[125I]Aminobenzyladenosine, a New Radioligand with Improved Specific Binding to Adenosine Receptors in Heart

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SUMMARY. The density of adenosine receptors in membranes derived from rat hearts is 25 times lower than the density of receptors in rat brain membranes. Consequently, adenosine radioligands which are useful in brain such as [3H]phenylisopropyladenosine, [3H]cydohexyladenosine, [3H]-2-chloroadenosine and [3H]-hydroxyphenylisopropyladenosine are of limited usefulness in heart, due to a high ratio of nonspecific to specific binding. We have synthesized a new radioligand, [125I]-N*-4-aminobenzyladenosine, which binds to rat heart membranes with one-sixth the nonspecific binding of the other radioligands. [125I]-N*-4-aminobenzyladenosine bound to rat ventricle membranes with a K<sub>D</sub> equivalent to that of [3H]-hydroxyphenylisopropyladenosine and a B<sub>max</sub> of 15.2 fmol/mg protein. [125I]-N*-4-aminobenzyladenosine bound with a higher affinity to brain (K<sub>D</sub> = 1.93 nM) than to heart membranes (K<sub>D</sub> = 11.6 nM). At the radioligand K<sub>D</sub>, 60% of the total [125I]-N*-4-aminobenzyladenosine bound to heart membranes was specifically bound. Iodination of aminobenzyladenosine increased its affinity for the adenosine receptor by 22-fold, possibly due to a steric or hydrophobic effect of iodine. The new ligand was found to be a full adenosine agonist based on its ability to inhibit cyclic adenosine monophosphate accumulation in isolated embryonic chick heart cells and rat adipocytes. [125I]-N*-4-Aminobenzyladenosine bound to a single affinity site and was displaced from cardiac and brain adenosine receptors by other adenosine analogues with a potency order of 1-phenylisopropyladenosine > 5'-N-ethylcarboxamide adenosine. These characteristics suggest that the radioligand binds to an R<sub>I</sub> adenosine receptor. (Circ Res 56: 279-284, 1985)

RADIOLIGAND binding to adenosine receptors in membranes derived from tissues rich in adenosine receptors (brain and testes) has been convincingly demonstrated using [3H]cyclohexyladenosine, [3H]phenylisopropyladenosine, and [3H]-2-chloroadenosine (reviewed by Daly, 1982). Other adenosine radioligands which have been shown to bind to adenosine receptors include [3H]-5'-N-ethylcarboxamide adenosine (Daly et al., 1979), [3H]diethylphenylxanthine (Yeung and Green, 1984), and [125I]-hydroxyphenylisopropyladenosine (HPIA) (see Munshi and Baer, 1982; Schwabe et al., 1982; Linden, 1984). Inotropic effects by adenosine and adenosine analogues on heart have been studied extensively (Shrader et al., 1977; Dobson, 1978; Belardinelli et al., 1982), but it has been difficult to characterize adenosine receptors in heart membranes because the sparsity of receptors results in a high ratio of nonspecific to specific binding. Our initial attempts to measure adenosine receptors in heart membranes using tritiated compounds were only marginally successful. For the purposes of this study, two iodinated radioligands, [125I]HPIA and new compound [125I]-N*-4-aminobenzyladenosine ([125I]ABA), were tested as cardiac adenosine receptor radioligands. Both compounds bound specifically to adenosine receptors with similar K<sub>D</sub>'s in the nanomolar range, but the latter compound displayed one-sixth of the lower nonspecific binding and is therefore the more useful radioligand.

Methods

Synthesis of N*-4-Nitrobenzyladenosine
The N*-4-nitrobenzyl derivative of adenosine was prepared by a modification of the method of Fleysher et al. (1969). Adenosine (10.7 g, 40 mmol) was added to 140 ml of dimethylformamide and stirred at 80°C under N<sub>2</sub> until a clear solution resulted. The solution was cooled to 21°C, and p-nitrobenzylbromide (25.92 g, 120 mmol; Aldrich) was added. After being stirred at room temperature under nitrogen for 24 hours, the resultant N<sub>1</sub>-substituted adenosine derivative was rearranged to the N<sub>*</sub>-substituted compound and crystallized. The structure of this and other compounds was confirmed by UV and NMR spectra.

