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Characterization of Agonist Radioligand Interactions with Porcine Atrial A_1 Adenosine Receptors

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SUMMARY

The agonist radioligand (−)-N^6-[^{125}I]-*p*-hydroxyphenylisopropyladenosine ([^{125}I]HPIA) was used to characterize adenosine recognition sites in porcine atrial membranes. [^{125}I]HPIA showed saturable binding to an apparently homogeneous population of sites with a maximum binding capacity of 35 ± 3 fmol/mg of protein and an equilibrium dissociation constant of 2.5 ± 0.4 nM. Kinetic experiments were performed to address the molecular mechanism of [^{125}I]HPIA binding in porcine atrial membranes. [^{125}I]HPIA apparently interacts with the cardiac adenosine receptor in a simple bimolecular reaction. A kinetically derived [^{125}I]HPIA dissociation constant (2.4 nM) was in good agreement with that parameter measured at equilibrium. Guanyl nucleotides negatively modulated [^{125}I]HPIA binding by increasing its rate of dissociation. This finding is consonant with the formation of a ternary complex in porcine atrial membranes, consisting of ligand, receptor, and guanyl nucleotide-binding protein. Prototypic adenosine receptor agonists and antagonists inhibited specific binding in a manner consistent with the labeling of an A_1 adenosine receptor. The results of these experiments suggest that the adenosine receptor present in porcine atrial membranes, as labeled by [^{125}I]HPIA, is of the A_1 subtype.

Adenosine has been identified as a putative neurotransmitter or neuromodulator in the central nervous system as well as a physiological regulator in several peripheral cell types (1, 2). Effects of adenosine on the myocardium are uniformly inhibitory in nature (3). Negative chronotropy was among the first physiological effects ascribed to the endogenous nucleoside, nearly six decades ago (4). Adenosine and its analogs also exert negative inotropic and dromotropic effects on isolated atrial, ventricular, or whole mammalian heart preparations (5–9). Consonant with its modulatory role in other tissues (1, 2), adenosine attenuates myocardial responsiveness to catecholaminergic stimulation (10, 11). This may serve as an important regulatory mechanism inasmuch as catecholaminergic stimulation has been shown to rapidly increase adenosine release from the myocardium (11). Physiological effects of adenosine on the isolated myocardium are competitively antagonized by xanthine derivatives, indicative of a receptor-mediated event (6, 9, 12). Cardioinhibitory effects of adenosine are believed to be mediated via an interaction with adenosine receptors of the A_1 subtype (3). Three general lines of evidence support this hypothesis. 1) Agonist rank order potencies in *in vitro* physiological studies are consistent with an A_1-mediated event (5–7). 2) Adenosine recognition sites in myocardial membranes have been directly radiolabeled using agonist probes [^{125}I]HPIA (13) and [^{125}I]aminobenzyladenosine (14) and an A_1-selective antagonist probe [^{3}H]DPCPX (15, 16). The rank order potency profile of agonists inhibiting specific binding of these radioligands is consistent with that of an A_1 adenosine receptor. 3) Activation of adenosine receptors on dispersed rat cardiac myocytes (17, 18) or in guinea pig myocardial membranes (19) inhibits catecholamine-stimulated and basal adenylyl cyclase activity, respectively. This finding is consistent with the presumed A_1 nature of the cardiac adenosine receptor (2). Adenosine also activates a potassium conductance in cardiac membrane patches that is analogous to that activated by acetylcholine in that it shows inward rectification and pertussis toxin sensitivity (20, 21). Both adenosine and acetylcholine receptors couple to this type of K^+ channel via an interaction with a guanyl nucleotide-binding protein, presumedly G_i (20, 21). Ac-

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ABBREVIATIONS: [^{125}I]HPIA, N^6-3([^{125}I]iodo-4-hydroxyphenylisopropyl)adenosine; APNEA, N^6-2-(4-aminophenyl)ethyladenosine; 2-CADO, 2-chloroadenosine; CPA, N^6-cyclopentyladenosine; CHA, N^6-cyclohexyladenosine; CV-1808, 2-phenylaminoadenosine; DMSO, dimethylsulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; [^{3}H]DPCPX, 8-cyclopentyl-1,3[^{3}H]dipropylxanthine; DPX, 1,3-diethyl-8-phenylxanthine; Gpp(NH)P, guanyl-5′-yl-imidodiphosphate; GTPγS, guanosine-5′-(3-*O*-thio)-triphosphate; HPIA, N^6-hydroxyphenylisopropyladenosine; IBMX, 3-isobutyl-1-methylxanthine; K_D, equilibrium dissociation constant; K_i, inhibition constant; k_{obs}, observed rate constant; K_{+1}, association rate constant; k_{-1}, dissociation rate constant; NECA, 5′-*N*-ethylcarboxamido)adenosine; PACPX, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine; PMSF, phenylmethanesulfonyl fluoride; (*R*)-PIA, N^6-[(*R*)-phenylisopropyl]adenosine; (*S*)-PIA, N^6-[(*S*)-phenylisopropyl]adenosine; *p*SPT, 8-(*p*-sulfophenyl)theophylline.

