Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4089–4093, June 1986
Neurobiology

[^{3}H]Xanthine amine congener of 1,3-dipropyl-8-phenylxanthine: An antagonist radioligand for adenosine receptors

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Communicated by Bernhard Witkop, January 27, 1986

ABSTRACT **An amine-functionalized derivative of 1,3-dipropyl-8-phenylxanthine has been prepared in tritiated form as a xanthine amine congener ([^{3}H]XAC) for use as an antagonist radioligand for adenosine receptors. [^{3}H]XAC has higher receptor affinity, higher specific activity, lower nonspecific membrane binding, and more favorable hydrophilicity than 1,3-diethyl-8-[^{3}H]phenylxanthine, the xanthine commonly used for adenosine receptor binding. In rat cerebral cortical membranes, [^{3}H]XAC exhibits saturable, specific binding with a K_d of 1.23 nM and a B_{max} of 580 fmol/mg of protein at 37°C. N^6-(R-Phenylisopropyl)adenosine is a more potent inhibitor of [^{3}H]XAC binding than is 5′-*N*-ethylcarboxamidoadenosine, indicating that binding is to an A_1-adenosine receptor. In the absence of GTP, the inhibition curves for adenosine agonists versus [^{3}H]XAC binding are biphasic, indicating that [^{3}H]XAC is binding to low- and high-affinity agonist states of the A_1 receptor. In the presence of GTP, adenosine analogs exhibit monophasic, low-affinity inhibition of binding of [^{3}H]XAC. Inhibition of [^{3}H]XAC binding by theophylline or by various 8-phenylxanthines is monophasic, and the potencies are commensurate with the potencies of these xanthines as adenosine receptor antagonists. The receptor sites in calf brain membranes exhibit a higher affinity (K_d = 0.17 nM) for [^{3}H]XAC, whereas sites in guinea pig exhibit a slightly lower affinity (K_d = 3.0 nM). Densities of [^{3}H]XAC binding sites are similar in brain membranes from all species.**

Adenosine receptors consist of two subtypes, designated the A_1 and A_2 receptors (1). The A_1 receptor is inhibitory to adenylate cyclase, whereas the A_2 receptor is stimulatory. However, adenosine receptors coupled to other effector systems, such as ion channels, may exist. Adenosine receptors relate to such diverse physiological effects as inhibition of lipolysis and neurotransmitter release (A_1), platelet aggregation (A_2), and vasodilation (A_2).

The use of radioligands has enabled measurement and/or localization of adenosine receptors in brain (2), myocardium (3), lung, (4), ileum (5), and other tissues. The N^6-substituted adenosines, including the N^6-cyclohexyl- (2), N^6-R-phenylisopropyl- (6), and N^6-cyclopentyl- (7) analogs, available as stable, tritiated radioligands with specific activities of 20–50 Ci/mmol (1 Ci = 37 GBq), have proven useful as agonist radioligands showing A_1-adenosine receptor selectivity. 5′-*N*-Ethylcarboxamidoadenosine (NECA), available as a stable, tritiated radioligand with a specific activity of 20–40 Ci/mmol, has been used as an agonist ligand for A_1 and A_2 adenosine receptors (8–13). However, the binding characteristics of [^{3}H]NECA to membranes containing only the A_2 receptors are not fully consonant with that expected of the A_2 receptors that are stimulatory to adenylate cyclase in these same membranes, since N^6-substituted adenosines do activate the cyclase in these membranes but are very weak antagonists of binding of [^{3}H]NECA (11–13). Similarly, certain xanthines are much weaker antagonists of [^{3}H]NECA binding than would be expected from their ability to block NECA activation of adenylate cyclase.

1,3-Diethyl-8-[^{3}H]phenylxanthine (2, 14–17), ([^{3}H]DPX) is the only antagonist radioligand used routinely for the study of adenosine receptors. It has associated problems of hydrophobicity, relatively low affinity (a K_d at rat cerebral cortical membranes of 60 nM), and low specific activity (typically, 13 Ci/mmol). A number of substituted 8-phenylxanthine derivatives (18–21) have been reported to have K_i values at A_1 receptors in the nanomolar range, comparable in affinity to the agonist analogs. Affinity increases upon successive substitution of the 1,3-dimethyl groups of theophylline (and its 8-substituted analogs) by *n*-propyl groups and with attachment of certain 8-aryl substituents. Unfortunately, due to the resulting increases in lipophilicity, attempts to use some of the most potent and selective xanthines as radioligands have been hampered by high filter binding and/or high nonspecific membrane binding (18).

