^{125}I-Labeled 8-Phenylxanthine Derivatives: Antagonist Radioligands for Adenosine A_1 Receptors†

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A series of 8-phenylxanthine derivatives has been synthesized with oxyacetic acid on the para phenyl position to increase aqueous solubility and minimize nonspecific binding and iodinatable groups on the 1- or 3-position of the xanthine ring. The structure–activity relationship for binding of these compounds to A_1 adenosine receptors of bovine and rat brain and A_2 receptors of human platelets was examined. The addition of arylamine or photosensitive aryl azide groups to the 3-position of xanthine had little effect on A_1 binding affinity with or without iodination, whereas substitutions at the 1-position caused greatly reduced A_1 binding affinity. The addition of an aminobenzyl group to the 3-position of the xanthine had little effect on A_2 binding affinity, but 3-aminophenethyl substitution decreased A_2 binding affinity. Two acidic 3-(arylamino)-8-phenylxanthine derivatives were labeled with ^{125}I and evaluated as A_1 receptor radioligands. The new radioligands bound to A_1 receptors with K_D values of 1–1.25 nM. Specific binding represented over 80% of total binding. High concentrations of NaCl or other salts increased the binding affinity of acidic but not neutral antagonists, suggesting that interactions between ionized xanthines and receptors may be affected significantly by changes in ionic strength. On the basis of binding studies with these antagonists and isotope dilution with the agonist [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine, multiple agonist affinity states of A_1 receptors have been identified.

Adenosine regulates cellular function in a number of peripheral tissues and brain, at least partially through receptors on the extracellular surface of cell membranes.[1] Two classes of adenosine receptors have been extensively characterized, A_1 and A_2 receptors, which inhibit and stimulate adenylate cyclase activity, respectively.[2] The A_1 receptor is thought to be involved in the mediation of central depressant effects of adenosine agonists.[3] Alkylxanthines such as theophylline and caffeine are adenosine receptor antagonists that bind to A_1 receptors of rat brain with K_D values of 5.9 and 24 μM, respectively.[4] Increased antagonist potency for the A_1 subclass of receptors is achieved by adding an 8-phenyl group to the xanthine ring.[5] Such compounds are very poorly soluble in water, but we found that solubility can be increased without greatly reducing binding affinity by adding acidic side chains to the para position of the 8-phenyl group.[6] For the purpose of synthesizing radioligands, we reasoned that acidic compounds might be preferable to nonpolar compounds since polar compounds are likely to exhibit minimal nonspecific binding.

Although a number of tritiated[7] and iodinated[4,8] agonist radioligands for adenosine A_1 receptors are available, the addition of GTP converts receptors in brain membranes[9] or receptors solubilized in cholate[10] or digitonin[11] into a state of low affinity for agonists. *N*-Ethylmaleimide (NEM) has a similar effect, apparently by interacting with G_i.[12] In contrast, the binding of antagonist radioligands is not diminished by uncoupling of receptors from G proteins;[9,13,14] hence, they may be preferable to agonists for following the various conformational states of adenosine A_1 receptors, particularly during receptor purification.

In this paper we describe the synthesis of new acidic high-affinity ^{125}I-labeled antagonist radioligands and photoaffinity labels. These compounds and the agonist [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine have been used to characterize multiple conformational states of adenosine A_1 receptors.

Chemistry

In order to evaluate the effects of arylamines, aryl azides, and iodination on the affinity of acidic 8-phenylxanthine derivatives for adenosine A_1 and A_2 receptors, 19 derivatives were synthesized (Figure 1). Novel ester precursors Ia–e (Figure 1) were prepared from the corresponding *N*-(4-nitroaralkyl)-5,6-diaminouracils by oxidative xanthine ring formation with the appropriate aldehyde in nitrobenzene, by use of methods derived from ref 23–25 and catalytic reduction of the nitro group to the amine. Iodinations of amino xanthines IIa–d were carried out with molecular iodine in aqueous sodium bicarbonate. The resulting iodoamines, IIIa–d, cochromatographed with the corresponding ^{125}I-labeled xanthines prepared with chloramine T and [^{125}I]sodium iodide. This confirmation of the structure of [^{125}I]-IIIa–d was required since the chloramine T iodinations of these xanthines resulted in other iodinated products (see the Experimental Section). Iodo azides IVa–d were also characterized and compared by HPLC to the corresponding ^{125}I-labeled xanthines to confirm the structures of the photoaffinity ligands.

6-(Aminobenzyl)adenosine (VIa) was synthesized in improved yield over previous methods[8] by preparing the triacetyl ribonucleoside Vb, which has much greater solubility than the unblocked ribonucleoside Va, allowing for

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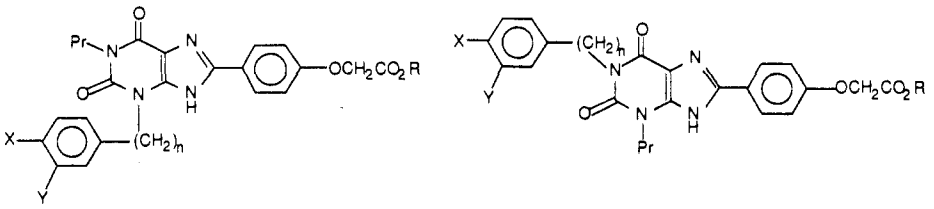
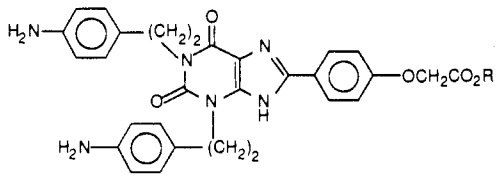
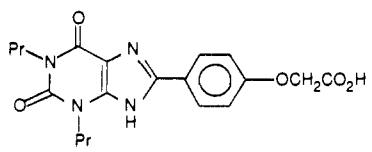


Ia: n = 1, X = NH_2, Y = H, R = Et
Ib: n = 2, X = NH_2, Y = H, R = Et
IIa: n = 1, X = NH_2, Y = H, R = H
IIb: n = 2, X = NH_2, Y = H, R = H
IIIa: n = 1, X = NH_2, Y = I, R = H
IIIb: n = 2, X = NH_2, Y = I, R = H
IVa: n = 1, X = N_3, Y = I, R = H
IVb: n = 2, X = N_3, Y = I, R = H

