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Purification and Characterization of (−)[^{125}I]Hydroxyphenylisopropyladenosine, an Adenosine *R*-Site Agonist Radioligand and Theoretical Analysis of Mixed Stereoisomer Radioligand Binding

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SUMMARY

(−)-N^6-(*R*-4-Hydroxyphenylisopropyl)adenosine (HPIA) was iodinated with NaI and trace ^{125}I. Mono- and diiodinated reaction products and the starting material were separated by high pressure liquid chromatography and the structures of the reaction products were verified by NMR. (−)-N^6-(*R*-Phenylisopropyl)adenosine (PIA), IHPIA, and I_2HPIA decreased rat atrial contractility with ED_{50} values of 24, 28, and 33 nM, respectively. The contractile effects of these compounds were competitively blocked by theophylline (K_I = 7.9 μM), but were not affected by adenosine deaminase. IHPIA also inhibited (−)isoproterenol-stimulated cyclic AMP accumulation in adipocytes with an ED_{50} (10 nM) and to an extent (83%) nearly identical to PIA. [^{125}I]HPIA prepared using carrier-free ^{125}I bound to adenosine receptors on membranes from rat cerebral cortex, adipocyte ghosts, and heart ventricles. Binding was inhibited stereospecifically by PIA and by other adenosine analogues and alkylxanthines. The K_D of [^{125}I]HPIA determined kinetically using brain membranes at 21° was 0.94 nM (K_1 = 2.55 × 10^7 M^{-1} min^{-1}; K_{-1} = 0.024 min^{-1}) in good agreement with the equilibrium determination of 1.94 nM. The density of adenosine receptors in brain membranes was found to be 871 fmol/mg of protein. When normalized to protein, the density of receptors in heart membranes and adipocyte ghosts, respectively, was found to be 39- and 2.3-fold less than in brain membranes. We conclude that [^{125}I]HPIA can be rapidly synthesized and purified, binds to adenosine *R*-sites and is an agonist radioligand resistant to adenosine deaminase. Computer modeling of the equilibrium binding resulting from the use of mixed stereoisomers of a radioligand indicates that the combined use of (−)[^{125}I]HPIA and (+)[^{125}I]HPIA would result in the generation of nonlinear Scatchard plots.

INTRODUCTION

Rall and Sattin (1) were the first to postulate that adenosine receptors exist. They did so in order to explain the stimulatory effects of adenosine on cyclic AMP formation in the brain. Subsequently, evidence of receptor-mediated effects produced by adenosine has been observed in many tissues including adipose tissue and heart. In adipocytes, adenosine decreases the concentration of cyclic AMP and inhibits lipolysis (2). Adenylate cyclase inhibition by adenosine *in vitro* was first demonstrated by Londos *et al.* (3) using a preparation of adipocyte plasma membranes. In the ventricular myocardium, adenosine antagonizes inotropic, electrophysiological, and metabolic effects produced by catecholamines, and attenuates cyclic AMP accumulation (4–6). Leung *et al.* (7) described adenosine receptor-mediated inhibition of myocardial adenylate cyclase in membranes prepared from guinea pig ventricles.

Initial attempts to directly characterize adenosine receptors using [^{3}H]adenosine were only partially successful (8). Schwabe and Trost (9) achieved better results characterizing adenosine receptors in rat brain using (−)[^{3}H]PIA[1] labeled to a specific activity of 26 Ci/mmol. Munshi and Baer (10) and Schwabe *et al.* (25) used carrier-free ^{125}I (>2000 Ci/mmol) to radiolabel (±)HPIA. A reaction product was shown to bind to brain homogenates and binding was displaced by unlabeled PIA or

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[1] The abbreviations used are: PIA, (−)-N^6(*R*-phenylisopropyl)-adenosine; HPIA, (−)-N^6-(*R*-4-hydroxyphenylisopropyl)adenosine; IHPIA, (−)-N^6-(*R*-3-iodo-4-hydroxyphenylisopropyl)adenosine; IBMX, isobutylmethylxanthine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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HPIA. The purpose of this study was: 1) to iodinate a single optical isomer of HPIA with carrier-free ^{125}I; 2) to fully characterize and identify the radioligand using the products of a preparative iodination; and 3) to use the radioligand as a probe for adenosine receptors in the brain and in other tissue types. The results confirm the identify of [^{125}I]HPIA and demonstrate that it is an adenosine agonist *R*-site radioligand resistant to adenosine deaminase. Computer modeling indicates that the use of mixed stereoisomers of radioligands can complicate the interpretation of binding data.

MATERIALS AND METHODS

HPIA was a kind gift of Drs. M. Senn and W. Kampe of Boehringer Mannheim. Analysis of this compound by HPLC revealed that a single peak contained 95% of the UV-absorbing material (271 nm). Ro7-2956 and 5′-*N*-(ethylcarboxamide) adenosine were gifts from Dr. P. F. Sorter of Hoffmann-LaRoche and Dr. M. L. Black of Warner Lambert, respectively. Adenosine deaminase and optical isomers of PIA were purchased from Boehringer Mannheim; cyclohexyladenosine was from Calbiochem; collagenase was from Worthington; and caffeine, theophylline, IBMX, 2-chloroadenosine, *S*-adenosyl-L-methionine chloride, and (−)isoproterenol hydrochloride were from Sigma.

Iodination of HPIA. For the preparative iodination of (−)HPIA, 1 mg was dissolved in 2 ml of 0.3 N KH_2PO_4, pH 7.5. To this solution was added 0.25 ml of 10 mM KI and 10 μCi of $Na^{125}I$. Iodination was initiated by the addition of 0.5 ml of chloramine-T (1.7 mg/ml in H_2O). After vortexing for 1 min, 1 ml of sodium bisulfite (10 mg/ml in H_2O) was added. The products were extracted five times with 0.5 ml of ethyl acetate, evaporated to dryness, and resuspended in HPLC chromatography buffer consisting of MeOH/50 mM ammonium formate, pH 8.0 (1:1). The iodination products were applied to a 4.5 × 20 mm octadecyl (C18) column and eluted isocratically at a flow rate of 1 ml/min using an IBM LC/9533. UV absorption was monitored at 271 nm, and fractions were collected at 30-sec intervals. Fractions containing IHPIA and I_2HPIA were partially dried under nitrogen, extracted into ethyl acetate, dried and redissolved in methanol for NMR and spectrophotometric scanning. To synthesize [^{125}I]HPIA for use in binding studies, 5 μl of 0.1 mM HPIA dissolved in MeOH (HPLC grade, Burdick and Jackson Laboratories) was evaporated to dryness under nitrogen and dissolved in 40 μl of 0.3 N KH_2PO_4, pH 7.5. One mCi of carrier-free $Na^{125}I$ (Amersham) was added followed by 10 μl of chloramine-T (1 mg/ml in H_2O). After vortexing for 60 sec, the iodination reaction was stopped by the addition of 50 μl of sodium bisulfite (5 mg/ml in H_2O). Reaction products containing 85–90% of the starting ^{125}I were extracted four times into 200 μl of HPLC grade ethyl acetate. [^{125}I]HPIA was purified by HPLC as described above.[2]

Atrial contractility studies. Rat left atria obtained from 300–400-g male Sprague-Dawley rats were stretched between platinum hooks in buffer composed of (in millimolar): NaCl, 120; KCl, 5; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; dextrose, 10; $NaHCO_3$, 25; $CaCl_2$, 1.5; pH 7.4, 30°. The buffer was bubbled with 95% O_2, 5% CO_2 and the atria were stimulated through point electrodes with 10-msec monophasic pulses at 2 times the threshold for contractions at a rate of 2 beats/sec. Tension was monitored using Grass FTOC3 force transducers and digitized at 1-msec intervals by a Trans Era 7050 analog to digital converter. Contractile parameters were computer monitored (Tektronix 4052) and stored on magnetic tape every 5 sec. Prior to the addition of adenosine analogs to atria, 0.2 unit/ml of adenosine deaminase was added to some tissues to hydrolyze endogenous adenosine released by the tissue.