We have reported a "functionalized congener" approach to adenosine receptor ligands (19, 21, 22). In the antagonist series, we have developed analogs of 1,3-dipropyl-8-phenylxanthine that have nanomolar affinities. In this series, distal alkyl amino groups on an attached chain cause an increase in potency. One such active analog, a xanthine amine congener (XAC), has served as a synthetic intermediate for many active conjugates (21). We now report that the synthesis of [^{3}H]XAC with high specific activity (103 Ci/mmol) provides a versatile antagonist radioligand for characterization of adenosine receptors.

MATERIALS AND METHODS

Materials. N^6-(R-Phenylisopropyl)adenosine (R-PIA) and NECA were purchased from Research Biochemicals (Wayland, MA). [^{3}H]R-PIA (49.9 Ci/mmol) and [^{3}H]DPX (13.4 Ci/mmol) were purchased from New England Nuclear. Theophylline, adenine, and inosine were from Sigma. 2′,5′-Dideoxyadenosine was from P-L Biochemicals, and dipyridamole was from Thomae (Biberach, F.R.G.). All other chemicals were of analytical grade or the best commercially available.

Synthesis and Purification of [^{3}H]XAC. A solution of 0.57 g (1.49 mmol) of 8-{[(4-carboxymethyl)oxy]phenyl}-1,3-diallylxanthine (**1**, prepared according to ref. 23) in ethanolic HCl (prepared by addition of 3 ml of thionyl chloride to 30 ml of absolute ethanol) was heated at 50°C for 10 min. Upon cooling and the addition of 50 ml of water, a white solid

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Abbreviations: XAC, xanthine amine congener (compound **3b**); DPX, 1,3-diethyl-8-phenylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; R-PIA, N^6-(R-phenylisopropyl)adenosine.

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precipitated. The product, 8-(4-{[(ethoxycarbonyl)methyl]oxy}phenyl)-1,3-diallylxanthine (**2**) (0.57 g, 93% yield) melted at 256–258°C. Analysis ($C_{21}H_{22}N_4O_5$): calc. 61.46% C, 5.40% H, 13.65% N; found 61.38% C, 5.43% H, 13.62% N.

The diallyl amine derivative **3a** was synthesized as described (23) or by the following alternative procedure. Compound **2** (0.40 g, 0.97 mmol) was added to ethylenediamine (20 ml). After solution was complete, the mixture was concentrated in a rotary evaporator (50°C water bath) to a small volume. Addition of methanol, ethyl ether, and petroleum ether precipitated 0.339 g of product 8-{4-[({[(2-aminoethyl)amino]carbonyl}methyl)oxy]phenyl}-1,3-diallylxanthine (**3a**) (83% yield), melting at 228–230°C with decomposition. Analysis ($C_{21}H_{24}N_6O_4 \cdot \frac{1}{2}$dimethylformamide$\cdot \frac{1}{2}H_2O$): calc. 57.50% C, 6.11% H, 19.37% N; found 57.46% C, 5.82% H, 19.63% N.

The diallyl precursor **3a**, dissolved in dimethylformamide, was reduced catalytically (20% Pd/C) under tritium gas as described (23). After filtration and removal of exchangeable tritium, the radiochemical purity of the crude product was judged to be 32%, with more polar impurities. A 1.0 mCi sample dissolved in dimethylformamide was purified by high-pressure liquid chromatography (Waters 440 system, Altex ODS 25 × 0.46 cm column, 0.8 ml/min, 65% MeOH/0.05 M sodium phosphate, pH 5, 320 nM UV detector). The product, **3b**, having a retention time of 28 min, contained 0.2 mCi of radioactivity. TLC (Merck silica gel 60, 0.2 mm, developed in chloroform/methanol/acetic acid, 85:10:5) monitored with a Berthold LB 2760 TLC scanner revealed a single radioactive spot with an R_f (= 0.16) identical to that of authentic XAC. After determination of molar concentration by the intensity of the UV absorption at 319 nm (ε = 34,300), the specific activity was calculated to be 103 Ci/mmol.