Ic: n = 1, X = NH_2, Y = H, R = Et
Id: n = 2, X = NH_2, Y = H, R = Et
IIc: n = 1, X = NH_2, Y = H, R = H
IId: n = 2, X = NH_2, Y = H, R = H
IIIc: n = 1, X = NH_2, Y = I, R = H
IIId: n = 2, X = NH_2, Y = I, R = H
IVc: n = 1, X = N_3, Y = I, R = H
IVd: n = 2, X = N_3, Y = I, R = H

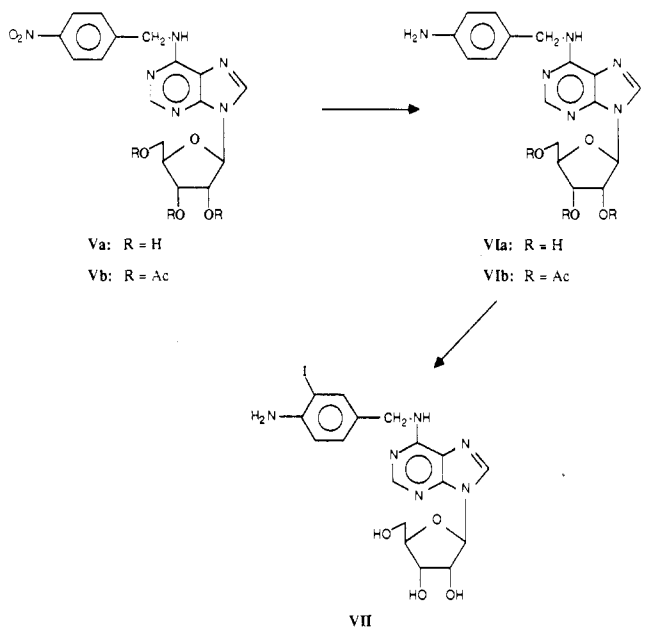


Ie: R = Et
IIe: R = H



VIII

Figure 1. Structure of arylamine-substituted 8-phenylxanthine derivatives. Details of the syntheses are described in the text.



Va: R = H
Vb: R = Ac
VIa: R = H
VIb: R = Ac
VII

Figure 2. Modified scheme for the synthesis of ^{125}I-labeled (aminobenzyl)adenosine. Details of the synthesis are described in the text.

more efficient hydrogenation of the nitro to the amine VIb (Figure 2). We found that it was preferable to work with the free amine VIa rather than the hydrochloride, due to ease of handling and greater stability.

Biological Evaluation

Structure–Activity Relationship of 8-Phenylxanthine Derivatives for Binding to Adenosine A_1 and A_2 Receptors. Arylamine-substituted and aryl azide substituted 8-phenylxanthine derivatives shown in Figure 1 were evaluated in order to determine their binding affinity for adenosine A_1 and A_2 receptors (Table I). The parent compound of this series, VIII, bound to A_1 receptors with higher affinity than to A_2 receptors, but it is important to note that the use of different tissues and species to evaluate these receptors may contribute to this difference. The substitution of either aminobenzyl or aminophenethyl groups for propyl at the 3-position of the xanthine ring did not greatly affect the affinity for A_1 receptors, nor did iodination of these groups. The substitution of an aminobenzyl group at the 1-position of the xanthine also did not reduce A_1 binding affinity (IIc), but iodination of that group (IIIc) or replacing 1-aminobenzyl with 1-aminophenethyl (IId) reduced A_1 binding affinity. The substitution of a 3-aminobenzyl or 3-azidobenzyl group with or without iodination did not decrease binding affinity for the A_2 receptor (IIa, IIIa, IVa), but 1-phenethyl groups decreased binding affinity (IIb, IIIb, IVb). The acidic compounds that bound to A_1 receptors with the highest affinities were all highly selective for the A_1 receptor. The data suggested that ^{125}I-labeled IIIa,b and IVa,b might make useful A_1 selective radioligands and photoaffinity labels, respectively.

^{125}I-Labeled 8-Phenylxanthine Derivatives as Radioligands for Adenosine A_1 Receptors. The antagonists IIa (BWA-118U) and IIb (BWA-827U) and the agonist VIa [(aminobenzyl)adenosine] were radioiodinated

Table I. Effects of Arylamine and Aryl Azide Substituents on the Binding Affinities of 8-Phenylxanthine Derivatives to A_1 and A_2 Adenosine Receptors[a]

8-phenylxanthine derivative[b]	A_1 K_D,[c] nM	A_2 K_i,[c] μM
VIII, 1,3-dipropyl	17 ± 2	0.8 ± 0.2
amines		
IIa, 3-benzyl	32 ± 13	1 ± 0.3
IIb, 3-phenethyl	60 ± 16	11.5 ± 2.7
IIc, 1-benzyl	44 ± 4	23 ± 7
IId, 1-phenethyl	2300 ± 1020	11.5 ± 4.4
IIe, 1,3-diphenethyl	3590 ± 820	>100
iodoamines		
IIIa, 3-benzyl	37 ± 20	0.7 ± 0.5
IIIb, 3-phenethyl	29 ± 5	5.3 ± 2.1
IIIc, 1-benzyl	160 ± 20	1.8 ± 0.3
IIId, 1-phenethyl	3100 ± 830	20 ± 11
iodo azides		
IVa, 3-benzyl	55 ± 19	2.1 ± 0.4
IVb, 3-phenethyl	24 ± 6	5.4 ± 0.2
IVc, 1-benzyl	960 ± 4	5.3 ± 0.6
IVd, 1-phenethyl	2860 ± 480	12.3 ± 2.5

[a] Data represent the mean ± SE, N = 2–4. [b] Refer to Figure 1 for structures. [c] K_D values for A_1 receptors were calculated on the basis of competition for [^{125}I]VII binding to bovine brain membranes. K_i for A_2 receptors were derived from inhibition of NECA-stimulated platelet adenylate cyclase activity; refer to the Experimental Section.