tivation of this K^+ channel may represent the molecular basis of adenosine-induced negative chronotropy (21). Adenosine also attenuates catecholaminergic-stimulated calcium conductance in bovine ventricular myocytes voltage-clamped in the whole cell configuration (22). Whether this is related to adenosine-mediated inhibition of adenylyl cyclase activity or coupling of cardiac adenosine receptors to calcium channels is unknown.

The objective of the present study was threefold: 1) to determine whether porcine atria represent an acceptable model for the characterization of cardiac adenosine receptors in terms of density and pharmacological specificity; 2) to directly address the kinetic mechanism of binding of an adenosine receptor agonist radioligand; and 3) to assess the influence of guanyl nucleotides on agonist equilibrium binding and dissociation kinetics. The results of our pharmacological characterization suggest that the porcine atrial adenosine receptor labeled by [^{125}I]HPIA is of the A_1 subtype. The kinetics of [^{125}I]HPIA binding were consistent with those expected for a simple bimolecular reaction. The homogeneous population of cardiac adenosine receptors labeled by [^{125}I]HPIA presumably represents binding to the high affinity form of the receptor, inasmuch as [^{125}I]HPIA binding is sensitive to negative modulation by guanyl nucleotides. Thus, the porcine atria may represent a suitable model for further characterization of cardiac adenosine receptors.

Experimental Procedures

Materials

[^{125}I]HPIA (1800–2200 Ci/mmol) was purchased from Amersham (Chicago, IL). (*R*)- and (*S*)-PIA, NECA, HPIA, GTPγS, Gpp(NH)p, GDP, GMP, PMSF, and adenosine deaminase were from Boehringer Mannheim (Mannheim, West Germany). CPA, CV-1808, DPCPX, PACPX, and *p*SPT were obtained from Research Biochemicals, Inc. (Wayland, MA). CHA, IBMX, and DPX were from Calbiochem (La Jolla, CA). 2-CADO, theophylline, caffeine and GTP were obtained from Sigma Chemical Company (St. Louis, MO). Ultrapure DMSO was obtained from Pierce Chemical Company (Rockford, IL). APNEA was synthesized by the method of Stiles *et al.* (23).

Membrane Preparation and Experimental Protocol

Radioligand binding. Membranes used in all binding assays were prepared as previously described (24). Immediately before binding assays, this P3 fraction was resuspended at a protein concentration of 1–2 mg/ml and incubated with 5 units/ml adenosine deaminase for 30 min at 22°. Adenosine deaminase-treated homogenates were used directly in binding assays.

All binding experiments were carried out at 37° in a final volume of 0.25 ml containing 25 mM imidazole (pH 7.4), 5 mM $MgCl_2$, and 0.3–0.5 mg of membrane protein. Nonspecific binding was defined as that occurring in the presence of 100 μM(*R*)-PIA, 100 μM 2-CADO, or 1 mM theophylline (which gave identical values). Concentrated stock solutions of insoluble adenosine receptor agonists and antagonists were prepared in 100% ultrapure DMSO, which was diluted such that the final concentration of DMSO in binding assays was 0.5%. Therefore, all binding experiments (except those with guanyl nucleotides) were carried out in 0.5% DMSO. Equilibrium binding experiments were carried out for 90–120 min and terminated by rapid filtration over GF/C filters using a Brandel Cell Harvester (M-24R; Gaithersburg, MD). GF/C filters were presoaked in 0.5% polyethyleneimine to reduce nonspecific binding. Kinetic experiments were carried out for varying time intervals and were terminated similarly. Additional saturation isotherms were carried out using the dilution method of Green (25). Filter-trapped radioactivity was quantified by use of a Beckman 4000 γ-counter at a counting efficiency of 75%. Protein concentration determined by the method of Lowry *et al.* (26).

Data analysis. Saturation and competition data were analyzed using Lundon I Saturation Analysis Software (Lundon Software, Cleveland, OH) and EBDA (Elsevier-Biosoft, Cambridge, UK), respectively. Kinetic data were analyzed by linear transformation of binding data to a natural logarithm plot:

$$\ln([B_{eq}-B_t]/[B_{eq}]) = -k_{obs}(t)$$

where B_{eq} and B_t are the amounts of [^{125}I]HPIA specifically bound at equilibrium and time t, respectively. Fitting kinetic data to this equation generates a plot with slope corresponding to $(-)k_{obs}$.