Preparation of Brain Membranes. Membranes from rat and guinea pig brain and calf cerebral cortex were prepared according to the method described by Whittaker (24). The animals were killed, and the brains were quickly removed and immediately placed in ice-cold 0.32 M sucrose. The tissue was homogenized at 4°C in a glass/Teflon homogenizer in 10 vol of 0.32 M sucrose. The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant was again centrifuged at 100,000 × *g* for 30 min. The pellets were resuspended in 10 ml of water, centrifuged at 100,000 × *g* for 30 min, and washed once with 50 mM Tris·HCl (pH 7.4) using the same centrifugation step. Finally, the membranes were resuspended in 50 mM Tris·HCl (pH 7.4), frozen in liquid nitrogen, and stored at −70°C. Protein was determined according to the Lowry method as described (4).

Radioligand Binding. The binding of [^{3}H]XAC to rat brain membranes was measured in a total volume of 1 ml containing 50 mM Tris·HCl (pH 7.4), 0.2 unit of adenosine deaminase, and ≈70–100 μg of membrane protein. The radioligand was routinely present in a final concentration of 0.5 nM. Other substances were added as indicated. Incubation was carried out at 37°C for 120 min. All assays were done in triplicate. Bound and free radioligand were separated by addition of 4 ml of ice-cold incubation buffer followed by rapid filtration through Whatman GF/B glass fiber filters that had been treated with 0.3% polyethylenimine for 60 min as described by Bruns *et al.* (25). The filters were washed twice with 5 ml of ice-cold incubation buffer. For filtration, a Brandel M-24R manifold (Brandel Instruments, Gaithersburg, MD) was used. Nonspecific binding of [^{3}H]XAC to the membranes was determined in the presence of 10 μm R-PIA. It amounted to 5–10% at 0.5 nM [^{3}H]XAC. The same amount of nonspecific binding was obtained with 5 mM theophylline. Nonspecific binding was not subtracted for data analysis of competition curves. Nonspecific adsorption of 0.5 nM [^{3}H]XAC to filters amounted to about 4% of the total radioactivity filtered with untreated filters and was reduced to <1% with polyethylenimine-treated filters.

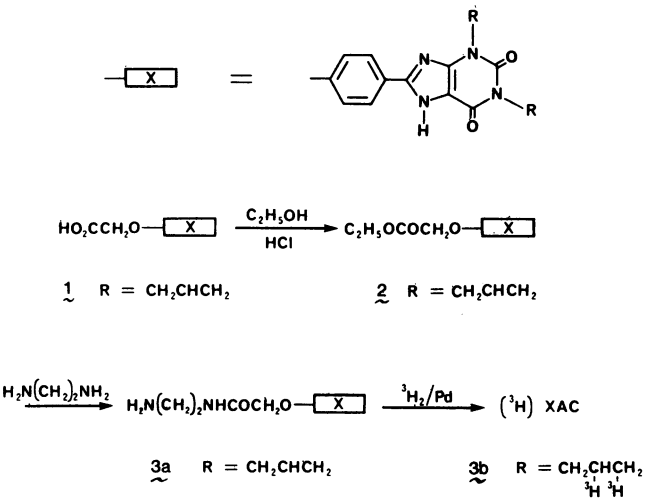

FIG. 1. Synthesis of [^{3}H]XAC by way of diallyl precursors.

Binding of [^{3}H]XAC, [^{3}H]DPX, and [^{3}H]R-PIA to calf cerebral cortex and guinea pig brain membranes was carried out in essentially the same way. The protein concentration was about 100 μg per tube. Nonspecific binding was determined in the presence of 5 mM theophylline for antagonist binding and 10 μM R-PIA for agonist binding.

Data Analysis. Competition binding data were analyzed by the computer program developed by Munson and Rodbard (26) providing parameter estimates by nonlinear curve-fitting. Slope factors were determined from Hill plots ("pseudo-Hill" coefficients).