Synthesis of N*-4-Aminobenzyladenosine (ABA)
N*-4-Nitrobenzyladenosine (NBA) (3.62K, 9 mmol) was dissolved in acetone and 10% NaOH (pH 10). Sodium dithionite (5.22 g, 30 mmol) was added, and the mixture was heated with stirring at 40-50°C for 1 hour. The product was cooled to room temperature and adjusted to pH 7-8 with HCl. The mixture was not allowed to become acidic, since hydrolysis in acid leads to formation of the...
free base (Jones and Robins, 1963). The product was extracted with ethyl acetate several times and dried by adding Na$_2$SO$_4$. After filtration, the product was concentrated and then crystallized by the addition of hexane and cooling to 4°C for 18 hours. The crystals were filtered, washed with ethyl acetate, and stored desiccated at −20°C.

### HPLC Method for Purification of N°-Benzyladenosine Derivatives

N°-Benzyladenosine derivatives were applied to a 4.5 × 20 mm octadecyl (C18) reverse phase column and eluted with chromatography buffer (MeOH/50 mM ammonium formate, pH 8; 1:1) isocratically at a flow rate of 1 ml/min using an IBM LC/9533. UV absorption of the eluate was monitored at 271 nm. Fractions of the column eluate were collected at 30-second intervals using a Gilson 201 fraction collector. The fractions containing $^{125}$I-labeled products were counted in a Beckman 5500 γ-counter.

### Preparative Iodination of ABA

One milligram of ABA was dissolved in 2.5 ml of 0.3 N KH$_2$PO$_4$ (pH 7.5). To this solution, 0.25 ml of 10 mM KI and 10 μCi Na $^{125}$I (Amersham) were added, followed by 0.5 ml of chloramine T (2 mg/ml in H$_2$O). After mixing for 60 seconds, the iodination reaction was stopped by the addition of 1 ml of sodium metabisulfite (10 mg/ml in H$_2$O). The product was extracted five times with 0.5 ml of ethyl acetate. The pooled extracts, which contained 60% of the added radioactive iodide, were evaporated to dryness under nitrogen and dissolved in chromatography buffer. IABA was separated from ABA and I²ABA by HPLC, as described above. There was a 1:1 ratio of iodine to ABA in IABA based on the ratio of OD:tracer $^{125}$I.

### Synthesis of Carrier-Free $^{125}$I]ABA

One milliliter of Na $^{125}$I, in 100 μl of 0.3 N KH$_2$PO$_4$ (pH 7.5) was added to 100 μl of 0.1 mM ABA, followed by 5 μl of chloramine T (1 mg/ml in water). The mixture was mixed for 60 seconds, and the iodination reaction was stopped with 50 μl of sodium metabisulfite (5 mg/ml in H$_2$O). The products were extracted five times with 200 μl of ethyl acetate, evaporated to dryness under nitrogen and dissolved in chromatography buffer. I²ABA was purified by HPLC, as described above.

### Radioligand Binding Assays

I$^{[125]$IHydroxyphenylisopropyladenosine ([$^{125}$I]HPIA) synthesis, binding assays, and protein determinations were conducted as described by Linden (1984). Equilibrium binding assays with either [$^{125}$I]ABA or [$^{125}$I]HPIA, were conducted for 2 hours at 21°C and terminated by filtration over 25-mm glass fiber filters (Gelman A/E). Nonspecific binding was assessed by the addition of 0.1 mM l-phenylisopropyladenosine (PIA). Membranes were preincubated with 1 U/ml of adenosine deaminase at 37°C for 20 minutes. Fifty microliters of membranes were added to 50 μl of radioligand prepared in Tris buffer (50 mM Tris-Cl, 15 mM MgCl$_2$, and 0.1 mM EDTA, pH 7.3, at 25°C). The radioligand concentrations were held constant between 0.1 and 25 μM.

### Preparation of Membranes

Rat brains or heart ventricles were homogenized with a Polytron tissue disruptor in 25 volumes of ice-cold HEPES-sucrose buffer (mm): HEPES, 10; EDTA, 10; benzamidine, 0.1; dithiothreitol, 1; sucrose, 292. The homogenates were centrifuged at 2,000 g for 20 minutes. The pellets were resuspended in 25 volumes of ice-cold sucrose-free HEPES buffer, incubated on ice for 15 minutes, and centrifuged at 20,000 g for 30 minutes. The final pellet was resuspended in five volumes of HEPES buffer containing 20% glycerol and frozen at −70°C. The brain membranes were diluted 10-fold with HEPES buffer for binding assays, heart membranes were used undiluted. Glycerol slightly reduced the amount (20%) but had no effect on the affinity of radioligand binding.