Membrane and adipocyte preparation. Ventricles from rat hearts were minced in 25 volumes of ice-cold 50 mM Tris buffer, pH 8.07 (at 5°), 1 mM EDTA, and 0.25 M sucrose. The tissue was homogenized using a Polytron tissue disruptor for 5 sec at setting 5, filtered through gauze, and centrifuged at 1000 × *g* for 10 min. The pellet was resuspended in 25 volumes of 50 mM Tris buffer with sucrose replaced by 0.4 N LiBr, stirred on ice for 45 min, and recentrifuged. The pellet was resuspended in 25 volumes of 1 mM Tris buffer (1 mM Tris base plus 0.25 mM EDTA) and recentrifuged. The final pellet was resuspended in 10 volumes of 1 mM Tris buffer/glycerol (4:1) and frozen at −70° in aliquots containing 400 μg of protein/ml.

Cerebral cortexes from rats were frozen between tongs precooled in liquid nitrogen. Tissues were stored under liquid nitrogen prior to homogenization in 25 volumes of ice-cold Tris/magnesium buffer (50 mM Tris, pH 7.9, at 5°, 0.5 mM EDTA, 15 mM $MgCl_2$), filtered through gauze, and centrifuged at 20,000 × *g* for 20 min. The pellet was resuspended and recentrifuged in Tris/magnesium buffer and the final pellet was resuspended in 25 volumes of Tris/magnesium buffer and frozen at −70° in aliquots containing 2 mg of protein/ml.

Fat cell ghosts were prepared freshly on the day they were used. Epididymal fat pads were collected in Ham's F12 medium buffered with 10 mM Na-Hepes, pH 7.4, and minced in the same medium supplemented with 1 mg/ml collagenase (Worthington) and 1% bovine serum albumin. After incubation for 45 min at 37° in a shaking water bath, tissue pieces were dispersed by repeated aspiration into a 10-ml plastic pipette, filtered through 100-μm nylon mesh, diluted 1:1 with Ham's F12 medium containing 1% bovine serum albumin and allowed to settle into two layers. The top layer containing adipocytes was washed with room temperature lysis buffer (2 mM Na-Hepes, pH 7.5, 2.5 mM $MgCl_2$, 2.5 mM ATP, and 0.2 mM $CaCl_2$). Adipocytes were lysed by being resuspended and vigorously mixed in ice-cold lysis buffer. After centrifugation at 250 × *g* for 2 min, the infranatant was collected; unlysed cells were subjected to a second exposure to lysis buffer. Pooled infranatant fractions containing fat cell ghosts were pelleted by centrifugation at 10,000 × *g* for 10 min, washed once, and resuspended in Tris/magnesium buffer, filtered through 20-μm nylon mesh and placed on ice until their use. The protein content of adipocyte membranes was 0.8–1.2 mg/ml. In some experiments, the cyclic AMP content of intact adipocytes was measured. Cells were washed once with Hepes-buffered Ham's F12 medium and incubated with various drugs in 0.2 ml for 5 min at 37°. The incubation was terminated by the addition of 0.8 ml of 0.1 N HCl. After centrifugation at 20,000 × *g* for 20 min, 0.25 ml of infranatants was acetylated and assayed for cyclic AMP by Gammaflow automated radioimmunoassay (11).

Binding assays. Membranes were preincubated at 37° for 20 min with 2 units/ml of adenosine deaminase in order to metabolize endogenous adenosine. The enzyme did not metabolize IHPIA as judged by

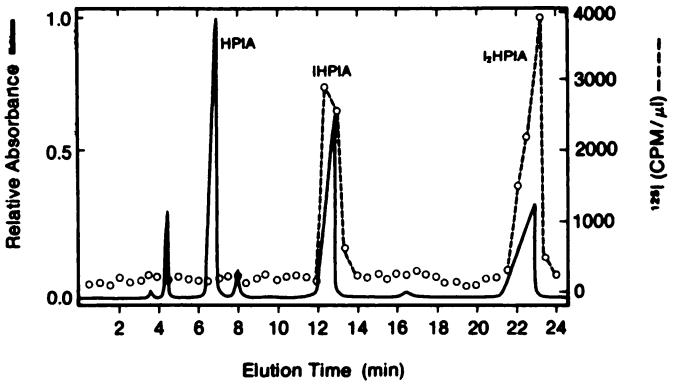

FIG. 1. *HPLC of the iodination products of HPIA*

HPIA was iodinated with KI containing trace ^{125}I and chromatographed by HPLC. UV absorption was continuously monitored at 271 nm. Two-μl aliquots of 0.5-ml fractions collected at 0.5-min intervals were counted in a gamma counter. The peak labeled *HPIA* co-migrated with the starting material. The other peaks were tentatively assigned structures based on the ratio of UV absorbance to ^{125}I.

[2] In order to clean the HPLC injection loop of contaminating ^{125}I after the application of millicurie amounts of ^{125}I-containing compounds, the loop was flushed several times with chloroform.