RESULTS

[^{3}H]XAC (Fig. 1, compound **3b**) was prepared by catalytic reduction of the 1,3-diallyl analog (**3a**) using tritium gas (23). One route to this precursor (23) was the direct coupling of excess ethylenediamine to 1,3-diallyl-8-*p*-(carboxymethyloxy)phenylxanthine (**1**). Alternatively, for preparation of larger quantites of **3a**, the carboxylic acid was esterified in ethanol, and the resulting ethyl ester (**2**) was aminolyzed in neat ethylenediamine. The incorporation of four tritium atoms per molecule led to a high specific activity of the reduction product, **3b**, which was then purified by high-pressure liquid chromatography. The radiochemical purity of [^{3}H]XAC was judged to be 96% (Fig. 2).

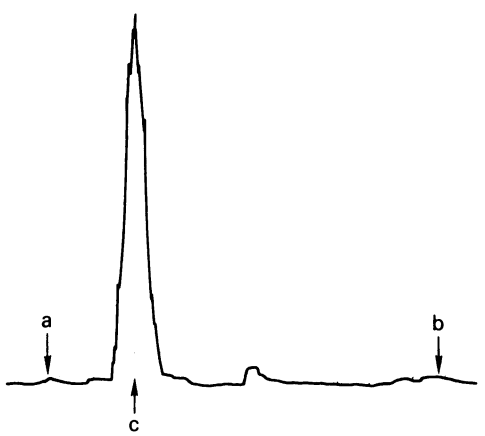

FIG. 2. TLC radiochromatogram showing positions of origin (a), solvent front (b), and XAC as reference (c).

The [^{3}H]XAC was tested as a radioligand in membrane preparations from rat brain. A representative saturation isotherm for [^{3}H]XAC binding is shown in Fig. 3*A*. Nonspecific binding increased linearly with [^{3}H]XAC concentrations. Specific [^{3}H]XAC binding was saturable with increasing concentrations of the radioligand. The Scatchard plot of the data is linear, indicating a homogeneous population of noninteracting binding sites with a K_d of 1.23 nM and a binding capacity (B_{max}) of 580 fmol/mg of protein. The addition of GTP (100 μM) did not affect [^{3}H]XAC binding (Fig. 3). In the presence of GTP, K_d and B_{max} were 1.22 nM and 590 fmol/mg of protein, respectively, and therefore nearly identical to the values obtained in the absence of GTP.

The pharmacological profile of [^{3}H]XAC binding sites was determined in competition experiments. The competition curves of the adenosine agonists R-PIA and NECA in the absence and presence of GTP are shown in Fig. 4. R-PIA is more potent than NECA in competing for [^{3}H]XAC binding. This order of potency is characteristic for A_1-adenosine receptors. In the absence of GTP, the competition curves of the agonists are shallow, with slope factors in the range of 0.48–0.58. Computer analysis revealed that a two-component binding model describes the data significantly better than a one-component model ($P < 0.001$). For R-PIA, a high-affinity state with a K_H of 1.8 nM and a low-affinity state with a K_L of 128 nM were obtained. The proportions of the high- and low-affinity states were 73% and 27%, respectively. The addition of GTP steepened the curve and shifted it to the right, as can be seen by an increase of the IC_{50} for R-PIA from 20.3 nM to 156 nM. The slope factor approaches unity. In the presence of GTP, only one affinity state for the agonist was obtained. The K_i value of R-PIA is 106 nM, which is close to the value of the low-affinity state in the absence of GTP. For NECA, nearly identical proportions of the high- and low-affinity state in the absence of GTP were obtained. K_H and K_L values for NECA were 8.4 nM and 1370 nM, respectively. Again, in the presence of GTP only one state of homogeneous affinity with a K_L of 1290 nM was obtained.

The competition curves for several xanthine antagonists are shown in Fig. 5. The curves are monophasic with slope factors near unity. XAC itself was the most potent antagonist tested. The K_i value of 2.8 nM versus binding of [^{3}H]XAC is in reasonable agreement with the K_d value derived from the saturation experiment with [^{3}H]XAC. XAC was about 10-fold more potent than the parent compound, 1,3-dipropyl-8-phenylxanthine. The latter was about 1.5-fold more potent than its carboxylic acid congener and about 2.5-fold more potent than 8-phenyltheophylline. Incorporation of *p*-sulfo substituents on the 8-phenyl moiety affords water-soluble derivatives but reduces the potency at [^{3}H]XAC binding sites by a factor of 4–12. Theophylline was much less potent than the 8-phenylxanthine derivatives. Dipyridamole, an adenosine uptake blocker, 2',5'-dideoxyadenosine, an agonist at the adenosine P-site, ATP, and the adenosine metabolites adenine and inosine inhibited [^{3}H]XAC binding only slightly at very high concentrations (Table 1).