Results

[^{125}I]HPIA bound saturably, reversibly, and with high affinity to an apparently homogeneous population of recognition sites with a B_{max} of 35 ± 3.0 fmol/mg of protein and a K_D of 2.5 ± 0.4 nM (Fig. 1). Parameter estimates for a two-site model could not be obtained for these data using an iterative curve fitting routine (Lundon I). A Scatchard-Rosenthal replot of saturation data was monophasic in the concentration range of [^{125}I]HPIA used (Fig. 1, *inset*). It was not possible to exceed the maximal concentration shown (approximately 4 nM) due to high levels of nonspecific binding with attendant unfavorable signal to noise ratios. At a ligand concentration equal to its K_D, approximately 35% of total [^{125}I]HPIA binding was specific. A Hill plot of saturation data yielded a slope of 1.0 ± 0.1 and an apparent K_D of 2.5 ± 0.6 nM (data not shown). At the concentrations of radioligand used, this binding presumably represents the labeling of the high affinity state of the porcine atrial adenosine receptor. Additional saturation experiments were performed using the dilution method of Green (25) to achieve greater fractional occupancy of adenosine receptors in equilibrium binding experiments. The binding of a fixed concentration of [^{125}I]HPIA (0.2 nM) was determined in the presence of 0.2 to 20 nM unlabeled HPIA in these experiments. Under these conditions, HPIA labeled a single population of recognition sites with a B_{max} of 49.1 ± 2.2 fmol/mg of protein and a K_D of

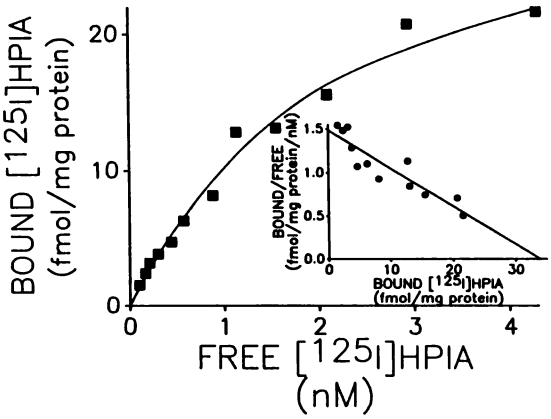

Fig. 1. [^{125}I]HPIA saturation isotherm in porcine atrial membranes. Nonspecific binding was defined as that occurring in the presence of 100 μM (*R*)-PIA. The fit shown was obtained using Lundon I Saturation Analysis software, which yielded a B_{max} of 35 ± 3 fmol/mg of protein and a K_D of 2.5 ± 0.4 nM. *Inset*, Scatchard-Rosenthal replot of saturation data. This fit was determined as above, generating identical binding parameters. Shown is a representative experiment, done in duplicate and replicated once. At a ligand concentration equal to its K_D, approximately 35% of total [^{125}I]HPIA binding was specific.

7.4 ± 0.8 nM (Fig. 2). A Scatchard-Rosenthal replot of these data was monophasic over this expanded concentration range (Fig. 2, *inset*). A Hill plot of these data yielded a slope of 1.09 ± 0.07 and an apparent K_D of 7.7 ± 0.4 nM (data not shown). Moreover, parameter estimates for a two-site model could not be obtained from these data using an iterative curve-fitting procedure (Lundon I). Therefore, a one-site model adequately described [^{125}I]HPIA equilibrium binding isotherms in all saturation experiments.

Kinetic experiments were undertaken to address the mechanism of [^{125}I]HPIA binding in porcine atrial membranes. In order to maintain pseudo-first order reaction conditions, ligand concentrations used were always 5-fold greater than that of receptor (approximately 50 pM). k_{obs} was determined at several ligand concentrations (Fig. 3A) and plotted versus [^{125}I]HPIA concentration (Fig. 3B). The latter plot was linear (r^2 = 0.99) with a slope of (1.9 ± 0.1) × 10^{-2} $min^{-1}nM^{-1}$ and a y intercept of (4.5 ± 0.2) × 10^{-2} min^{-1}. These parameters represent k_{+1} and k_{-1}, respectively: dividing k_{-1} by k_{+1} yields a kinetically derived K_d of 2.4 nM, which is in excellent agreement with that parameter determined in equilibrium binding experiments (2.5 ± 0.4 nM; above and Fig. 1). The dependence of k_{obs} on [^{125}I]HPIA concentrations greater than 4 nM could not be determined due to diminished signal to noise ratios.