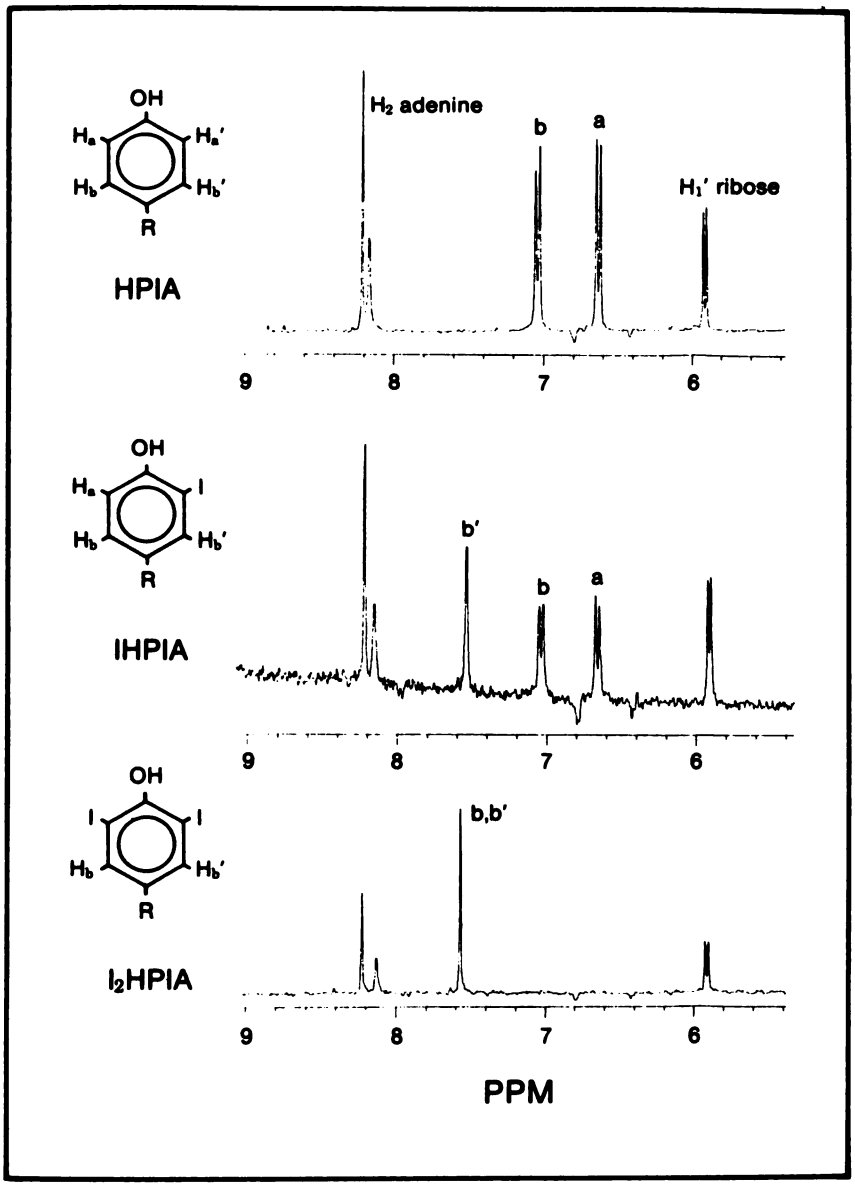

FIG. 2. *NMR of HPIA, IHPIA, and I_2HPIA*

Fractions corresponding to the three major peaks shown in Fig. 1 were dissolved in methanol and analyzed. Methanol residual methyl peaks were set to 3.28 ppm. R = N^6-isopropyladenosine.

bioassay using atrial strips and by HPLC. Concentrates of [^{125}I]HPIA were evaporated to dryness under nitrogen and taken up in Tris/magnesium buffer. For equilibium binding assays, 50 μl each of membranes and radioligand were incubated for 2 hr at 21°. Assays were terminated by twice adding 4 ml of wash solution (10 mM KH_2PO_4, pH 7.4, 0.1 mM EGTA, and 0.5 mM $MgCl_2$) to test tubes containing membranes and decanting them over 25-mm glass fiber filters (Schleicher and Schuell No. 30). The filters were washed with an additional 8 ml of wash solution and counted in a Beckman Gamma 5500 counter at 73% efficiency. Radioligand concentrations were calculated assuming carrier-free ^{125}I with correction for isotope decay from the date of radioligand synthesis. Since [^{125}I]HPIA bound to membranes with K_D values in the nanomolar range, equilibrium binding assays required isotope dilution with unlabeled IHPIA. For these studies, [^{125}I]HPIA was held constant at 20–40 pM and unlabeled IHPIA was added at concentrations ranging between 1 and 50 nM. Nonspecific binding to adenosine receptors was assessed by the addition of 100 μM (−)PIA. Substituting 100 μM IHPIA or 1 mM IBMX for PIA resulted in equivalent measures of nonspecific binding. Radioligand dissociation constants and receptor density were calculated using a nonlinear least squares fitting algorithm (12, 13) to the untransformed binding data assuming a single class of receptors: $B = R_T \cdot H/(K_D + H)$, where B = specifically bound radioligand, R_T = receptor number, K_D = radioligand dissociation constant, and H = free radioligand concentration. Parameters were initially estimated using Scatchard (14) analysis. Fifty mM HCl was used as a vehicle for PIA stocks solutions (10 mM).[3]

The second order rate constant for the formation of receptor ligand complex, K_1, was calculated from kinetic binding data by calculating least squares linear regression fit of Eq. 1 versus time:

$$[\ln[B_e(H - B \cdot B_e/R_T)/(H \cdot (B_e - B))]]/(H \cdot R_T/B_e - B_e) \qquad (1)$$

where B_e = specifically bound radioligand at equilibrium. H is the

[3] Some sources of dimethyl sulfoxide, recommended as a vehicle for PIA by the manufacturer, contained impurities which inhibited [^{125}I]HPIA binding to brain membranes.

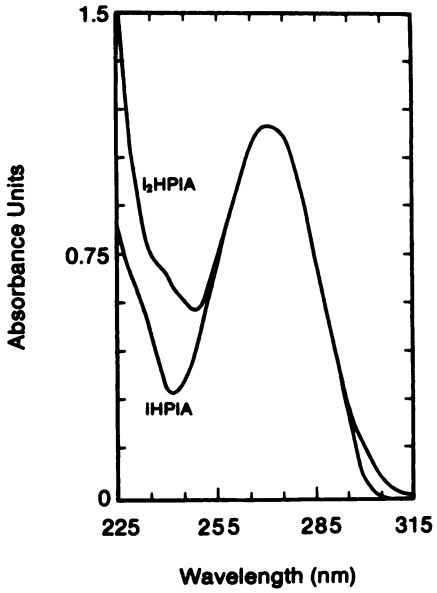

FIG. 3. *UV scans of IHPIA and I_2HPIA*

Compounds dissolved in 100% methanol were scanned using a Beckman DU-8 spectrophotometer at a rate of 20 nm/min using a slit width of 0.5 nm and a path length of 1 cm. The concentrations of IHPIA and I_2HPIA, respectively, were 57 and 55 μM as determined from trace ^{125}I. The λ_{max} for both compounds was 271 nm. Molar extinction coefficients, ϵ, for IHPIA and I_2HPIA, respectively, were calculated to be 20,600 and 21,350 M^{-1} cm^{-1} at 271 nm.

initial free concentration of radioligand, B is the concentration of specifically bound radioligand at each time point, and R_T is the receptor concentration previously determined by equilibrium binding analysis.

The IC_{50} values for drugs, i.e., the concentrations required to inhibit specific binding by 50%, were calculated at the x axis intercepts of Hill plots in which $\log[\%I/(100 - \%I)]$ was plotted against log dose where $\%I$ represents the per cent inhibition of specific binding. IC_{50} values were also calculated by Hill analysis of inhibition of atrial contractility and inhibition of cyclic AMP accumulation in adipocytes. In these cases $\%I$ represented the percentage of the maximal drug-induced inhibitory response.