Previous studies have shown that 8-phenylxanthine derivatives have higher affinities to A_1 receptors of bovine brain compared to those in rat brain (2, 18). Saturation experiments

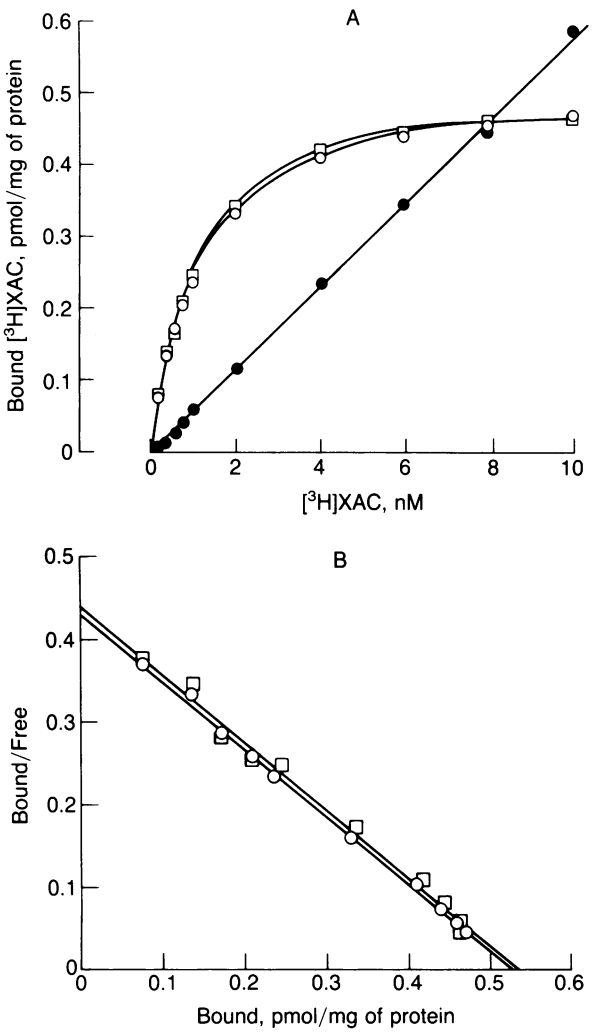

FIG. 3. Saturation of [^{3}H]XAC binding to rat brain membranes. (*A*) Specific binding of [^{3}H]XAC in the absence (○) and presence (□) of 100 μM GTP and nonspecific binding (●) were determined for 120 min at 37°C. Values are means of a typical experiment done in triplicate. (*B*) Scatchard plots of the same data. In the absence of GTP (○), K_d was 1.23 nM and B_{max} was 580 fmol/mg of protein; in the presence of GTP (□), values of 1.22 nM for K_d and 590 fmol/mg of protein for B_{max} were obtained.