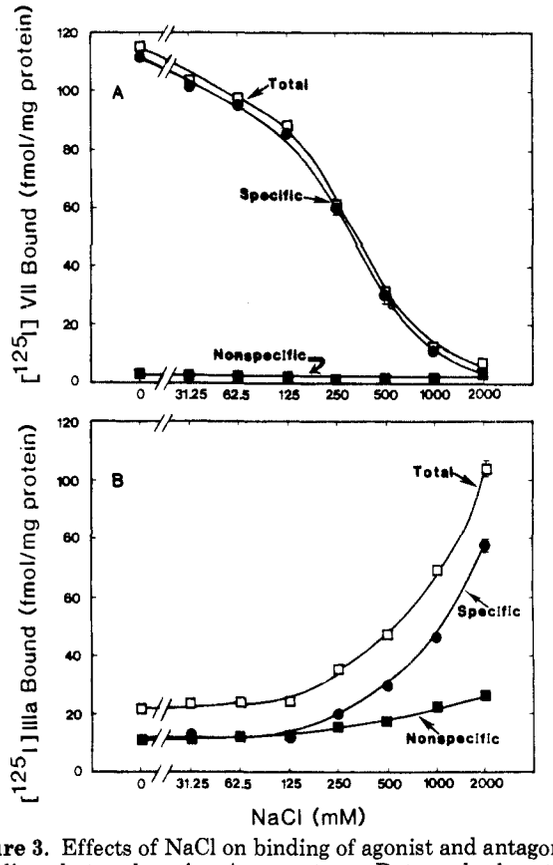


Figure 3. Effects of NaCl on binding of agonist and antagonist radioligands to adenosine A_1 receptors. Rat cerebral cortical membranes (42 μg of protein/100-μL assay volume) were incubated in the presence of 2.5 mM $MgCl_2$ with (A) 107 pM [^{125}I]-VII ([^{125}I]ABA) or (B) 76 pM [^{125}I]-IIIa and various concentrations of NaCl as indicated. Each point represents the mean ± SE of triplicate determinations, error bars are omitted where they are smaller than the symbols. Compared to 0 NaCl, 2 M NaCl decreased specific binding of the agonist by 96.5% and increased specific antagonist binding by 8-fold.

with carrier-free ^{125}I as described in the Experimental Section. Since the membranes used for binding assays had been pretreated with chelator, high affinity agonist binding was dependent upon the addition of a divalent cation.[14] Initially, binding assays to rat brain membranes were conducted with conditions optimized for agonist binding, i.e., with 2.5 mM $MgCl_2$ and without addition of NaCl. Under these conditions the specific binding of the agonist [^{125}I]-VII and of the antagonist [^{125}I]-IIIa represented 95% and 50%, respectively of total binding (Figure 3, 0 NaCl). It was found that adding NaCl or other salts (KCl, NH_4Cl, or $NaHCO_3$, not shown) decreased specific binding of the agonist and increased specific binding of antagonists (Figure 3). On the basis of equilibrium binding experiments, 1 M NaCl was found not to change the number of antagonist radioligand binding sites in rat brain membranes (500–600 fmol/mg of protein, $N = 3$) but to increase radioligand binding affinity by 20–25-fold. The effect of varying the concentration of NaCl on [^{125}I]-VII and [^{125}I]-IIIa binding is shown in Figure 4. NaCl had two distinct effects: at a lower concentration (EC_{50} = 250 mM, Figure 3a) it inhibited agonist binding and had little effect on antagonist binding; at higher concentration (>500 mM, Figure 3b) it nonsaturably increased antagonist binding. The ratio of specific to nonspecific binding for antagonist was maximized (80%) by the addition of 1–2 M NaCl.

In order to compare A_1 receptors labeled by agonist and antagonist radioligands, single batches of rat brain membranes were prepared and incubated with several radioligands (Figure 4). Binding conditions were optimized for each radioligand, i.e., agonist binding was measured in the presence of $MgCl_2$ and without NaCl, and antagonist binding was measured in the presence of 1 M NaCl. K_D values for two antagonists, [^{125}I]-IIIa and [^{125}I]-IIIb, were 1.0 and 1.25 nM, respectively. There was good agreement in the determination of the number of binding sites detected by the various radioligands; B_{max} for [^{125}I]-IIIa, [^{125}I]-IIIb, and [^{125}I]-VII were 530, 516, and 506 fmol/mg of protein, respectively. A small difference between the number of high affinity agonist binding sites and antagonist binding sites might result if not all receptors are in a conformation necessary to bind agonists with high affinity.

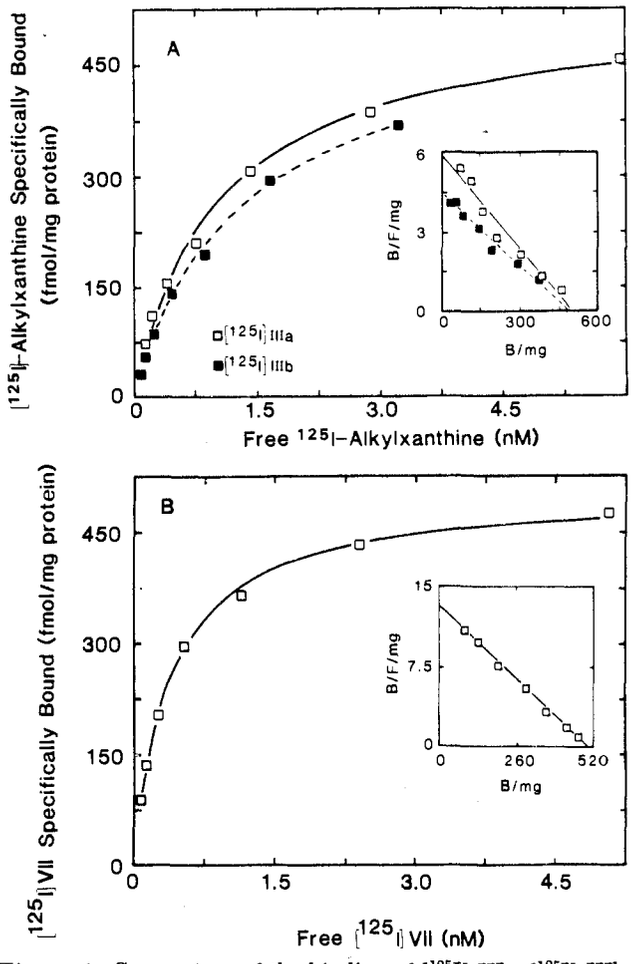


Figure 4. Comparison of the binding of [^{125}I]-IIIa, [^{125}I]-IIIb, and [^{125}I]-VII to rat cerebral cortical membranes. Specific binding of antagonist radioligands (A) measured in the presence of 1 M NaCl and the agonist radioligand [^{125}I]-VII (B) measured in the absence of NaCl were determined as described in the text. Each assay (100 μL) contained 47 μg of protein. The insets show Scatchard plots derived from these data. Binding parameters (B_{max}, fmol/mg of protein, and K_D, nM) were calculated to be [^{125}I]-IIIa, 530, 1.0; [^{125}I]-IIIb, 516, 1.25; [^{125}I]-VII, 506, 0.39. Similar radioligands binding characteristics were found in two to four additional experiments.