Dissociation constants (K_I) were calculated for competitive inhibitors of radioligand binding from IC_{50} values with correction for the concentrations of radioligand and receptor (15):

$$I_f = IC_{50} - R_T + (R_T/2)\cdot[H/(K_D + H) + K_D/(K_D + H + R_T/2)] \quad (2)$$

$$K_I = I_f/[1 + H/K_D + (R_T/K_D)\cdot(K_D + H/2)/(K_D + H)] \quad (3)$$

where I_f = free concentration of inhibitor, H = free concentration of radioligand in the absence of inhibitor, and K_D is the dissociation constant of the radioligand.

Theoretical analysis of the binding of radioligand containing mixed stereoisomers. The equilibrium binding of radioligand composed of two stereoisomers which bind with different affinities to a single receptor is given by:

$$F_1 = L_1/K_1/(1 + L_1/K_1 + L_2/K_2) \quad (4)$$

$$F_2 = L_2/K_2/(1 + L_1/K_1 + L_2/K_2) \quad (5)$$

$$L_T/2 = L_1 + F_1\cdot R \quad (6)$$

$$L_T/2 = L_2 + F_2\cdot R \quad (7)$$

where F_i = the fractional occupancy of the receptor by each optical isomer of the ligand, L_i = the free concentration of each ligand, K_i = the dissociation constant of each ligand, L_T is the total ligand concentration, R is the total receptor concentration, and the total amount of each stereoisomer is assumed to be $L_T/2$.

Algebraic manipulation of these formulae yields a cubic equation:

$$Ax^3 + Bx^2 + cx + D = 0 \quad (8)$$

where $x = L_1$; $T = L_T/2$; $C_1 = T\cdot K_1$; $C_2 = K_2 - K_1$; $A = C_2$; $B = R\cdot C_2 - T\cdot C_2 + K_1\cdot C_2 + 2\cdot C_1$; $C = C_1\cdot(R - 2\cdot T + K_1) - T\cdot K_1\cdot C_2$; $D = -T\cdot K_1\cdot C_1$. This equation was solved for L_1 by interpolation using Newton's

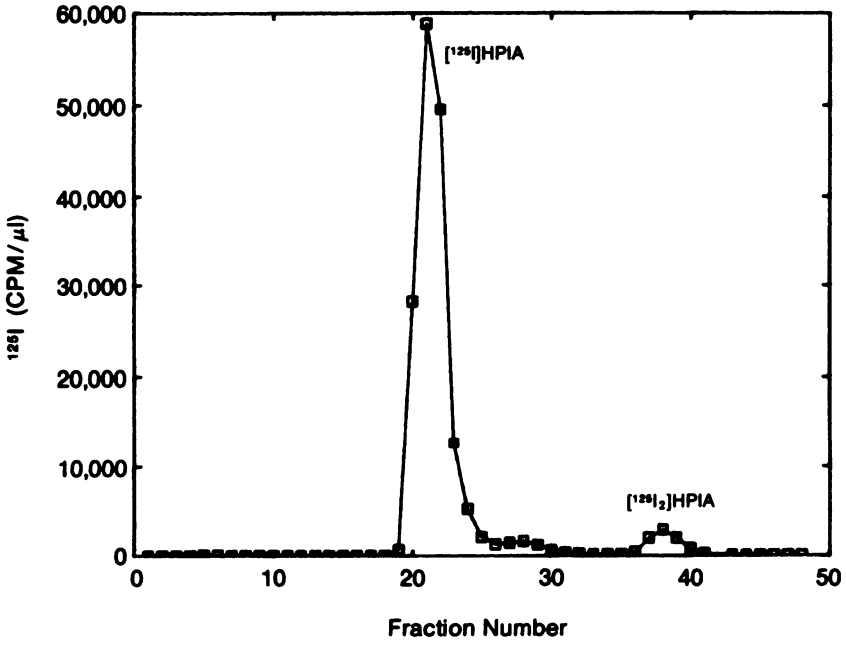

FIG. 4. *HPLC of [^{125}I]HPIA*

HPIA was iodinated with carrier-free ^{125}I and chromatographed by HPLC as described in the legend for Fig. 1. Peaks 20–23 were pooled and stored at −20° in chromatography buffer and used for binding studies. Rechromatography after 60 days of storage revealed that 97% of the ^{125}I was retained as [^{125}I]HPIA.

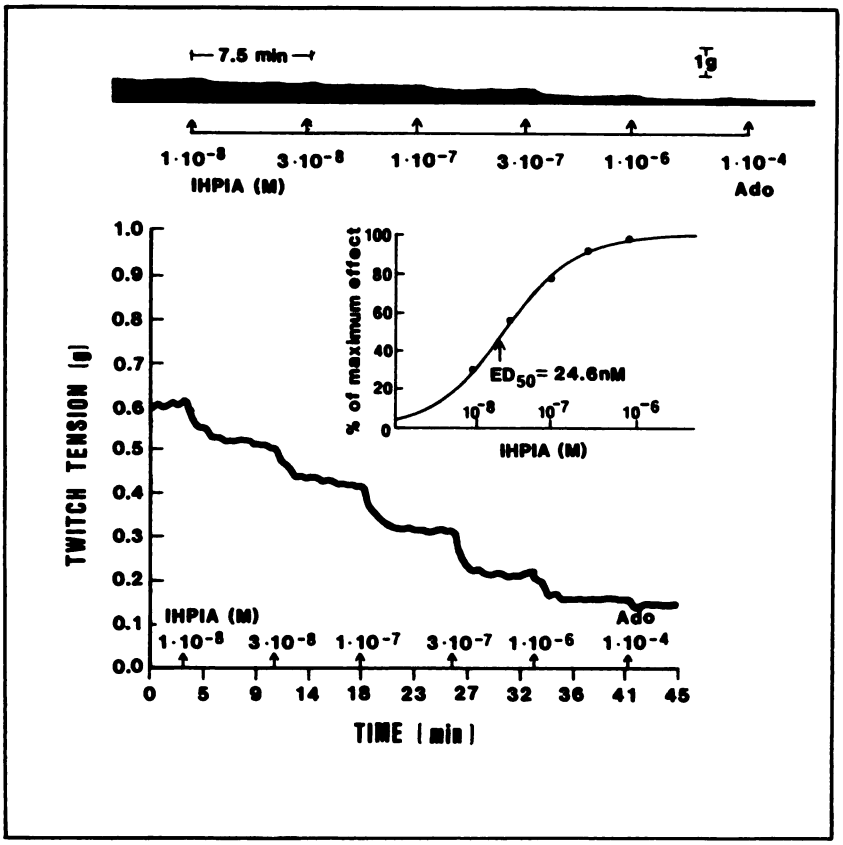

FIG. 5. *The effect of IHPIA on rat atrial contractility*

An isolated rat atrium was exposed to cumulative additions of IHPIA at 10-min intervals. Top: twitches recorded on Beckman RT-11 oscillographic recorder. Bottom: data from the same atrium simultaneously recorded by computer monitoring as described in Materials and Methods. Middle: semilog dose inhibition curve of the contractile data. *Ado*, adenosine.

TABLE 1

Effects of adenosine analogues on the contractility of rat atria

Atria were exposed to cumulative additions of drugs at 10-min intervals. Data are expressed as means ± standard error ($n = 3$).