### Preparation of Adipocytes and Heart Cells

Epidymal fat pads from 200 to 300-g male Sprague-Dawley rats were minced in Eagle’s spinner-modified minimal essential medium buffered with 10 mM Na-HEPES (pH 7.4) containing 1% bovine serum albumin and 1 mg/ml collagenase. After incubation at 37°C for 45 minutes in a shaker bath, loosely adherent cells were separated upon being pipetted through 250 μm nylon mesh. The cells were resuspended in HEPES medium and maintained at 21°C until used. Ventricles obtained from 10-day-old embryonic white Leghorn chicks were dispersed in Ca$^{++}$- and Mg$^{++}$-free phosphate-buffered saline containing 0.05% collagenase and 0.01% trypsin. Cells were harvested at 5-minute intervals. Cells were washed and maintained at 21°C in HEPES-buffered (10 mM, pH 7.4) Ham’s F12 medium until used.

### cAMP Measurements

Adipocytes and heart cells were incubated in a volume of 100 μl with 1 U/ml adenosine deaminase, 1 mM Ro7-2956 (a phosphodiesterase inhibitor), 1 mM ascorbate, and in some cases adenosine analogues or 1 μM (−)-isoproterenol for 90 seconds at 37°C. Cells were lysed by the addition of 400 μl of 0.1 N HCl. Succinyl cAMP tryosyl methyl ester was iodinated and purified by paper and anion exchange chromatography as described by Brooker et al. (1979). Tissue extracts of cAMP were acetylated and assayed by Gammaflo automated radioimmunoassay (Brooker et al., 1976).

### Results and Discussion

#### Physical Properties of NBA, ABA, and IABA

Alkylation of adenosine with p-nitrobenzylbromide gave the N°-substituted derivative which was rearranged in base to give the N°-substituted compound, NBA. Both NBA and its reduced products,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Properties of N°-Nitrobenzyl- °Aminobenzyl, and °lodoaminobenzyl Adenosines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>UV$_{max}$* (n × 10$^{-5}$)</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Adenosine</td>
<td>260 (15.1)</td>
</tr>
<tr>
<td>Nitrobenzyladenosine</td>
<td>269 (22.4)</td>
</tr>
<tr>
<td>Aminobenzyladenosine</td>
<td>268 (20.2)</td>
</tr>
<tr>
<td>Iodoaminobenzyl adenosine</td>
<td>269 (20.6)</td>
</tr>
</tbody>
</table>

* Wavelength of maximum UV absorption (nm).
† Extinction coefficient (m$^{-1}$ cm$^{-1}$).
ABA and IABA, could be readily resolved from each other by HPLC. The UV absorption spectra of NBA and ABA agreed with previously published results (Table 1). NMR confirming the structures of ABA and IABA are illustrated in Figure 1.

**Binding of[^125]I]HPIA and[^125]I]ABA to Heart Membranes**

Figure 2 compares the specific and nonspecific equilibrium binding of[^125]I]HPIA and[^125]I]ABA to heart membranes. Both radioligands bound with about the same affinity, but[^125]I]ABA displayed one-sixth the nonspecific binding.

![Figure 2](http://circres.ahajournals.org/content/111/3/281.f2)

**Figure 2.**[^125]I]ABA and[^125]I]HPIA binding to heart membranes.[^125]I]ABA (120 pm) and[^125]I]HPIA (145 pm) were incubated with the same preparation of rat ventricle membranes (460 μg) for 2 hours at 21°C. The concentrations of both radioligands were less than 10% of their Kᵦ, in which case the ratio of specifically bound to free radioligand is proportional to the receptor affinity for the radioligand. Since this ratio was approximately equal for both radioligands, we conclude that they have nearly identical Kᵦ. In contrast, nonspecific[^125]I]HPIA binding was 6.2 times greater than nonspecific[^125]I]ABA binding. Data points represent the mean ± SEM, n = 4.
Figure 3 shows the results of $^{125}$IABA equilibrium-binding assays to brain and heart membranes. Specific radioligand binding to heart membranes exceeded nonspecific binding at radioligand concentrations below its $K_D$. $^{125}$IABA bound with higher affinity to heart membranes ($K_D = 1.93$ nM) than heart membranes ($K_D = 11.6$ nM), possibly reflecting a difference in receptors. It is notable in this regard that $^3$Hcyclohexyladenosine binds with higher affinity to bovine ($K_D = 0.7$ nM) than guinea pig brains.