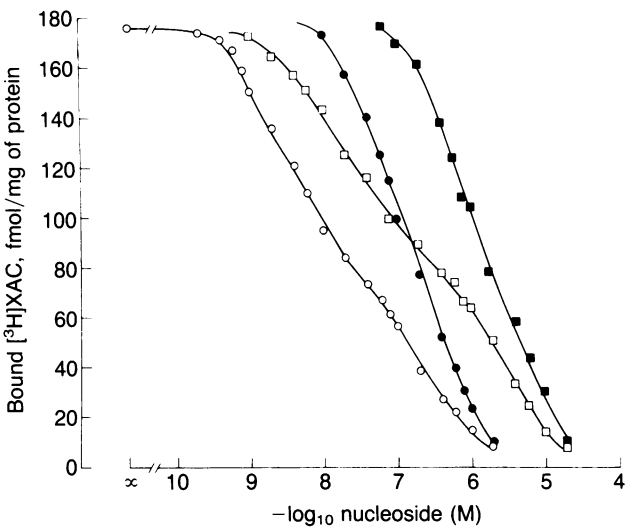

FIG. 4. Competition for [^{3}H]XAC binding to rat brain membranes by adenosine analogs. Binding of [^{3}H]XAC in the absence (open symbols) and presence (closed symbols) of 100 μM GTP was determined at 37°C. Slope factors were 0.58 and 0.99 for R-PIA (●, ○) and 0.48 and 0.94 for NECA (■, □) in the absence and presence of GTP, respectively. Analysis of the curves gave the following estimates: R-PIA without GTP, K_H = 1.8 nM; K_L = 128 nM; R_H = 73%; R_L = 27%. NECA without GTP, K_H = 8.4 nM; K_L = 1370 nM; R_H = 72%; and R_L = 28%. In the presence of GTP, only one affinity state was detected (R_L = 100%). K_L values were 106 nM for R-PIA and 1290 nM for NECA. Values are means of a typical experiment done in triplicate.

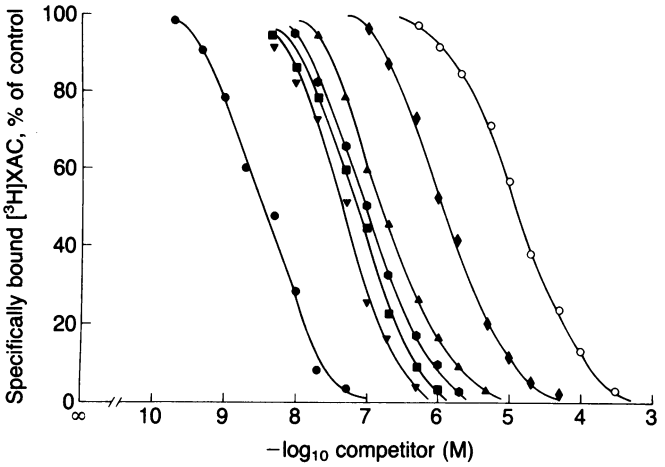

FIG. 5. Competition for [^{3}H]XAC binding to rat brain membranes by adenosine antagonists. Binding of 0.5 nM [^{3}H]XAC was measured for 120 min at 37°C. Slope factors were 1.03 for 1,3-dipropyl-8-[$H_2N(CH_2)_2NHCOCH_2O$-phenyl]xanthine (XAC, ●); 1.10 for 1,3-dipropyl-8-phenylxanthine (▼); 0.99 for 1,3-dipropyl-8-(HO_2CCH_2O-phenylxanthine (■); 0.98 for 8-phenyltheophylline (●); 1.09 for 1,3-dipropyl-8-(*p*-sulfophenyl)xanthine (△); 1.01 for 8-(*p*-sulfophenyl)theophylline (◆); and 0.92 for theophylline (○). Mean values of three experiments are shown.

with [^{3}H]XAC confirm these observations. [^{3}H]XAC binds to calf cerebral cortex membranes with a K_d of 0.17 nM (Table 2), which is lower than that in rat brain by a factor of about 7. The affinity of [^{3}H]DPX is lower than that of [^{3}H]XAC by a factor of about 32. Binding of the adenosine agonist [^{3}H]R-PIA revealed a single binding site with a K_d of 0.5 nM in calf brain membranes. With all radioligands, nearly the same maximal number of binding sites was obtained. In contrast to rat and calf brain, guinea pig brain has been reported to have lower affinities for adenosine agonists and antagonists (2). The K_d for [^{3}H]XAC binding in guinea pig brain membranes is 3 nM (Table 2) and, therefore, about 2.5-fold higher than that in rat brain. The K_d values of [^{3}H]DPX and of [^{3}H]R-PIA in guinea pig brain are 70 nM and 4.9 nM, respectively (Table 2).

DISCUSSION

A variety of radioactive adenosine analogs have proven satisfactory as agonist ligands for A_1-adenosine receptors. However, the only antagonist ligand routinely used for characterization of adenosine receptors—namely, [^{3}H]DPX (2)—has several shortcomings: it has relatively low affinity for adenosine receptors, relatively high proportion of nonspecific binding, and relatively low specific activity. Indeed, it has not proven satisfactory as a ligand in human and guinea pig brain membranes (14).