Drug	ED_{50}	Hill coefficient	Efficacy[a]
	nM		%
PIA	23.7 ± 5.8	0.91 ± 0.18	71.5 ± 6.1
IHPIA	28.0 ± 4.1	1.30 ± 0.21	77.6 ± 3.4
IHPIA + 0.2 unit/ml AD[b]	24.8 ± 3.1	1.01 ± 0.18	75.1 ± 2.2
I_2HPIA	33.0 ± 4.0	0.86 ± 0.24	80.3 ± 2.2
PIA + 10 μM theophylline[c]	53.9 ± 1.9[d]	0.95 ± 0.17	75.9 ± 4.0
IHPIA + theophylline[c]	55.0 ± 5.1[d]	1.11 ± 0.11	75.0 ± 4.1

[a] Per cent decrease in developed tension caused by a maximally effective concentration of drug.

[b] AD, adenosine deaminase, added 10 min before the first dose of IHPIA, had no effect on basal atrial contractility.

[c] Theophylline, added 10 min before the first dose of PIA or IHPIA, had no effect on basal atrial contractility.

[d] Greater than PIA or IHPIA alone by the Tukey test ($p < 0.01$).

method [$X' = X - F(x)/F'(x)$]. Other parameters are: $L_2 = T \cdot L_1 \cdot K_2/(C_1 + C_2 \cdot L_1)$; $F_1 = (T - L_1)/R$; $F_2 = (T - L_2)/R$. Bound radioligand is given by $R \cdot (F_1 + F_2)$; free radioligand is given by $L_1 + L_2$.

RESULTS

Characterization of HPIA and its iodination products. After iodination of HPIA, two major new UV-absorbing peaks were detected by HPLC in addition to the starting material. Trace ^{125}I added during the iodination reaction was confined exclusively to the two new peaks. From the ratio of ^{125}I:UV absorbance in the new peaks, they were tentatively assigned structures corresponding to mono- and diiodinated products (Fig. 1). The HPLC eluate containing the three major UV-absorbing peaks shown in Fig. 1 were analyzed by NMR from which the molecular structures were confirmed (Fig. 2). Spectrophotometric scanning of IHPIA and I_2HPIA revealed UV absorption maxima at 271 nm (Fig. 3). From the specific activity of trace ^{125}I contained in these compounds, molar extinction coefficients were calculated for both compounds. The results of iodination and HPLC of HPIA with carrier-free ^{125}I are shown in Fig. 4. Since there was a large excess of HPIA over ^{125}I, the bulk of the product was monoiodinated. Fractions 20–23 were pooled and stored at −20° for use in binding assays.

Effects of adenosine analogs on atrial contractility and adipocyte cyclic AMP metabolism. The addition of adenosine analogs to isolated electrically driven rat left atria resulted in a time- and dose-dependent decrease in developed tension. The results of a typical experiment are illustrated in Fig. 5, and a summary of results is presented in Table 1. There was no significant difference in the potencies of (−)PIA, IHPIA, and I_2HPIA and all three compounds also had similar maximal effects. Adenosine deaminase had no effect on the potency of IHPIA, indi-

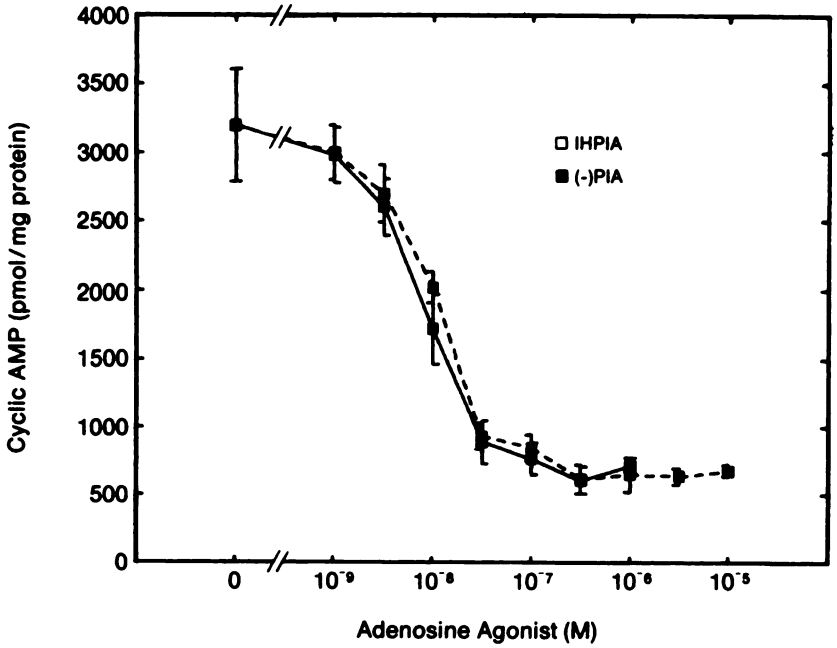

FIG. 6. *Effects of (−)PIA and IHPIA on (−)isoproterenol-stimulated cyclic AMP accumulation in adipocytes*

Adipocytes were freshly prepared and assayed for cyclic AMP content as described in Materials and Methods. The cells were incubated for 5 min at 37° with 1 mM Ro7-2956, 1 mM ascorbate, 1 μM (−)isoproterenol, and 0.2 unit/ml adenosine deaminase. Data are expressed as means ± standard error (n = 5). The ED_{50} values for IHPIA and PIA, respectively, were calculated by Hill analysis to be 9.3 and 11.2 nM. At maximally effective concentrations, both compounds reduced the cyclic AMP content of adipocytes by 83%.

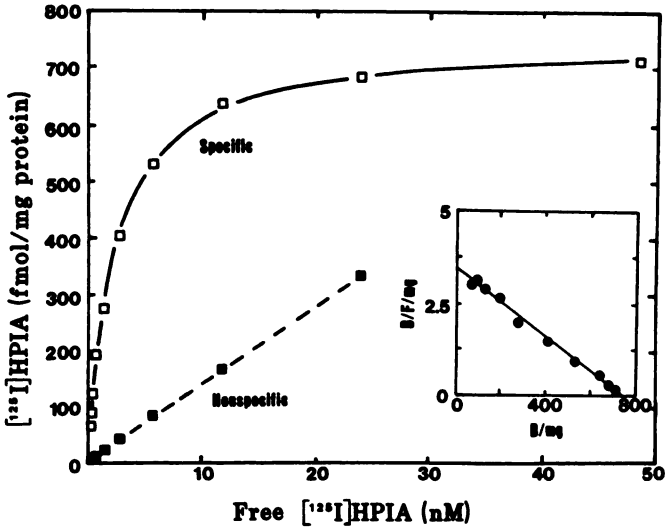

FIG. 7. *Equilibrium binding of [125I]HPIA to brain membranes*

Assays were conducted at 21° for 2 hr in a volume of 0.1 ml on membranes pretreated with adenosine deaminase as described in Materials and Methods. Each point is the mean of three closely agreeing replicates. Inset: Scatchard analysis of specific radioligand binding. The line was calculated by linear regression analysis (r = 0.997) B, [125I]HPIA specifically bound (fmol/mg of protein); F, free concentration of radioligand (in femtomoles); R_T = 751 fmol/mg protein; K_D = 2.3 nM.