Effects of NaCl on the Binding of Various Ligands to Adenosine A_1 Receptors. In order to further investigate the nature of the effects produced by high concentrations of salt, the binding of several 8-phenylxanthine derivatives was investigated in the absence and presence of 1 M NaCl by competition for [^{125}I]-VII or [^{125}I]-IIIa, respectively. The results indicate that high salt increases the binding affinity of acidic more than neutral antagonists. Figure 5 shows a comparison between 8-phenyltheophylline and the oxyacetic acid IIb for binding to

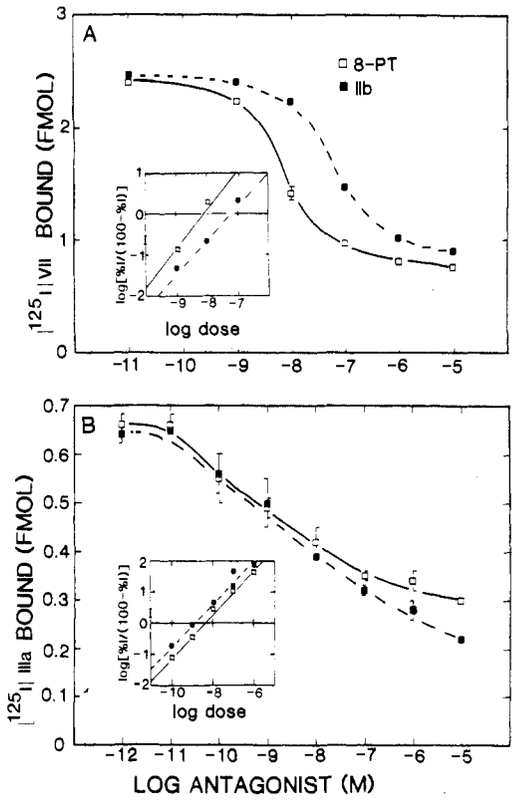


Figure 5. Effect of NaCl on the binding of 8-phenyltheophylline and IIb to adenosine A_1 receptors of bovine brain. Membranes (44 μg of protein/100 μL assay volume) were incubated with 188 pM [^{125}I]-VII and no NaCl (A) or 40 pM [^{125}I]-IIIa with 1 M NaCl (B) and various concentrations of 8-phenyltheophylline (8-PT) or IIb. The IC_{50} values of these compounds were calculated from Hill plots shown as insets. IC_{50} values (nM) and Hill coefficients were as follows: For 8-PT without NaCl, 8.7, 0.94; with NaCl, 4.3, 0.35. For IIb without NaCl, 60.5, 0.82; with NaCl, 1.4, 0.34. The results are typical of three experiments.

bovine brain A_1 receptors. The IC_{50} of 8-phenyltheophylline was hardly changed in the absence and presence of 1 M NaCl (8.7 vs 4.3 nM), whereas the inclusion of NaCl decreased the IC_{50} of IIb by 43-fold from 60 to 1.4 nM. This pattern of a selective effect of salt on the binding affinity of acidic antagonists was also observed in rat brain membranes (Table II). The inhibition curves of antagonist vs radiolabeled antagonist (Figure 5b) had very low Hill coefficients. These data suggest that antagonists bind to more than one affinity state when assayed in the presence of 1 M NaCl.

The addition of 1 M NaCl reduced the binding affinity of several agonists by over 1000-fold (Table II). With use of [^{125}I]-VII at concentrations between 0.1 and 5 nM, increasing concentrations of NaCl were found to decrease the number of high affinity agonist radioligand binding sites without affecting the K_D of the remaining receptors. We reasoned that some of the receptors might be converting to new conformational states with low affinity for the agonist and that these sites might be detected by raising the concentration of radioligand. This was achieved by isotope dilution experiments to avoid the use of very high amounts of radioactivity. By increasing the concentration of VII above 10 nM, it was possible to demonstrate a low affinity receptor conformation induced by the addition of NaCl. Addition of 500 mM NaCl decreased the number of high-affinity [^{125}I]-VII binding sites (K_D < 1 nM) from 506 fmol/mg of protein (Figure 4) to 35 fmol/mg of protein (Figure 6). At this NaCl concentration, most of the receptors (377 fmol/mg of protein) converted to a

Table II. Effect of NaCl on the IC_{50} Values of Various Ligands for Binding to Adenosine A_1 Receptors of Rat Brain Membranes[a]

	IC_{50}, nM		
ligand	1 M NaCl	0 NaCl	ratio −/+ NaCl
acidic antagonists			
IIa (BW-A118u)	3.4 ± 0.6	64 ± 8	19
IIb (BW-A827u)	3.4 ± 0.6	37 ± 9	11
IIc (BW-A184u)	5.2 ± 2.2	44 ± 4	8
IIIa (BW-A522u)	3.0 ± 0.6	37 ± 7	12
neutral antagonists			
8-phenyltheophylline	8.8 ± 0.4	18 ± 4	2.0
1,3-diethyl-8-phenylxanthine	32 ± 3	48 ± 4	1.5
agonists			
(*R*)-N^6-(phenylisopropyl)-adenosine	1150 ± 280	0.9 ± 0.2	8×10^{-4}
N^6-cyclopentyladenosine	720 ± 110	0.35 ± 0.1	5×10^{-4}
NECA	2460 ± 410	2.4 ± 0.4	10×10^{-4}

[a] Binding in the absence and presence of NaCl was assessed by competition for [^{125}I]VII and [^{125}I]IIIa binding, respectively, as described in the Experimental Section, N = 2–3.