[^{3}H]XAC overcomes many of the limitations of the [^{3}H]DPX radioligand. The K_d value in rat brain membranes is 50-fold lower than that of [^{3}H]DPX, and the specific activity is 8-fold higher. At concentrations of [^{3}H]XAC required for competitive binding studies, the proportion of nonspecific binding is relatively low. Presumably, the higher hydrophilicity of [^{3}H]XAC, compared to DPX and other nonfunctionalized 1,3-dialkyl-8-phenylxanthines, is responsible in part for favorable reduction in nonspecific membrane binding. For [^{3}H]DPX binding to rat brain membranes, >25% of the total binding was nonspecific at a concentration equal to 15% of its K_d (27). In contrast, with [^{3}H]XAC, only 5–10% binding was nonspecific at a concentration of 0.5 nM—i.e., 40% of its K_d value. A problem of filter binding was still evident with [^{3}H]XAC but has been solved in part by pretreatment of the glass fiber filters with polyethylenimine according to the method of Bruns *et al.* (25). Finally, the high receptor affinity of [^{3}H]XAC makes it feasible to use a multiple filtration apparatus in binding assays. A multiple filtration apparatus had not proved satisfactory with [^{3}H]DPX as radioligand (unpublished data).

Table 2. Radioligand binding to A_1-adenosine receptors of calf, rat, and guinea pig brain membranes

Tissue	K_d, nM	B_{max}, pmol/mg of protein
Calf cerebral cortex		
[^{3}H]XAC	0.17	0.67
[^{3}H]DPX	5.5	0.72
[^{3}H]R-PIA	0.52	0.67
Rat brain		
[^{3}H]XAC	1.2	0.58
[^{3}H]DPX*	68	1.22
[^{3}H]R-PIA	1.0	0.53
Guinea pig brain		
[^{3}H]XAC	3.0	0.82
[^{3}H]DPX†	70	50‡
[^{3}H]R-PIA	4.9	0.75

Binding of the radioligands was measured at 37°C. Saturation of radioligand binding was measured by using at least eight different concentrations of the radioligands. K_d and B_{max} values were obtained from the corresponding Scatchard plots.

*Values for [^{3}H]DPX binding to rat brain membranes are from ref. 27.

†Values for [^{3}H]DPX binding to guinea pig brain membranes are from ref. 2. [^{3}H]DPX binding was measured at 0°C.

‡B_{max} in this case refers to pmol/g of wet weight.

Table 1. Competition for [^{3}H]XAC binding to rat brain membranes

Compound	K_i, nM
1,3-Dipropyl-8-[$H_2N(CH_2)_2NHCOCH_2O$-phenyl]xanthine (XAC)	2.8 (1.9–4.2)
1,3-Dipropyl-8-phenylxanthine	30 (24–38)
1,3-Dipropyl-8-(HO_2CCH_2O-phenyl)xanthine	44 (39–48)
8-Phenyltheophylline	74 (54–102)
1,3-Dipropyl-8-(*p*-sulfophenyl)xanthine	130 (85–198)
8-(*p*-Sulfophenyl)theophylline	920 (700–1,210)
Theophylline	7,600 (5,100–11,600)
Dipyridamole	19,400 (13,100–28,000)
Inosine	>100,000 (15.3%)
2′,5′-Dideoxyadenosine	>100,000 (10.1%)
ATP	>100,000 (8.0%)
Adenine	>100,000 (3.1%)

Data are presented as geometric means with 95% confidence limits in parentheses from three experiments. For K_i values >100 μM, the percentage inhibition of [^{3}H]XAC binding at 100 μM is given in parentheses.

The K_d value for [^{3}H]XAC binding to rat brain membranes is 1.23 nM, with an apparent density of binding sites of 580 fmol/mg of protein. The K_i value of 2.8 nM for XAC in antagonism of [^{3}H]XAC binding is in reasonable accord with the K_d value. The K_d value for [^{3}H]XAC binding is nearly identical to the K_i value for XAC (1.2 nM) for inhibition of N^6-cyclohexyl[^{3}H]adenosine binding in rat brain membranes (11). The presence of GTP has no effect on the saturation curve for [^{3}H]XAC (Fig. 3) as has been reported previously for another antagonist, [^{3}H]DPX. In contrast, guanyl nucleotides have marked effects on binding of agonist radioligands by increasing the proportion of low-affinity agonist binding sites (27, 28). Competition studies versus binding of [^{3}H]XAC with the adenosine analogs R-PIA and NECA and with the series of xanthines (Table 1) displayed the order of potency expected for A_1-adenosine receptors.