TABLE 2

Summary of equilibrium binding data using [125I]HPIA as a probe for adenosine receptors

Binding studies were performed in triplicate using six to eight ligand concentrations. Data are presented as the means ± standard error.

Tissue	R_T	Adenosine receptors		
		K_D	r[a]	Specific binding[b]
	fmol/mg protein	*nM*	r[a]	
Brain	871 ± 31	1.94 ± 0.74	0.982	0.86
Adipose	379 ± 11	2.95 ± 0.84	0.981	0.68
Heart	22 ± 8	3.11 ± 0.95	0.959	0.28

[a] The aveage correlation coefficients of least squares linear regression fits to Scatchard plots.

[b] The average ratio of specific binding to total binding measured at the radioligand K_D.

cating that this compound is resistant to the enzyme. The addition of 10 μM theophylline alone to atria produced no inotropic response, indicating that at this concentration the methylxanthine does not produce significant phosphodiesterase inhibition. Ten μM theophylline shifted the ED_{50} of (−)PIA and IHPIA to the right 2.3-fold without affecting the maximum responses, suggesting that theophylline is a competitive adenosine receptor antagonist. Using the formula dose ratio −1 = [theophylline]/K_I, the K_I for theophylline was calculated to be 7.9 μM. In order to determine if IHPIA acts as an adenosine agonist in adipose tissue, its ability to reduce (−)isoproterenol-stimulated cyclic AMP accumulation in intact adipocytes was examined. As illustrated in Fig. 6, the inhibitory effects of (−)PIA and IHPIA on adipocyte cyclic AMP accumulation were virtually indistinguishable both in terms of potency and efficacy.

[125I]HPIA binding to tissue homogenates. Equilibrium binding assays were conducted to assess the density and affinity for [125I]HPIA of adenosine receptors present in

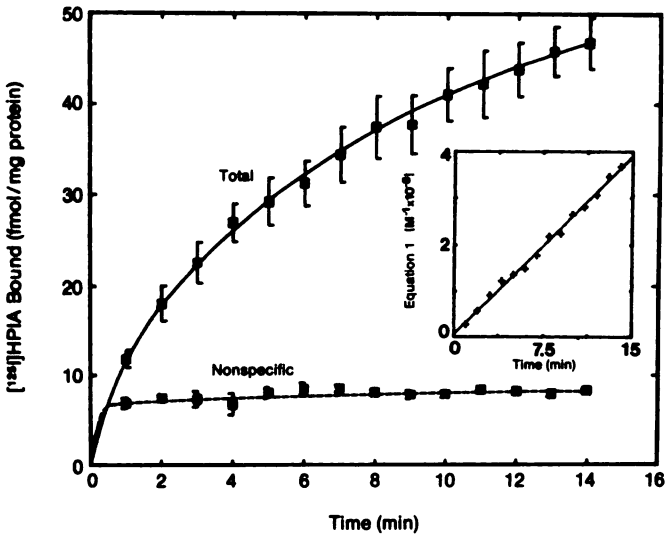

FIG. 8. *Kinetics of [^{125}I]HPIA binding to brain membranes*

Test tubes containing 0.75 mg/ml of brain membranes and 233 pM [^{125}I]HPIA were incubated in the absence (□) or presence (■) of 100 μM (−)PIA. Aliquots (100 μl) were removed at various times and washed by filtration; each point is the mean ± standard error of three determinations. Inset: the data was transformed according to Eq. 1 (see Materials and Methods) and fit with a straight line by linear regression analysis (r = 0.998). H, the initial concentration of free radioligand, was assumed to be equal to the total radioligand concentration (233 pM). R_T, calculated using the receptor density shown in Table 2, was 653 pM. Specific binding, B, was calculated from the difference between total and nonspecific binding at each time point. Specific binding at equilibrium, B_e, measured after 2 hr of incubation was 49 fmol/mg of protein. The second order rate constant, K_1, given by the slope of the fit line, was 2.55×10^7 M^{-1} min^{-1}.

membranes derived from brain, adipose tissue, and heart. A typical experiment performed on membranes derived from brain is illustrated in Fig. 7. Binding parameters from the three tissue types are summarized in Table 2. The affinity of [^{125}I]HPIA for adenosine receptors did not vary significantly with tissue type. Membranes derived from brain and adipose tissue had considerably more adenosine receptors than membranes derived from heart.

[^{125}I]HPIA binding to adenosine receptors was further characterized using brain membranes. The kinetics of [^{125}I]HPIA binding to and dissociation from brain adenosine receptors was determined from experiments illustrated in Figs. 8 and 9. From the ratio of rate constants, K_{-1}/K_1, the kinetically determined K_D was calculated to be 0.94 nM, which is close to the equilibrium determination of 1.94 nM (Table 2).

Competition with [^{125}I]HPIA for binding to adenosine receptors by adenosine analogs and methylxanthines. [^{125}I]HPIA binding to adenosine receptors could be inhibited by other compounds known to bind to *R*-sites. Binding was inhibited stereospecifically by PIA (Fig. 10). Dissociation constants for adenosine analogs were calculated from the ED_{50} values of competition assays using Eqs. 2 and 3 in Materials and Methods. These are, in ascending nanomolar order: (−)PIA, 1.1; cyclohexyladenosine, 1.2; 5′-*N*-(ethylcarboxamide)adenosine, 2.1; 2-chloroadenosine, 3.5; (+)PIA, 16.2; *S*-adenosyl-L-methionine, 220. Methylxanthines also competed with [^{125}I]HPIA for binding to adenosine receptors with a potency order IBMX > theophylline > caffeine (Fig. 11). The apparent K_I for theophylline as an inhibitor of PIA