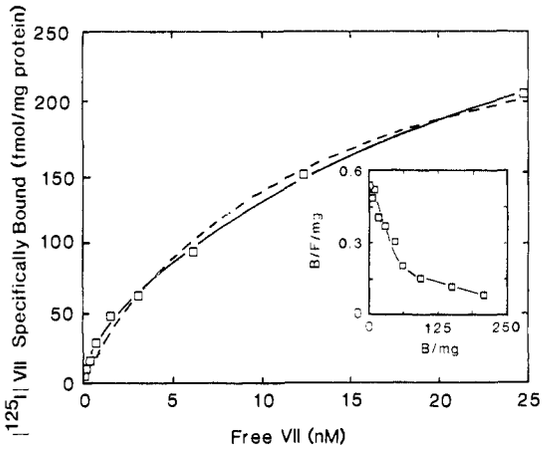


Figure 6. Effect of NaCl on the binding of VII to adenosine A_1 receptors in rat cerebral cortical membranes. Specific binding of the angonist radioligand, [^{125}I]-VII was assessed by isotope dilution with VII in medium containing 2.5 mM $MgCl_2$ and 500 mM NaCl. Binding data were fit to a two-site model (solid line) significantly better than by a one-site model (dashed line). Binding parameters averaged from this and two identical experiments were (B_{max}, fmol/mg of protein; K_D, nM) for the high- and medium-affinity receptor conformations, respectively: B_{max} = 35 ± 3, K_D = 0.67 ± 0.15; B_{max} = 377 ± 7, K_D = 29 ± 3.

low affinity state with a K_D for [^{125}I]-VII of 29 nM. The remaining receptors (ca. 100 fmol/mg of protein) could not be detected by agonist binding and presumably converted to an even lower affinity state, which could only be detected by competition for antagonist radioligand binding (Figure 7).

On the basis of competition between the agonist VII and the antagonist radioligand [^{125}I]-IIIa, two affinity states of receptors for VII were detected in the presence of 1 M NaCl, a low-affinity state (K_D = 25 nM) and a very low affinity state (K_D = 1.27 μM, Figure 7). The addition of 100 μM guanosine 5′-(β,γ-imidotriphosphate) (Gpp(NH)p) or 1 mM NEM in combination with NaCl resulted in a shift of nearly all of the receptors into the lowest agonist affinity receptor conformation (Figure 7).

Discussion

A series of 8-phenylxanthine derivatives containing iodinatable arylamine substituents on the 1- or 3-position of the xanthine ring have been synthesized. Oxyacetic acid substituents were also incorporated into these molecules

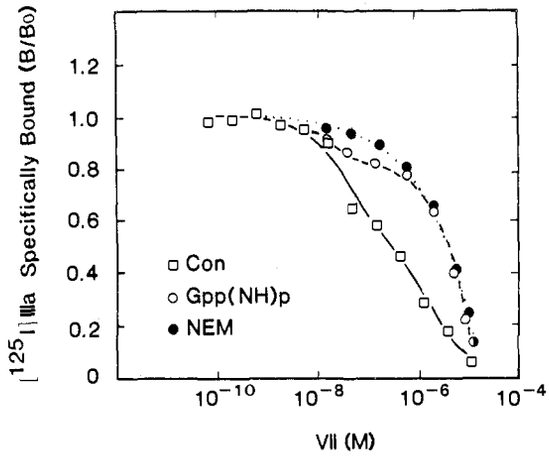


Figure 7. Binding of VII to medium and low affinity conformations of adenosine A_1 receptors. Inhibition of antagonist radioligand binding to rat brain membranes by the agonist VII was measured in medium containing 1 M NaCl alone (con) or NaCl plus either 100 μM Gpp(NH)p or 1 mM NEM. Agonist binding to two states of adenosine receptors was assessed as described in the text. K_D values for the two binding sites were 25 nM and 1.27 μM, respectively. The number of receptors found to be in the low affinity state were: control, 49%; Gpp(NH)p, 91%; NEM, 100%. The results are typical of three experiments.

at the para phenyl position to enhance their water solubility. By comparison to the 1,3-dipropyl compound, the 3-arylamine derivatives had similar binding affinities for adenosine A_1 receptors whether or not these groups were iodinated. Due to multiple iodination products, it was necessary to confirm the structure of the radioligands by synthesizing their nonradioactive counterparts. Thus, we have verified the structures of (arylamino)-8-phenylxanthines, which were radioiodinated for use as the first ^{125}I-labeled antagonist radioligands for adenosine A_1 receptors.[14] The corresponding azide compounds are photoactive and have been utilized as the first antagonist photoaffinity labels for adenosine A_1 receptors.[14,15]

The binding of acidic antagonists was optimized by the addition of high concentrations (>1 M) of salts (NaCl, NH_4Cl, or KCl), which increased the binding affinity of these antagonists by over 10-fold. When assayed in the presence of 1 M NaCl, the K_D values of two (3-iodoaryl)-amines ([^{125}I]-IIIa and [^{125}I]-IIIb) were 1 and 1.5 nM, respectively, and binding to rat brain A_1 receptors was 80% receptor specific. In contrast to its effect on acidic antagonist binding, 1 M NaCl had little effect on the binding affinity of neutral 8-phenylxanthine derivatives. The most likely explanation for the selective effect of salts on the binding affinity of negatively charged 8-phenylxanthines is that charge repulsion between them and the negative charges in the phospholipid environment of receptors is diminished by raising the ionic strength of the solvent.[16] This may account for the fact that xanthines that bind to adenosine receptors with the highest affinity in the absence of high salt are either neutral or positively charged. High-affinity neutral compounds include 8-(2-amino-4-chlorophenyl)-1,3-dipropylxanthine, which binds to rat brain membranes with a K_D of 2.5 nM,[17] and 8-cyclopentyl-1,3-dipropylxanthine, which binds with a K_D of 0.42 nM.[18] Positively charged compounds include xanthine amino conjugates, which bind with K_D as low as 1.2 nM.[19,20]

The effect of NaCl to enhance specific acidic antagonist binding, which apparently results from masking of charge repulsion between the ligands and sites on the membranes, should not be confused with an effect of NaCl on agonist binding, which occurs at much lower concentrations, apparently by modifying the conformational state of the receptor. We examined the effects of NaCl, $MgCl_2$, Gpp(NH)p, and NEM on agonist binding to adenosine A_1 receptors in order to better understand the various conformational states of the receptor. There has been disagreement about whether there are two or three agonist binding conformations of adenosine receptors.[9,13,21] In membranes pretreated with 10 mM EDTA to chelate tightly bound divalent cations, multiple adenosine A_1 receptor conformations can be demonstrated. A high-affinity (K_D for [^{125}I]-VII = 0.39 nM) binding state is demonstrable in the presence of $MgCl_2$ (without other salts). Guanine nucleotides or NEM convert the receptor into a low affinity state(s) presumably due to their effects on G proteins. At physiological concentrations, $MgCl_2$ and NaCl have reciprocal effects on agonist binding. Maguire et al.[22] have suggested that intracellular [Mg^{2+}] may act as a physiological regulator of adenylate cyclase activity. Our data are consistent with the possibility that Mg^{2+} concentration in a physiologically important pool could regulate the affinity of A_1 receptors for adenosine. NaCl and other salts at concentrations between 100 and 500 mM decrease high affinity agonist binding by converting the adenosine A_1 receptor from a high affinity to a lower affinity conformation (K_D for VII = 25 nM). NaCl does not act entirely by uncoupling receptors from G proteins since Gpp(NH)p and NEM could reduce binding affinity further when added to 1 M NaCl (K_D for VII = 1.27 μM). On the basis of competition studies with antagonist radioligands, it is apparent that almost all adenosine A_1 receptors are converted to the very low affinity conformation by a combination of NaCl and Gpp(NH)p or NEM (Figure 7). The low affinity agonist binding site may represent receptors dissociated from guanine nucleotide binding proteins and influenced by NaCl. Such uncoupled receptors can be detected by antagonist but not by agonist radioligands.