The competition curves for the agonists R-PIA and NECA versus binding of [^{3}H]XAC are biphasic in the absence of GTP (Fig. 4). The high-affinity state has an apparent affinity of 1.8 nM for R-PIA, whereas the low-affinity state has an affinity of 128 nM. The high-affinity state for agonists accounts for 70–80% of the inhibition of [^{3}H]XAC binding by R-PIA. Similar results have been reported for antagonism of binding of [^{3}H]DPX by R-PIA in rat brain membranes in the absence of GTP (27). The presence of GTP, although having no effect on binding of [^{3}H]XAC results in monophasic inhibition curves for R-PIA and NECA versus binding of [^{3}H]XAC. This conversion by GTP of all adenosine receptors to a conformational state with low affinity for agonists has been reported (27, 28).

The affinity for [^{3}H]XAC for binding sites is highest in calf brain membranes, intermediate in rat brain, and lowest in guinea pig brain (Table 2). Such a profile is commensurate with data on agonist and antagonist binding in bovine, rat, and guinea pig brain membranes (2, 14, 27). In guinea pig brain membranes, the K_d value for [^{3}H]XAC is 3 nM (Table 2). Guinea pig and human brain membranes have been shown to exhibit relatively low affinity at 0°C in such membranes (2, 14). The higher affinity of [^{3}H]XAC permits its use at 37°C in guinea pig and, presumably, human brain membranes. In calf cerebral cortex [^{3}H]XAC exhibits a K_d of 0.17 nM (Table 2), indicative of a much higher affinity for brain A_1-adenosine receptors of this species, as reported for DPX and other 8-phenyl substituted xanthines (2, 14, 18).

[^{3}H]XAC is clearly a very satisfactory antagonist ligand for A_1-adenosine receptor in brain membranes. The high affinity and relatively low level of nonspecific binding of [^{3}H]XAC suggests the use of this ligand for autoradiography. In this regard it is worth noting that the bioavailability and *in vivo* stability of XAC are comparable to theophylline (ref. 29 and unpublished data). The high specific activity available by virtue of having four sites of tritium incorporation could be particularly valuable in visualizing low-density, peripheral A_1-adenosine receptor sites. It is expected that, as with [^{3}H]DPX, and unlike the tritiated adenosine analogs (28), binding studies with [^{3}H]XAC will be satisfactory at 0°C as well as at 37°C, which may be of value for autoradiographic studies.

[^{3}H]XAC is also potentially useful in measuring binding to A_2-adenosine receptors. XAC does have a 40-fold margin of A_1 receptor selectivity in *in vitro* screening of A_1 and A_2 brain receptors (19) and apparent A_1 selectivity in reversing the cardiovascular effects of NECA (29). However, XAC is a potent antagonist (K_i of 25 nM) of A_2 receptor stimulation of adenylate cyclase by NECA in human platelet membranes. It is less potent (K_i of 83 nM) in a similar protocol with rat PC 12 membranes (30). Further studies will be required to establish the usefulness of [^{3}H]XAC as a radioligand for A_1 receptors in peripheral tissues and as a radioligand for A_2 receptors in tissues or cells with high density of such receptors, such as striatum, platelets, and PC 12 cells.

Since XAC is an intermediate in the synthesis of many conjugates, the tritiated form may also serve as the prototypical member *and* synthetic starting material for a series of new radioligands. With the option of attaching various prosthetic groups—e.g., an iodinatable *p*-hydroxyphenylpropionyl group at the ϵ position of D-lysine as a "carrier" (30)—other radioisotopes such as ^{125}I could be included.

D.U. is on leave from the Pharmakologisches Institut der Universität Heidelberg, F.R.G., with support of the Deutsche Forschungsgemeinschaft (Uk 4/1-1).

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