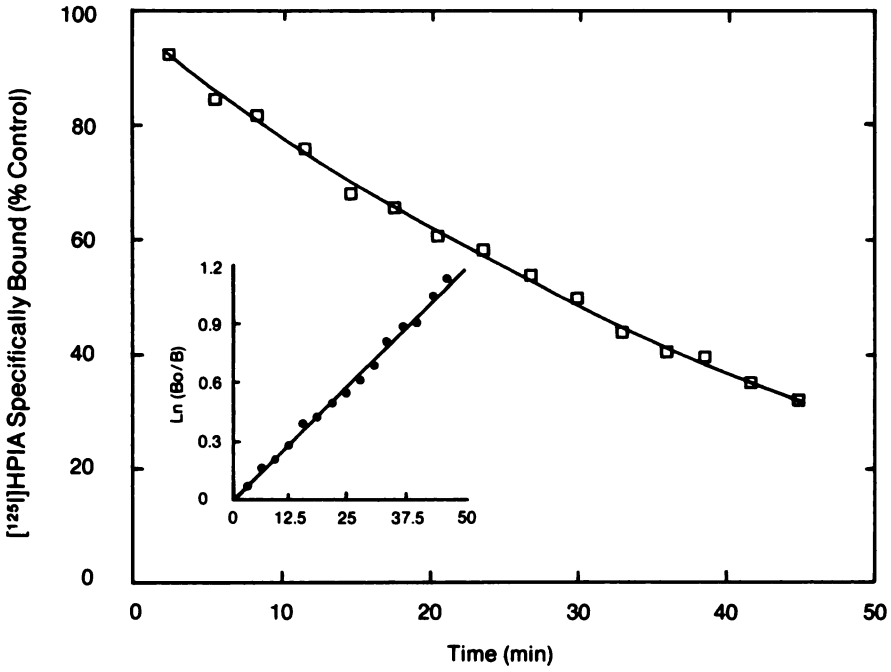

FIG. 9. *Kinetics of [^{125}I]HPIA dissociation from brain membranes*

Brain membranes were preincubated with [^{125}I]HPIA for 2 hr at 21°. Dissociation of the radioligand from receptors was monitored at 3-min intervals following the addition of 100 μM (−)PIA. Nonspecific binding, measured 2 hr after the addition of (−)PIA, was subtracted from each point to calculate specific binding. Each point represents the mean of three determinations. Inset: $\ln(B_0/B)$ is plotted as a function of time where B_0 represents specific binding 3 min after the addition of (−)PIA and B represents specific binding at various time points thereafter. The line was fit by linear regression analysis (r = 0.996). The rate constant for dissociation, K_1, given by the slope of the fit line, is 0.024 min^{-1}. The $t_{1/2}$ for dissociation ($\ln 2/K_{-1}$) is 28.9 min.

contractile effects in atria, 7.9 μM, agrees well with its K_I as an inhibitor of [125I]HPIA binding to brain membranes, 5.9 μM. The nomethylxanthine phosphodiesterase inhibitor Ro7-2956 (1 mM) decreased specific [125I]HPIA binding to brain adenosine receptors by only 26%.

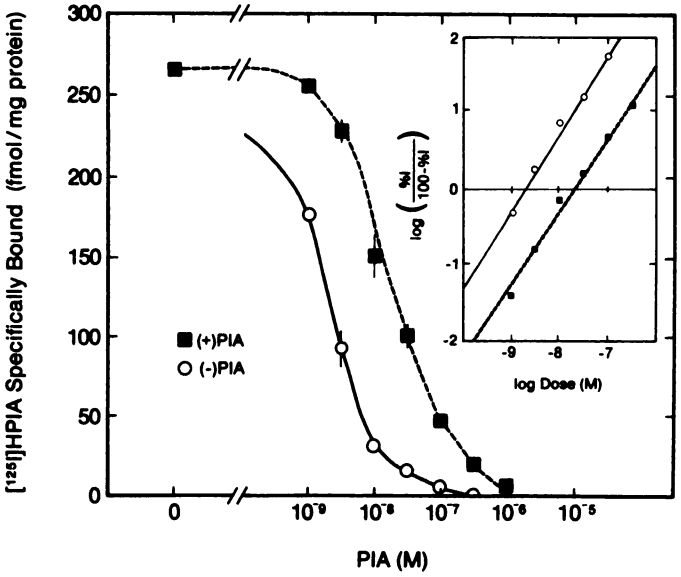

FIG. 10. *Competition for [125I]HPIA binding to brain membranes by optical isomers of PIA*

Brain membranes containing 17 fmol of adenosine receptors (19.5 μg of protein) were incubated with 125 pM [125I]HPIA and various concentrations of PIA in a volume of 100 μl. Specific binding was calculated by subtracting nonspecific binding (22 fmol/mg of protein) from the mean of triplicate determinations of total binding. Inset: Hill plots of the same data. IC_{50} values for (−)PIA and (+)PIA, respectively, were 1.32 and 19.8 nM; Hill coefficients were 1.00 and 0.98.

Computer modeling of the binding of mixed stereoisomers of a radioligand to a single population of receptors. In order to assess the advantage of using a single stereoisomer of [125I]HPIA for binding studies versus using a mixed stereoisomer, theoretical binding of a mixed stereoisomer radioligand was evaluated using Eqs. 4–8 in Materials and Methods. For the purposes of this analysis, we assumed that both isomers exist in equal concentrations and that their K_D values differ by 10-fold, which approximates the differences in K_D of the stereoisomers of PIA for the adenosine receptor. As shown in Fig. 12A, binding of the mixed stereoisomer results in a curvilinear concave down Scatchard plot. The degree of curvature and the slope of the Scatchard plot depend on the relative values of K_D and R_T. If R_T is well below both K_D values, the Scatchard plot approaches linearity and the apparent $K_D = 2 \cdot K_1 \cdot K_2/(K_1 + K_2)$. Figure 12B illustrates how the ED_{50} of the radioligand varies depending on the value of R_T. For the case of IHPIA, approximate values of K_1 and K_2 are 2 and 20 nM. Assuming a receptor density of 1 pmol/mg of protein, R_T would be 1 nM if the protein concentration in the assay were 1 mg/ml. This corresponds to $R_T = 0.5$ (relative to K_1) in Fig. 12B.

DISCUSSION

IHPIA has been shown to share several pharmacological features which have been described previously for PIA. These include *R*-site agonist properties in heart

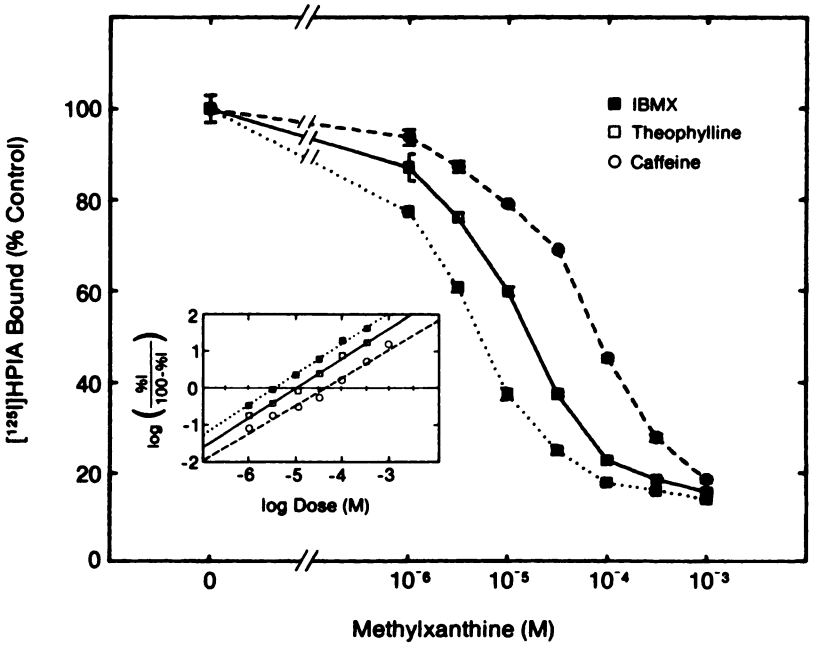

FIG. 11. *Competition for [125I]HPIA binding to brain membranes by methylxanthines*

Brain membranes containing 85.9 fmol of adenosine receptors (99 μg of protein) were incubated with 120 pM [125I]HPIA and various concentrations of methylxanthines in a volume of 100 μl. Radioligand binding in the presence of 1 mM IBMX was equivalent to nonspecific binding as assessed by the addition of 100 μM (−)PIA. Each point is the mean ± standard error of three determinations. Inset: Hill plots of specifically bound radioligand. IC_{50} values for IBMX, theophylline, and caffeine, respectively, were 3.73, 9.05, and 36.2 μM. Hill coefficients were 1.05, 0.85, and 0.78. Dissociation constants for IBMX, theophylline, and caffeine, respectively, calculated using Eqs. 2 and 3 in Materials and Methods, were 2.42, 5.87, and 23.5 μM.