Green[21] concluded that there are three affinity states of adenosine receptors on the basis of studies on the binding of [^{3}H]N^6-(phenylisopropyl)adenosine ([^{3}H]PIA) to rat hippocampal membranes. He found that at 37 °C NaCl decreases the affinity of binding sites for [^{3}H]PIA, but Scatchard plots remain linear, whereas we found that at 21 °C addition of NaCl results in curvilinear Scatchard plots, which can be fit to a two-site binding model. The explanation for the difference between our results and those of Green[21] may be that at 37 °C binding reflects an equilibrium between high- and low-affinity receptor states

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whereas at 21 °C receptors may not readily interconvert between these two states. Lohse and co-workers[9] found one or two affinity states for [^{3}H]PIA binding to rat brain synaptosomal membranes assayed in the absence or presence, respectively, of GTP. Their assays were conducted in the absence of added monovalent cations, hence they did not detect the lower affinity binding states, which only occur in the presence of high salt or combinations of salt and guanine nucleotides or salt and *N*-ethylmaleimide (Figure 7). The availability of high affinity ^{125}I-labeled antagonist radioligands should simplify the process of studying conformations of the adenosine A_1 receptor, which bind agonists with low affinity.

Experimental Section

^{1}H NMR spectra for all compounds, recorded with a Varian XL-200 or XL-300 instrument in DMSO-d_6 solution, were consistent with the proposed structures. Chemical ionization (CH_4) mass spectra, provided by Oneida Research Services with a Finnegan triple quadrupole, Model 4500 TSQ, gave parent molecular ions for all compounds. Elemental analyses were provided by Atlantic Microlabs, Inc., and in all cases agreed with predicted values within ±0.4% except where noted. Melting points are uncorrected and were determined with a Meltemp apparatus. Xanthine products were homogeneous by thin-layer chromatography (silica gel, Whatman type MK6F) when developed with 7:3:0.1 $CHCl_3$/MeOH/H_2O. Adenosine derivatives were homogeneous by thin-layer chromatography with 7:3 $CHCl_3$/MeOH.

2-[4-[3-(4-Aminophenethyl)-1,2,3,6-tetrahydro-2,6-dioxo-1-propyl-9*H*-purin-8-yl]phenoxy]acetic Acid (IIb). A 3-mL portion of 1 N NaOH was added to a suspension of Ib (0.44 g, 0.9 mmol) in 4% EtOH (25 mL). The solution that formed on warming to 70 °C was filtered, cooled, and brought to pH 5 by addition of 1 N HCl. The precipitate was filtered and washed with H_2O, then triturated in boiling EtOH (20 mL), cooled, filtered, and washed with Et_2O. The solid (0.31 g) was recrystallized from 1:1 EtOH/DMF (40 mL) to give IIb as a pale yellow solid (0.13 g, 31%), mp slow dec starting at 240 °C. Anal. ($C_{24}H_{25}N_5O_5$) C, H, N.

The following were prepared by procedures analogues to that used in the synthesis of IIb. IIa, in 24% yield, mp 261 °C dec. Anal. ($C_{23}H_{23}N_5O_5 \cdot {}^5/_4H_2O$) C, H, N. IIc, in 74% yield, mp >300 °C. Anal. ($C_{23}H_{23}N_5O_5 \cdot {}^3/_4H_2O$) C, H, N. IId, in 77% yield, mp >280 °C. Anal. ($C_{24}H_{25}N_5O_5 \cdot {}^3/_4H_2O$) C, H, N. IIe, in 57% yield, mp >300 °C. Anal. ($C_{29}H_{28}N_6O_5 \cdot {}^3/_2H_2O$) C, H, N.

2-[4-[3-(4-Amino-3-iodophenethyl)-1,2,3,6-tetrahydro-2,6-dioxo-1-propyl-9*H*-purin-8-yl]phenoxy]acetic Acid (IIIb). A 4-mL sample of 1 N NaOH was added to a suspension of Ib (0.49 g, 1.0 mmol) in H_2O (100 mL). The solution, which formed on heating to near reflux (indicating saponification of Ib to IIb), was cooled and neutralized to pH 7 with 1 N HCl (slightly more than 3 mL). To the slightly hazy solution were added alternatively in 1-mL portions over 4 h a 0.1 M ethanolic solution of iodine (10 mL) and a 0.1 M aqueous solution of sodium bicarbonate (10 mL). The hazy solution was stirred at ambient temperature overnight and brought to pH 2 with 1 N HCl, and the dark precipitate was filtered, washed with H_2O, and extracted twice with refluxing EtOH (150 mL). Concentration of the ethanol extract to 15 mL gave IIIb as a pinkish beige solid (0.2 g, 34%), mp 258 °C dec. Anal. ($C_{24}H_{24}IN_5O_5$) C, H, N, I.

The following were prepared by procedures analogous to that used in the synthesis of IIIb: IIIa, in 50% yield, mp 254–256 °C. Anal. ($C_{23}H_{22}IN_5O_5 \cdot H_2O$) C, H, N, I. IIIc, in 44% yield, mp >300 °C. Anal. ($C_{23}H_{22}IN_5O_5$) C, H, N, I. IIId, in 4% yield, mp slow dec beginning at 205 °C. Anal. ($C_{24}H_{24}IN_5O_5$) C, H, N.