To determine the effects of guanyl nucleotides on [^{125}I]HPIA dissociation kinetics, dissociation of [^{125}I]HPIA was initiated by addition of 2-CADO (100 μM), GTP (300 μM), or the combination of 2-CADO and GTP to an equilibrated incubate (Fig. 4). [^{125}I]HPIA dissociation induced by 2-CADO was described by a monoexponential equation yielding a k_{-1} of (1.6 ± 0.2) × 10^{-2} min^{-1}. However, biphasic dissociation kinetics were observed when dissociation was initiated by simultaneous addition of 2-CADO and GTP. Initially, a rapid dissociation occurred in which approximately 66 ± 9% of specifically bound [^{125}I]HPIA dissociated, yielding a k_{-1R} of 1.2 ± 0.1 min^{-1}. This burst phase was followed by a slower dissociation [k_{-1S} = (1.5 ± 0.2) × 10^{-2} min^{-1}], which was estimated to account for the remaining 34 ± 2% [^{125}I]HPIA specifically bound. The mean

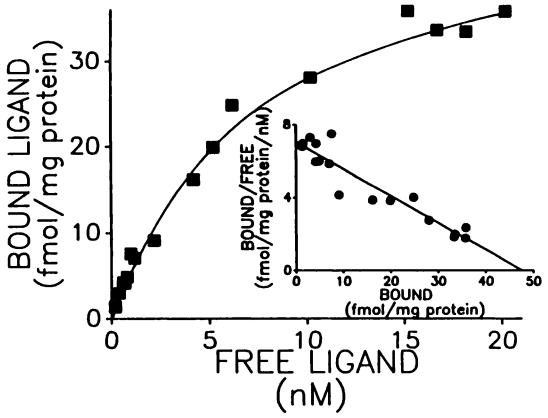

Fig. 2. HPIA saturation isotherm in porcine atrial membranes. The concentration of [^{125}I]HPIA was fixed at 0.2 nM and varying concentrations of unlabeled HPIA were added (0.2–20 nM). The fit shown was obtained using Lundon I Saturation Analysis software, which generated a B_{max} of 49.1 ± 2.2 fmol/mg of protein and a K_D of 7.4 ± 0.8 nM. *Inset;* Scatchard-Rosenthal replot of saturation data. This fit was determined as above, generating similar parameter estimates. Shown are the pooled results of three individual experiments, each usnig different membrane preparations.

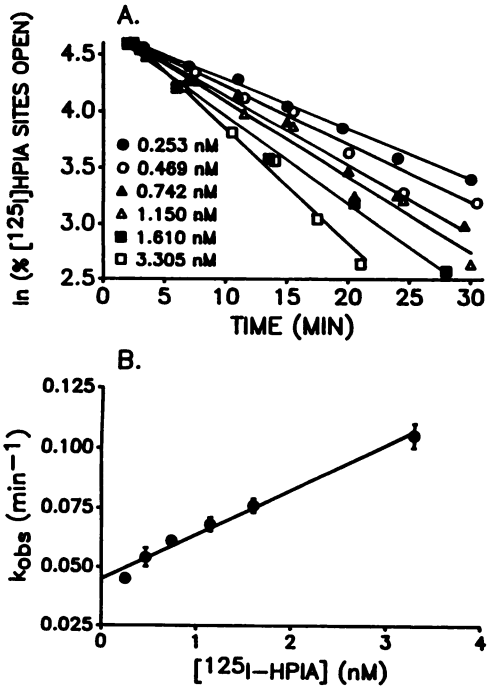

Fig. 3. A, [^{125}I]HPIA association experiments in porcine atrial membranes. Experiments were carried out with a receptor concentration of 50 pM and indicated ligand concentrations. Binding data were linearly transformed to a ln (% receptors open) versus time plot. *Lines* drawn represent best fits as determined by linear regression. Slopes of these lines correspond to (−)k_{obs} for that concentration of [^{125}I]HPIA. This yielded the following values for k_{obs}: 0.253 nM, (4.5 ± 0.2)×10^{-2} min^{-1}; 0.469 nM, (5.4 ± 0.4) × 10^{-2} min^{-1}; 0.742 nM, (6.1 ± 0.2) × 10^{-2} min^{-1}; 1.15 nM, (6.8 ± 0.3) × 10^{-2} min^{-1}; 1.61 nM, (7.6 ± 0.3) × 10^{-2} min^{-1}; 3.305 nM, (10.5 ± 0.5) × 10^{-2} min^{-1}. Experiments were conducted on the same day using the same membrane preparations. These findings were replicated twice under similar conditions. B, k_{obs} as function of [^{125}I]HPIA concentration in porcine atrial membranes. The *line* drawn represents the best fit as determined by linear regression (r^2 = 0.99). The slope [(1.9 ± 0.2) × 10^{-2} $min^{-1}nM^{-1}$] and y intercept [(4.5 ± 0.2) × 10^{-2} min^{-1}] of this plot represent k_{+1} and k_{-1}, respectively.