![Figure 4](image1.png)

**Figure 4.** Displacement of $^{125}$IABA from brain and heart adenosine receptors by PIA and NECA. Brain (72 µg) and heart membranes (451 µg) were incubated with 80 pM $^{125}$IABA and various concentrations of PIA or NECA. The $ED_{50}$ for PIA, calculated from the x-axis intercepts of the Hill plot shown in the inset, were 0.92 and 2.7 nM for brain and heart, respectively. The $ED_{50}$ for NECA were 8.1 and 2.7 nM for brain and heart, respectively. Hill coefficients ranged between 0.7 and 0.9. Data points represent the mean ± SEM, $n = 3$.

![Figure 5](image2.png)

**Figure 5.** Displacement of $^{[3]H}$PIA from brain adenosine receptors by ABA and 1ABA. Brain membranes (185 µg protein) were incubated with 160 pM $^{[3]H}$PIA and various concentrations of ABA and 1ABA. The $ED_{50}$ for 1ABA, 1.79 nM, was 22 times lower than the $ED_{50}$ for ABA, 39 nM. Hill coefficients were 0.85 and 0.88 for ABA and 1ABA, respectively; $n = 3$. Similar results were attained in a second experiment.

![Figure 6](image3.png)

**Figure 6.** Effect of adenosine analogues on the cAMP content of adipocytes. Freshly prepared adipocytes (70 µg protein) were incubated with 1 U/ml adenosine deaminase, 1 mM ascorbate, 1 mM Ro7-2956, and adenosine analogues without (top) or with (bottom) 1 µM 1-isoproterenol for 90 seconds, and then lysed with HCl (see Methods). In the basal condition, 100% corresponds to 24 pmol/mg protein; in the 1-isoproterenol-stimulated condition, 100% corresponds to 121 pmol/mg protein. Data points represent the mean ± SEM, $n = 4$. 

\[ \text{Adenosine Agonist (M)} \]

\[ \text{Brain} \]

- PIA
- NECA

\[ \text{Heart} \]

- PIA
- NECA
(KD = 6 nM; Bruns et al., 1980). The ability of PIA and 5'-N-ethylcarboxamide adenosine (NECA) to compete with [125I]ABA for binding to brain and heart membranes is illustrated in Figure 4. In both cases, NECA was less potent than PIA, indicative of binding to R receptors (Yeung and Green, 1983).

The high affinity of [125I]ABA for adenosine receptors was surprising in view of the relatively low affinity of N*-benzyladenosine compared to N*-substituted isopropyl and phenyl compounds (Daly, 1982). Displacement of [125I]HPIA by ABA or IABA from binding to brain membranes (Fig. 5) indicates that the iodinated compound has a much greater affinity for adenosine receptors in brain than the uniodinated compound. The increase in affinity upon iodination may be due to a steric or hydrophobic effect of the iodine atom.

**Effect of ABA and IABA on Adipocyte and Heart Cell Cyclic AMP Content**

The addition of three adenosine analogs, PIA, IABA, and AB, to isolated adipocytes resulted in a 40 and 80% decrease, respectively, in the basal- and I-isoproterenol-stimulated cAMP content of isolated adipocytes (Fig. 6). These experiments confirm that IABA is a full adenosine agonist with an efficacy equivalent to PIA. Also, as was the case in radioligand binding assays, IABA was found to be over 20 times more potent than ABA. PIA and IABA also decreased isoproterenol-stimulated cAMP accumulation in embryonic chick heart cells (Fig. 7). The maximum response of heart cells to adenosine agonists, 30%, was considerably less than the maximum response in adipocytes. In the absence of isoproterenol, adenosine analogs has no detectable effect on the cAMP content of heart cells. Assays were conducted in the presence of 1 mM Ro7-2956 and 1 U/ml of adenosine deaminase. cAMP levels for basal, PIA-treated (1 µM for 90 seconds), and IABA-treated heart cells were 11.74 ± 0.39, 11.10 ± 0.37, and 12.17 ± 0.41 pmol/mg protein, respectively (mean ± SEM, n = 10).

**Additional Uses of [125I]ABA**

In brain and testes, the density of adenosine receptors is in excess of 1 pmol/g wet weight, and currently available radioligands are adequate for measuring R1 receptors. However, [125I]ABA may represent an important improvement in detection of adenosine receptors in other tissues which contain fewer than 0.3 pmol/g wet weight of adenosine receptors. These include heart, lung, kidney, adrenal, fat, stomach, small intestine, pancreas, thyroid, skeletal muscle, vas deferens, ovary, and submandibular gland (Murphy and Snyder, 1981).

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**References**


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