2-[4-[3-(4-Azido-3-iodophenethyl)-1,2,3,6-tetrahydro-2,6-dioxo-1-propyl-9*H*-purin-8-yl]phenoxy]acetic Acid (IVb). A clear solution of IIIb (0.111 g, 0.188 mmol) in glacial acetic acid (30 mL, warmed nearly to reflux to effect solution and then cooled to ambient temperature) plus H_2O (15 mL added gradually) was treated first with a solution of sodium nitrite (0.020 g, 0.29 mmol) in H_2O (0.5 mL) and then with a solution of sodium azide (0.015 g, 0.23 mmol) in H_2O (0.5 mL). The resulting fine suspension was diluted gradually with H_2O (40 mL), allowed to stand for several minutes, filtered, washed well with H_2O and then EtOH, and dried in vacuo to give IVb as a beige solid (0.052 g, 45%), which was shielded from light, mp 198–200 °C dec. Anal. ($C_{24}H_{22}IN_7O_5$) C, H, N, I.

The following were prepared by procedures analogous to that used in the synthesis of IVb: IVa, in 15% yield, mp slow dec starting at 185 °C. Anal. ($C_{23}H_{20}IN_7O_5$) C, H, N. IVc, in 28% yield, mp slow dec starting at 170 °C. Anal. ($C_{23}H_{20}IN_7O_5$) C, H, N, I. IVd, in 56% yield, mp >250 °C. Anal. Calcd for $C_{24}H_{22}IN_7O_5$: C, 46.84; H, 3.60; N, 15.93; I, 20.62. Found: C, 47.80; H, 3.78; N, 15.13; I, 19.49. MS of IVd indicated possible contamination by diiodinated derivative $C_{24}H_{21}I_2N_7O_5$.

6-[(4-Aminobenzyl)amino]-9-β-D-ribofuranosyl-9*H*-purine (VIa). To an ice-cold solution of 6-[(*p*-nitrobenzyl)amino]-9-β-D-ribofuranosylpurine (Va,[26] 11.65 g, 30.0 mmol) in anhydrous pyridine (50 mL) under nitrogen atmosphere was added acetic anhydride (16.0 mL, 170 mmol). The pale yellow solution was stirred at 0 °C for 20 min and then at ambient temperature for 3 h. After addition of 5 mL of methanol, the mixture was evaporated to dryness, evaporated twice with toluene (100 mL) plus methanol (10 mL) to remove pyridine, dissolved in CH_2Cl_2 (250 mL), washed twice with dilute aqueous sodium bicarbonate, H_2O, and saturated NaCl, dried over $MgSO_4$, and evaporated to dryness. The residue was flash chromatographed (CH_2Cl_2/MeOH). Fractions containing product were combined, evaporated to dryness, and evaporated again with toluene (2 × 200 mL) and then absolute EtOH (2 × 200 mL) to give the triacetyl ribonucleoside Vb as a solid foam (13.35 g, 84%). A solution of this material in methanol (500 mL) was divided equally between two 500-mL Parr bottles, and each was hydrogenated at ≤40 psi over palladium on carbon (5%, 0.80 g) for 4 h. Catalyst was removed by filtration. The nearly colorless filtrate was placed under nitrogen, shielded from light, and treated with methanolic ammonia (ca. 4 N, 400 mL) added in four portions over 1.5 h. After crystallization on stirring overnight, the product (8.36 g) was recrystallized first from 90% methanol and then from 90% ethanol to give VIa as a colorless solid (7.41 g, 87%), mp 205–206 °C. Anal. ($C_{17}H_{20}N_6O_4$) C, H, N.

6-[(4-Amino-3-iodobenzyl)amino]-9-β-D-ribofuranosyl-9*H*-purine (VII). A 4.7-mL portion of 1 N HCl was added to a suspension of VIa (4.25 g, 11.4 mmol) in 50% EtOH (250 mL) at 55 °C. The resulting solution was cooled to ambient temperature and treated with solid iodine (3.61 g, 14.2 mmol) in ca. 0.5-g portions over 24 h with stirring to allow solvation of I_2 between additions. A total of 10 mL of 1 N NaOH was added between 5 and 34 h as stirring at ambient temperature was continued for a total of 5 days. The mixture was diluted with H_2O (50 mL), treated with sodium bisulfite (13.8 g), and brought to pH 7 with 1 N NaOH (17 mL). The volume was reduced to 100 mL by evaporation, and the precipitated solid was filtered, washed with water, and dried (5.26 g). The crude product thus obtained was purifed by flash chromatography (CH_2Cl_2/MeOH) and recrystallized from 25% EtOH several times to give VII as a nearly white solid (0.3 g, 5.3%), mp 187–194 °C dec. Anal. ($C_{17}H_{19}IN_6O_4$) C, H, N, I.

Radioiodination of 8-Phenylxanthine Derivatives. ^{125}I-labeled derivatives were prepared from corresponding arylamines by the method of Hunter and Greenwood.[27] Two milliCuries of Na^{125}I and 50 nmol of 8-phenylxanthine derivative were mixed in 0.1 mL of 0.3 M KPO_4, pH 7.5, with 5 μL of chloramine T (1 mg/mL in H_2O) for 60 s. Reactions were quenched by the addition of 50 μL of sodium metabisulfite (5 mg/mL in H_2O). Products were extracted four times into 200 μL of ethyl acetate. The pooled extracts, which contained 50–60% of the starting ^{125}I, were evaporated to dryness under nitrogen, and the resulting residues were dissolved in chromatography buffer (MeOH/5 mM KPO_4 (pH 6), 1:1) and purified by isocractic HPLC over a 4.5 × 250 mm, 5.0 μm C 18 column at a flow rate of 1 mL/min. UV absorption (310 nm) and γ radiation (Beckman Model 170 radioisotope detector) were continuously monitored in the eluate. Figure 8 illustrates chromatographic purification of a typical

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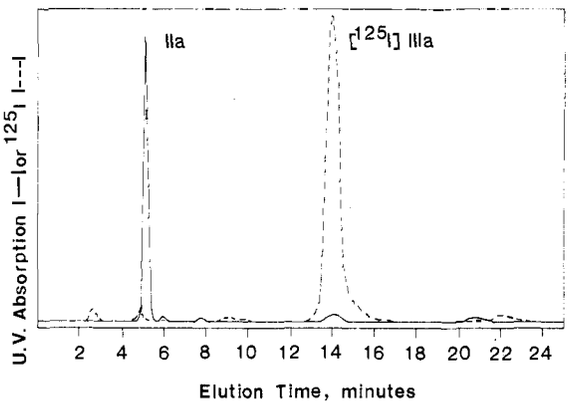

Figure 8. Purification of [^{125}I]-IIIa. IIa was iodinated with carrier-free ^{125}I and chromatographed isocratically by HPLC as described in the text. UV absorption was monitored at 310 nm and ^{125}I was monitored by an in-line γ-detector. Fractions containing purified [^{125}I]-IIIa were pooled, diluted in methanol, and stored at −20 °C.