value for k_{-1S} is nearly identical to the off-rate determined by addition of 2-CADO in the absence of GTP. Finally, dissociation initiated by addition of 300 μM GTP alone occurs very rapidly (k_{-1} = 1.3 ± 0.13 min^{-1}) and is essentially complete within 1 min. Approximately 53 ± 6% of specifically bound [^{125}I]HPIA was sensitive to dissociation induced by GTP alone. Both the rate and extent of [^{125}I]HPIA dissociation initiated by GTP were very similar to that observed in the rapid phase of dissociation induced by the combination of 2-CADO and GTP. These kinetic data are consistent with the formation of a ternary complex in porcine atrial membranes, consisting of ligand, receptor, and guanyl nucleotide-binding protein, which possesses high affinity for agonists. GTP presumedly causes a dissociation of this complex resulting in loss of high affinity agonist binding sites (27). The affinity of the guanyl nucleotide-binding protein-uncoupled receptor for [^{125}I]HPIA would appear to be sufficiently low such that this interaction could not be measured by the methodology used (rapid filtration).

To further characterize guanyl nucleotide modulation of [^{125}I]HPIA binding in porcine atrial membranes, titration experiments for several guanyl nucleotides were performed. Guanyl nucleotides negatively modulated specific binding of [^{125}I]HPIA in porcine atrial membranes with the following rank order

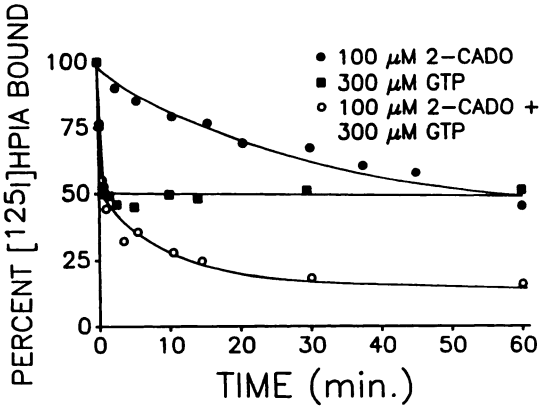

Fig. 4. Dissociation of specifically bound [^{125}I]HPIA from porcine atrial membranes. A total of 0.25–0.5 nM [^{125}I]HPIA was allowed to equilibrate with porcine atrial membranes (receptor concentration, 50 pM) for 60 min, at which time dissociation was initiated as indicated. k_{-1} was determined to be (1.6 ± 0.2) × 10^{-2} min^{-1} and 1.3 ± 0.13 min^{-1} for 2-CADO and GTP alone, respectively. For the combination of 2-CADO and GTP; 66 ± 9%[^{125}I]HPIA dissociated with a k_{-1} of 1.2 ± 0.1 min^{-1} and 34 ± 2% dissociated with a k_{-1} of (1.5 ± 0.2) × 10^{-2} min^{-1}. Parameters used in curve fitting were determined using the least squares curve fitting routine KINETIC (Elsevier-Biosoft). Shown is a representative experiment, which was replicated twice.

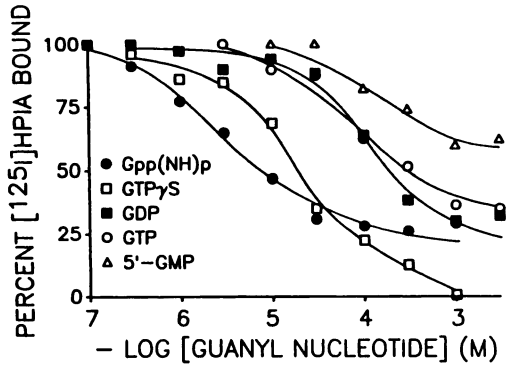

Fig. 5. Guanyl nucleotides inhibiting the specific binding of [^{125}I]HPIA in porcine atrial membranes. Receptor and [^{125}I]HPIA concentration were approximately 40 and 500 pM, respectively. Shown is a representative experiment, which was replicated two to four times. Titration curves drawn represent best fits as determined by EBDA software. Parameter estimates (IC_{50} ± standard error) were also obtained using EBDA and were as follows (μM): Gpp(NH)p, 2.5 ± 0.6; GTPγS, 19.4 ± 3.0; GDP, 231 ± 102; GTP,376 ± 112; 5′-GMP, >3000.

