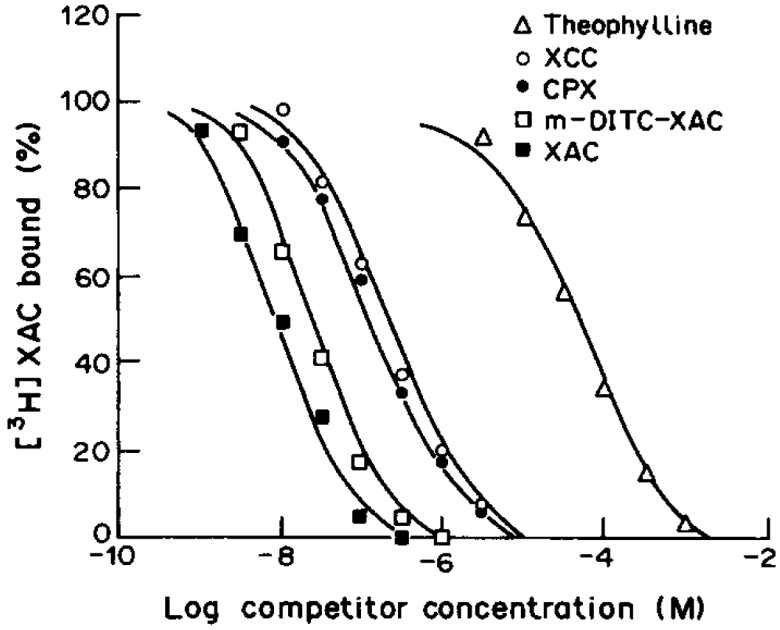


Fig. 4.

Inhibition of binding of [^{3}H]XAC to rabbit striatal A_2-adenosine receptors by various adenosine receptor ligands. The binding was carried out at 24°C for 60 min. [^{3}H]XAC was present at a concentration of 1.0 nM in 0.05 M tris buffer at pH 7.4 in the presence of 10 mM $MgCl_2$ and 50 mM CPX.

Table 1

Affinity, expressed as K_i values in nM, of adenosine receptor agonists and antagonists at central A_2-adenosine receptors in rabbit (vs [^{3}H]XAC) and in rat (vs [^{3}H]NECA) striatum, unless noted

Compound	K_i (nM) at rabbit A_2-adenosine receptors	K_i (nM) at rat A_2-adenosine receptors	Ratio‡
Antagonists			
(1) *N*,*N*^{6}-Me_2-XAC	5.3±1.2	5.0	0.94
(2) *N*^{6}-Me XAC	7.0±0.63	9.3*	1.33
(3) p-DITC-XAC	7.2±1.6	321	46
(4) XAC	7.5±0.46	63	8.4
(5) m-DITC-XAC	15.6±1.6	343	22
(6) *N*-Ac-XAC	21.1±2.9	530	25
(7) 2-thio-CPX	30.7±5.9	314	10.2
(8) CPX	179±11.8	340	1.90
(9) XCC	196±31	2200	11.2
(10) DPSPX	277±79.2	11,000*	39.7
(11) PSPT	30,090±1504	15,300	0.51
(12) Theophylline	38,250±244	25,300	0.66
(13) 8-*p*-Sulfophenylcaffeine	> 100,000	150,000*	
Agonists			
(14) NECA	70.8±5.2	10.3	0.15
(15) CGS21680	79.1±4.7	15	0.19
(16) APEC	169±34.7	5.7 12†	0.034
(17) PAPA-APEC	279±52.0	28†	0.10
(18) ADAC	860±48.5	210	0.24
(19) R-PIA	3216±253	124 410†	0.039
(20) S-PIA	4688±159	1820 3020†	0.39

Binding of [^{3}H]XAC (1.0 nM) was carried out for 60 min at 24°C, in the presence of 0.05 M Tris buffer at pH 7.4, 10 mM $MgCl_2$ and 50 mM CPX. K_i values are the mean ± SD of at least 3 experiments done in triplicate. K_i values in the rat are from Bruns *et al.* (1986), Daly and Jacobson (1989), Jacobson *el al.* (1989a, 1989c) and Shamim *et al.* (1989). ND = not determined.

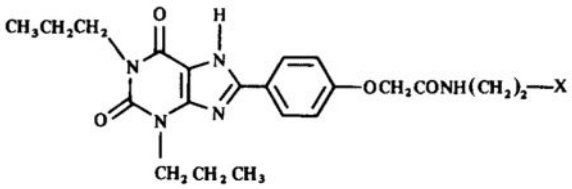
*Versus NECA stimulation of adenylate cyclase in rat PC 12 pheochromocytoma cell membranes.

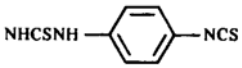
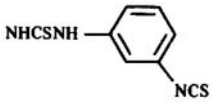
†Versus binding of [^{3}H]CGS21680 in rat striatum.

‡Ratio of K_i-values at rat A_2-receptors vs K_i-values at rabbit A_2-receptors.

Table 2

Structures of XAC derivatives assayed for affinity at rabbit A_2-adenosine receptors



X =	Compound
NH_2	4
$NHCH_3$	2
$N(CH_3)_2$	1
$NHCOCH_3$	6
	3
	5