[^{125}I]iodoarylamine, [^{125}I]-IIIa. In this and all cases, the ^{125}I-labeled product cochromatographed with the corresponding ^{127}I-containing compound. Iodination of this and other arylamine-containing 8-phenylxanthines resulted in additional unidentified iodinated products, which did not bind to adenosine receptors. These were eluted from the HPLC column with 100% MeOH and discarded.

Preparation of Brain Membranes. Cerebral cortices of bovine or Sprague–Dawley rat brains were homogenized (Brinkmann) for 10 s in 10 volumes of ice-cold buffer A (10 mM HEPES, 10 mM EDTA, 1 mM dithiothreitol, 10% (w/v) sucrose, 10 μg/mL phenylmethanesulfonyl fluoride, and 0.1 mM benzamidine, pH 7.4). The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 500g for 10 min. The supernatant and top layer of bifurcated pellets were collected and recentrifuged at 40000g for 30 min. The resulting pellets were resuspended in 10 volumes of ice-cold buffer A lacking sucrose and stirred at 4 °C for 30 min. After recentrifugation at 40000g for 30 min, the pellet was resuspended in 5 volumes of ice-cold buffer B consisting of 10 mM HEPES, 1 mM EDTA, and 0.1 mM benzamidine, pH 7.4, aliquoted in fractions containing 5 mg/mL of protein, and stored at −70 °C.

Radioligand Binding Assays. For equilibrium binding assays, membranes were diluted with buffer B so that the final protein content was 0.5 mg of protein/mL and incubated with radioligands in 100 μL of buffer B supplemented with 2.5 units/mL adenosine deaminase, 2.5 mM $MgCl_2$, and various concentrations of other salts for 2–3 h at 21 °C. Assays tubes generally contained 0.1–0.3 nCi of radioligands ([^{125}I]-VII, [^{125}I]-IIIa, or [^{125}I]-IIIb). In some cases radioligands were diluted with the corresponding ^{127}I-containing compounds, 0.1–100 nM. Membranes were trapped on Whatman GF/C glass fiber filters with a modified cell harvester (Brandel) and washed three times with 4 mL of ice-cold 10 mM Tris·HCl, pH 7.4, with or without 0.5 M NaCl for antagonist and agonist radioligands, respectively. Nonspecific binding was determined by the addition of 100 μM PIA, 1 mM theophylline, or 1 μM IIIa with equivalent results. The K_D values of the antagonists were calcualted from their IC_{50} values to inhibit [^{125}I]-VII binding to bovine brain membranes with correction for the concentrations of radioligands and receptor.[28]

Radioligand binding parameters were estimated by the method of Scatchard[29] and then fit to one- or two-site nontransformed equations by Marquardt's method as described previously.[30] Equilibrium radioligand binding was fit to the equation

$$B_i/B_{max} = \sum_{i=1}^{n} f_i[L]/(K_{D_i} + [L])$$

where i denotes the number of different affinity states of the receptor, K_D denotes the dissociation constant(s) of the radioligand, B denotes bound radioligand, f is the fraction of receptors in each state, and [L] denotes the free radioligand concentration. Inhibition of the binding of a radioligand, L, by a competing compound, I, was fit to the equation

$$B/B_{max} = \sum_{i=1}^{n} f_i[L]/[K_D(1 + I/K_i) + [L]]$$

where B denotes bound radioligand in the presence of a competitive compound, I, and K_i denotes the dissociation constant(s) for a competing compound. [L] was approximated by [L_T]. Appropriate values for n were selected by F tests evaluated at $p < 0.01$. Proteins were determined from fluorescamine fluorescence.[30] All figures are representative of at least three experiments.

Adenylate Cyclase Assays. The binding affinity of adenosine receptor antagonists to A_2 receptors was calculated on the basis of their ability to inhibit 1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuranuronamide (NECA) stimulated adenylate cyclase activity in membranes prepared from human platelets. Three units of outdated platelet-rich plasma were centrifuged at 250g for 10 min. The pellet was discarded, and the platelets in the supernatant were pelleted by centrifugation at 16000g for 10 min. The cells were resuspended in 100 mL of 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, and 20 mM EGTA and washed a second time by centrifugation. The cells were resuspended in 100 mL of ice-cold lysing buffer (5 mM EGTA adjusted to pH 7.5 with Tris base), disrupted by sonication, and centrifuged at 40000g for 30 min. The pellet was resuspended in 30 mL of lysing buffer and frozen in 1-mL aliquots at −70 °C. Adenylate cyclase activity was assayed in 0.1 mL of buffer containing 25 μg of platelet membrane protein, 50 mM Na-HEPES (pH 7.6), 125 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM 4-(3,4-dimethoxybenzyl)-2′-imidazolidinone (Ro7-2956),[31] 10 μM Gpp(NH)p, 0.1 mM ATP, 2.5 mM phosphoenolpyruvate, 5 units/mL adenosine deaminase, and 50 units/mL pyruvate kinase. Assays were terminated after 5 min at 37 °C by the addition of 0.4 mL of 50 mM HCl. The cyclic AMP that formed was acetylated by the addition of 22.5 μL of 3.5:1 triethylamine/acetic anhydride. Following centrifugation at 2000g for 10 min, acetyl cyclic AMP in the supernatant was detected by automated radioimmunoassay.[32] NECA increased adenylate cyclase activity 3-fold with an ED_{50} of 0.4 μM. The potency of adenosine antagonists to inhibit the NECA response was calculated from the formula K_i = [antagonist]/(dose ratio − 1), where dose ratio refers to the ratio of ED_{50}'s of NECA in the presence and absence of antagonist.

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Registry No. Ia, 112533-60-5; Ib, 112533-54-7; Ic, 112533-61-6; Id, 112533-62-7; Ie, 112533-63-8; IIa, 112533-56-9; IIb, 112533-55-8; IIc, 112533-57-0; IId, 112533-58-1; IIe, 112533-59-2; IIIa, 112533-64-9; IIIa (^{125}I), 112533-72-9; IIIb, 112548-91-1; IIIc, 112533-65-0; IIId, 112533-66-1; IVa, 112533-68-3; IVb, 112533-67-2; IVc, 112533-69-4; IVd, 112533-70-7; Va, 40297-54-9; Vb, 112533-71-8; VIa, 95523-13-0; VII, 98866-49-0.

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