potency: Gpp(NH)p > GTPγS > GDP = GTP ≫ 5′-GMP (Fig. 5). 5′-GMP was essentially ineffective as a negative modulator of [^{125}I]HPIA binding in concentrations up to 3 mM. With the exception of GTPγS, maximal inhibition of [^{125}I]HPIA binding by guanyl nucleotides was approximately 75%. GTPγS, however, inhibited 100% of [^{125}I]HPIA specific binding. These data provide further evidence for the formation of a ternary complex in porcine atrial membranes that is sensitive to negative modulation by guanyl nucleotides.

In order to address the pharmacological specificity of the porcine atrial adenosine receptor, the rank order potency profile for a series of adenosine receptor ligands as inhibitors of [^{125}I]HPIA binding was determined (Fig. 6; Table 1). CPA and (*R*)-PIA were the most potent inhibitors of [^{125}I]HPIA binding in porcine atrial membranes. (*R*)-PIA was 22-fold more potent than its diastereomer (*S*)-PIA. CV-1808, an adenosine A_2 receptor-selective ligand, was approximately 3 orders of magnitude less potent than A_1-active ligands. Overall adenosine receptor agonist rank order potency was follows: CPA > (*R*)-PIA > HPIA > CHA > APNEA > NECA > (*S*)-PIA > 2-CADO ≫ CV-1808. This agonist rank order potency profile is consistent with the labeling of an A_1 adenosine receptor in porcine atrial membranes. With the exception of HPIA and CV-1808, indirect

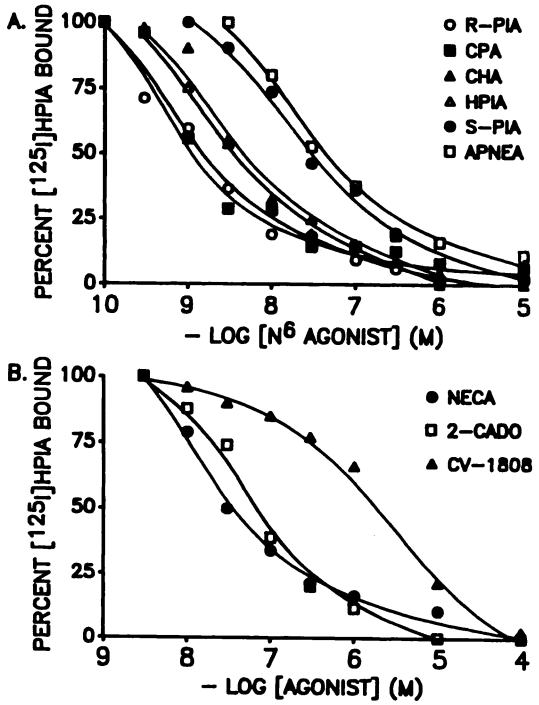

Fig. 6. A, N^6-adenosine receptor agonists inhibiting the specific binding of [^{125}I]HPIA in porcine atrial membranes. B, Non-N^6-substituted adenosine receptor agonists inhibiting the specific binding of [^{125}I]HPIA in porcine atrial membranes. Experimental conditions and curve fitting for A and B are as described in legend to Fig. 5. Shown are representative experiments, which were replicated 3 to 6 times.

TABLE 1

Adenosine receptor agonists and antagonists inhibiting the specific binding of [^{125}I]HPIA in porcine atrial membranes

Receptor and [^{125}I]HPIA concentration were approximately 40 and 500 pM, respectively. Parameter estimates were determined using EBDA and represent the mean ± standard error of three to six experiments (agonists) or two or three experiments (antagonists).

Compound	K_i	Slope factor
	nM	
Agonists		
CPA	1.04 ± 0.14	0.94 ± 0.30
(*R*)-PIA	1.38 ± 0.60	0.82 ± 0.17
HPIA	2.95 ± 0.40	0.78 ± 0.06
CHA	3.61 ± 0.60	0.83 ± 0.16
APNEA	25.4 ± 0.1	1.04 ± 0.23
NECA	27.1 ± 12.5	0.83 ± 0.14
(*S*)-PIA	30.0 ± 12.4	0.83 ± 0.14
2-CADO	64.5 ± 12.2	0.86 ± 0.16
CV-1808	2,310 ± 355	0.74 ± 0.06
Antagonists		
DPCPX	2.4 ± 0.2	0.70 ± 0.04
PACPX	22.0 ± 4.1	0.98 ± 0.14
IBMX	2,770 ± 343	0.82 ± 0.08
DPX	4,096 ± 593	1.00 ± 0.11
*p*SPT	4,479 ± 586	0.99 ± 0.11
Theophylline	45,888 ± 7295	1.02 ± 0.13
Caffeine	90,130 ± 25,283	1.15 ± 0.30

Hill slopes for agonist competition curves did not differ significantly from unity.