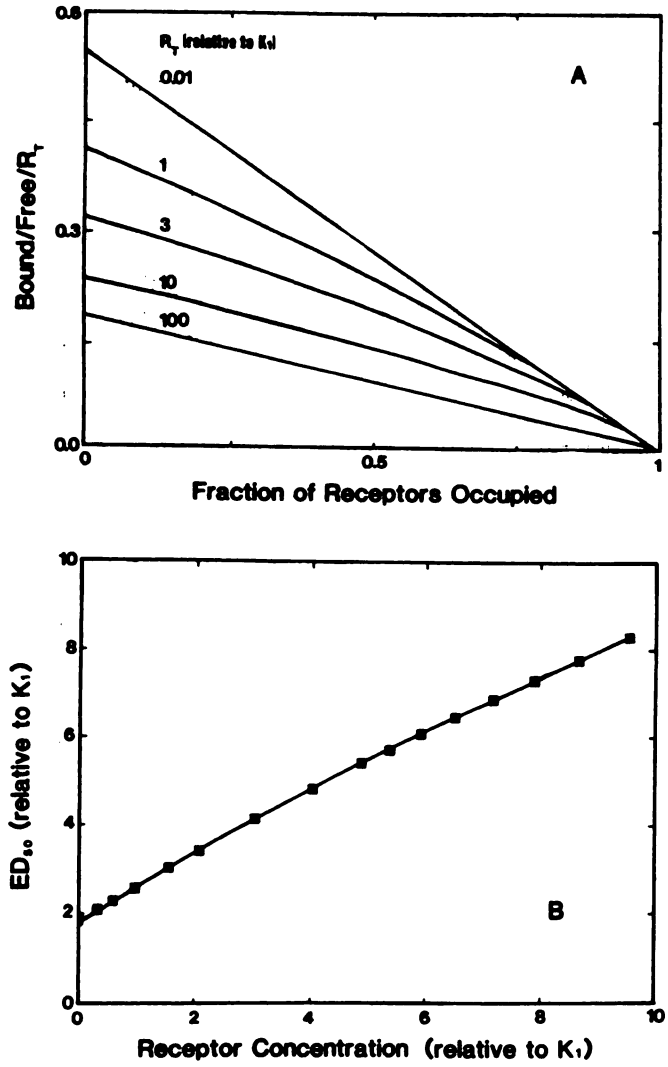

FIG. 12. *Theoretical binding of a radioligand containing mixed stereoisomers to a single receptor*

The data were analyzed using Eqs. 4–8 in Materials and Methods assuming equal concentrations of both stereoisomers. The relative values for the K_D (K_1 and K_2) were set to 1 and 10, respectively. A: Scatchard plots of theoretical binding for different values of R_T as indicated. B: ED_{50} (i.e., the concentration of radioligand required to occupy half of the receptors) is plotted as a function of receptor concentration.

(16) and adipose tissue (17) and resistance to degradation by adenosine deaminase (18). Methylxanthines inhibited the binding of [^{125}I]HPIA to brain membranes with the same potency order with which methylxanthines have been reported to block the effect of PIA: IBMX > theophylline > caffeine (19, 20). These findings conform to the generalization of Londos *et al.* that N^6-substituted adenosine derivatives retaining an intact ribose ring act on methylxanthine-sensitive "ribose-site" (R-site) extracellular receptors and bind poorly to internal sites which require an unsubstituted purine group for binding to the "purine-site" (21, 22). The similarity in the potency of IHPIA and (−)PIA both as negative inotropic agents, and an inhibitors of cyclic AMP accumulation in adipocytes suggests that the addition of hydroxyl and iodine to the phenyl group of (−)PIA has little effect on its binding affinity or pharmacology. As a radioligand, [^{125}I]HPIA appears to be similar to [^{3}H]PIA (9) and [^{3}H]cyclohexyladenosine (23). In brain membranes, [^{3}H]PIA and [^{125}I]HPIA bound to about the same number of specific receptor sites with similar affinities and kinetics.

Apparently, there are two types of extracellular R-site adenosine receptors designated $R_i(A_1)$ which decrease adenylate cyclase activity, and $R_a(A_2)$ which stimulate adenylate cyclase activity (24). [^{125}I]HPIA clearly binds to R_i receptors since adenosine receptors in adipose tissue (17, 24) and heart (6, 16) have been characterized as R_i. While both R_a and R_i receptors have been reported in brain, displacement of [^{125}I]HPIA by nanomolar concentrations of cyclohexyladenosine and the stereospecifically displayed by PIA are indicative of binding to R_i rather than R_a receptors (23). R_a receptors were probably not occupied by IHPIA at the concentrations used for this study, 50 nM. The kinetics of binding of [^{125}I]HPIA to R_i receptors is noteworthy. The value of the forward rate constant $K_1 = 2.55 \times 10^7$ M^{-1} min^{-1} at 21°, is small for a typical second order reaction limited by diffusion through water. Schwabe and Trost (9) reported a similar small K_i (6.58×10^6 M^{-1} min^{-1} at 37°) for [^{3}H]PIA binding to brain membranes. These data suggest either that the chemical interaction between the receptor and radioligand is slow, or [^{125}I]HPIA or [^{3}H]PIA in the vicinity of adenosine receptors is not in rapid equilibrium with the radioligands in the bulk aqueous phase or high affinity binding of these agonists to R_i receptors requires a kinetically slow interaction between the receptor and another protein, e.g., a guanine nucleotide-binding protein.

The method described here for the synthesis and purification of [^{125}I]HPIA results in a good yield of ^{125}I incorporation (>85%), rapid purification (1 hr), and radioligand stability (>60 days). We anticipate the [^{125}I]HPIA will be a useful radioligand with which to characterize R-type adenosine receptors from many tissues.

The theoretical analysis of the binding of a mixed stereoisomer radioligand points out some problems with their use for binding assays. The apparent dissociation constant depends on the ratio of K_D values even if the receptor concentration is well below both K_D. If the receptor concentration is raised to the point where a significant fraction of both stereoisomers bind, then Scatchard plots become markedly curvilinear and the ED_{50} of the radioligands varies with R_T. Therefore, the use of a single optimal isomer of [^{125}I]HPIA or other radioligands is preferable to the use of mixed stereoisomers for the accurate and straightforward analysis of binding parameters.

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