Prototypic adenosine receptor antagonists inhibited the binding of [^{125}I]HPIA in porcine atrial membranes (Fig. 7; table 1). DPCPX and PACPX, adenosine A_1-selective ligands, were the most potent antagonist inhibitors of [^{125}I]HPIA specific binding. DPCPX was approximately 10-fold more potent as an inhibitor of [^{125}I]HPIA specific binding than was PACPX. Overall rank order potency of antagonists inhibiting the binding of [^{125}I]HPIA in porcine atrial membranes was as follows: DPCPX > PACPX > IBMX > DPX > *p*SPT > theophylline > caffeine. Indirect Hill slopes for DPCPX and IBMX were unexpectedly but reproducibly less than unity. The reasons for this observation are unclear. Interestingly, these compounds represent two of the most lipophilic adenosine receptor ligands used in these experiments.

Discussion

The number of porcine atrial membrane recognition sites labeled by [^{125}I]HPIA is very similar to that previously reported for the adenosine receptor antagonist [^{3}H]DPCPX (35 ± 3 and 32±1 fmol/mg of protein, respectively) (16). At the concentrations of radioligand used in the present study, [^{125}I]HPIA labeled only the high affinity state of the adenosine receptors. Thus, the labeling of equivalent numbers of recognition sites in porcine atrial membranes by [^{125}I]HPIA and [^{3}H]DPCPX suggests that including 5 mM $MgCl_2$ in the incubate and carrying the reaction out at 37° resulted in a quantitative conversion of adenosine receptors from a low to a high affinity state. This was also demonstrated using a dilution procedure for equilibrium saturation experiments. Employing concentrations of HPIA up to 20 nM, we were unable to detect a second, presumably lower affinity, recognition site. Thus, under these incubation conditions, [^{125}I]HPIA apparently labels the high affinity recognition state of the porcine atrial adenosine receptor.

The relationship between k_{obs} of association and [^{125}I]HPIA concentration has not previously been evaluated. The demonstration of a linear dependence of k_{obs} on [^{125}I]HPIA concentration suggests a simple bimolecular association reaction. From these data it may be inferred that under the assay conditions used the porcine atrial A_1 adenosine receptor population exists

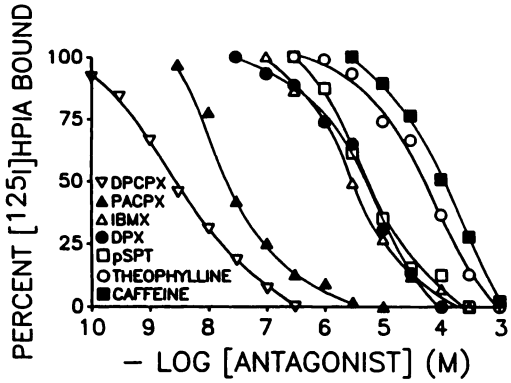

Fig. 7. Adenosine receptor antagonists inhibiting the specific binding of [^{125}I]HPIA in porcine atrial membranes. Experimental conditions and curve fitting are as described in legend to Fig. 5. Shown is a representative experiment, which was replicated 1 to 3 times.

precoupled with a G protein. However, the possibility of a ligand-induced isomerization of the porcine atrial adenosine receptor cannot be completely excluded at the present time. Such a phenomenon would yield values of k_{obs} that were hyperbolically dependent on [^{125}I]HPIA concentration (28–31). In the present study, concentrations of [^{125}I]HPIA used in association experiments may have generated k_{obs} values that merely represented the initial linear phase of a rectangular hyperbola. To more rigorously characterize such a possibility would require use of [^{125}I]HPIA concentrations considerably higher than those used. Unfortunately, use of [^{125}I]HPIA concentrations greater than 4.0 nM severely compromises the signal to noise ratio and therefore were not feasible. Nonetheless, excellent agreement exists between saturation- and kinetically-derived affinities, assuming a simple bimolecular reaction.

Guanyl nucleotides negatively modulated the specific binding of [^{125}I]HPIA in porcine atrial membranes in both kinetic and equilibrium experiments (Figs. 4 and 5). This is consistent with guanyl nucleotides and adenosine receptor agonists participating in negative heterotropic binding interactions (27). Simultaneous addition of GTP and 2-CADO to agonist-occupied receptors resulted in a very rapid dissociation of [^{125}I]HPIA, followed by a slow terminal dissociation with a rate constant nearly identical to that measured when dissociation was initiated by addition of 2-CADO alone. Dissociation initiated by addition of GTP (300 μM) was characterized by a similar burst phase but lacked a terminal (slow) dissociation phase. Approximately 53% of specifically bound [^{125}I]HPIA rapidly dissociated upon addition of this concentration of GTP. This finding was not unexpected inasmuch as the concentration of GTP used in these experiments approximated its IC_{50} as a negative modulator of [^{125}I]HPIA binding in equilibrium experiments.

In studies carried out at equilibrium, Gpp(NH)p and GTPγS were observed to be the most potent negative modulators of [^{125}I]HPIA binding in porcine atrial membranes (Fig. 5). GTP and GDP were essentially equipotent and 5′-GMP was devoid of efficacy as a modulator of [^{125}I]HPIA binding. The finding that GTP and GDP were equipotent may be related to the GTPase activity of porcine atrial membranes. These experiments were carried out at 37° in 5 mM $MgCl_2$ with a high protein concentration (300–500 μg of protein/assay tube) and without a GTP-regenerating system. These incubation conditions may contribute to low negative modulatory potencies that were observed for hydrolyzable guanyl nucleotides. With the exception of GTPγS, maximal inhibition of [^{125}I]HPIA binding was approximately 75–80%. The remaining 20–25% of [^{125}I]HPIA bound is insensitive to negative modulation by guanyl nucleotides. The reasons for this insensitivity are not known but may involve diffusional barriers within the membrane, creating a subpopulation of high affinity agonist recognition sites that are inaccessible to guanyl nucleotides. The finding that GTPγS inhibits 100% of [^{125}I]HPIA binding was unique and possibly related to differing partitioning properties for this nonhydrolyzable ligand.

Adenosine agonists inhibited the specific binding of [^{125}I]HPIA in a manner consistent with the labeling of an A_1 adenosine receptor (Fig. 6; Table 1) (32, 33). This hypothesis is supported by the rank order potency of adenosine analogs as inhibitors of [^{125}I]HPIA binding [(*R*)-PIA > NECA ≥ (*S*)-PIA], the finding that CV-1808 (An A_2-selective ligand) is approximately 3 orders of magnitude less potent than other A_1-active

ligands, and the potency of A_1-selective antagonists as inhibitors of the specific binding of [^{125}I]HPIA (see below). The cardiac adenosine receptor derived from porcine atria appears to be similar to that of bovine ventricular origin in that the potency of N^6-substituted agonists is enhanced over that of non-N^6-substituted agonists (NECA and (*S*)-PIA are essentially equipotent) (15).

Adenosine receptor antagonists were also useful in the classification of the porcine atrial membrane adenosine receptor. DPCPX and PACPX were the most potent inhibitors of [^{125}I]HPIA binding (DPCPX was approximately 10-fold more potent than PACPX). However, a discrepancy exists between the K_I of DPCPX as an inhibitor of [^{125}I]HPIA binding and both its K_D from [^{3}H]DPCPX saturation experiments and its K_I as an inhibitor of [^{3}H]DPCPX binding in the same tissue (2.4 ± 0.2 versus 0.4 ± 0.05 or 0.52 ± 0.24 nM) (16). This inconsistency is most likely due to incubation conditions of the competition experiments (5 mM $MgCl_2$). Yeung and co-workers (34) observed decreased potency (2–10-fold) of IBMX as an inhibitor of (*R*)-[^{3}H]-PIA binding in cholate-solubilized rat brain membranes when competition experiments were carried out in the presence of $MgCl_2$. $MgCl_2$ (3 mM) increased the amount of (*R*)-[^{3}H]-PIA bound and the affinity of adenosine receptors for this agonist (34). Mg^{2+} apparently promotes and/or stabilizes the formation of the ternary complex (27). Therefore, in the presence of 5 mM $MgCl_2$, the potency of antagonists as inhibitors of [^{125}I]HPIA binding may be reduced over that observed in its absence.

In summary, radioligand binding experiments suggest that the adenosine receptor present in porcine atrial membranes is of the A_1 subtype. [^{125}I]HPIA appears to label a single population of high affinity recognition sites. The mechanism of [^{125}I]HPIA binding to porcine atrial membrane adenosine receptors is apparently a simple bimolecular reaction. Cardiac adenosine receptors are similar to other rhodopsin-like hormone and neurotransmitter receptors in that agonist binding is subject to negative heterotropic regulation by guanyl nucleotides. Thus, porcine atria appear to represent an acceptable model for further characterization of cardiac adenosine receptors.

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