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Agonist Radioligand Interactions with the Solubilized Porcine Atrial A_1 Adenosine Receptor

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SUMMARY

Porcine atrial adenosine receptors have been solubilized using a detergent system consisting of digitonin and sodium cholate and characterized with the agonist radioligand N^6[^{125}I]hydroxyphenylisopropyl) adenosine [^{125}I]HPIA. ^{125}IHPIA labeled an apparently homogeneous population of solubilized recognition sites with a B_{max} of 88 ± 4 fmol/mg of protein and a K_D of 1.4 ± 0.1 nM. Solubilization resulted in a 2.5-fold enrichment of adenosine receptor specific activity and an enhanced signal to noise ratio over that observed for porcine atrial membrane preparations. Solubilized cardiac adenosine receptors were relatively stable and exhibited many of the properties of membrane-bound receptors. The rank order potency of adenosine receptor agonists inhibiting the binding of [^{125}I]HPIA was consistent with the labeling of a solubilized A_1 adenosine receptor. Association rate experiments suggested that the interaction of [^{125}I]HPIA with solubilized cardiac adenosine receptors was consistent with that of a simple bimolecular reaction. The dissociation constant calculated from kinetic data (0.73 nM) was in good agreement with that determined by equilibrium binding measurements (1.4 nM). The interaction of cardiac A_1 adenosine receptors and guanine nucleotide binding protein(s) G protein(s) was retained in this detergent system. Addition of guanosine-5′-*O*-(3-thio)triphosphate to an equilibrated mixture of solubilized cardiac adenosine receptors and [^{125}I]HPIA resulted in a rapid and complete dissociation of [^{125}I]HPIA. This dissociation was resolved into two kinetic phases, which appear to arise from two populations of independent, noninterconvertible receptor-G protein complexes that display differing sensitivities to guanine nucleotides. The A_1 adenosine receptor-G protein complex solubilized in digitonin/cholate appears to provide an excellent system by which agonist radioligand-receptor-G protein interactions can be further studied.

A_1 adenosine receptors in myocardial membranes mediate cardioinhibitory effects of adenosine and its structural congeners (1–5). Transduction mechanisms associated with cardiac adenosine receptor activation include activation of an inward rectifying potassium channel (6) and inhibition of adenylyl cyclase activity (7–10).[1] The former seems likely to represent the molecular mechanism of adenosine receptor-mediated negative chronotropy (6). Inhibition of adenylyl cyclase activity is not temporally correlated with negative chronotropy in the developing atria of the embryonic chick[1] but may be involved in adenosine receptor-mediated negative inotropy or catecholamine antagonism, inasmuch as adenosine attenuates catecholaminergic-stimulated positive inotropy (11) and calcium influx (12).

Molecular properties of membrane-bound rat (8, 13), bovine (14, 15), chick (16), and porcine (17, 18) cardiac adenosine receptors have been characterized. Solubilization of brain, but not cardiac, A_1 adenosine receptors has previously been reported (19–23). Solubilized bovine (19) and rat (21–23) brain adenosine receptors display high affinity agonist binding that is sensitive to negative modulation by guanine nucleotides, indicating that receptor-G protein interactions are preserved in detergent extracts of brain membranes. Guanine nucleotide-sensitive agonist binding to detergent-solubilized receptors has also been reported for the muscarinic receptor of rat myocardium (24) and rat striatal D-1 dopamine receptors (25). These findings are in contrast to results obtained for detergent-solubilized porcine atrial muscarinic receptors (26) or a variety of catecholaminergic receptors (27–29) that do not display guanine nucleotide-sensitive high affinity agonist binding. Thus,

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[1] Blair, T. A., M. Parenti, and T. F. Murray. Development of physiological sensitivity to adenosine analogs in embryonic chick heart: role of A_1 adenosine receptors and adenylyl cyclase inhibition. *Mol. Pharmacol.* in press (1989).

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; B_{max}, maximum binding capacity; 2-CADO, 2-chloroadenosine; CPA, N^6-cyclopentyladenosine; CV-1808, 2-phenylaminoadenosine; DTT, dithiothreitol; Gpp(NH)p, guanine-5′-yl-imidodiphosphate; GTPγS, guanosine-5′-(3-*O*-thio)-triphosphate; [^{125}I]HPIA, N^6-3([^{125}I]iodo-4-hydroxyphenylisopropyl)adenosine; K_D, equilibrium dissociation constant; k_{+1}, association rate constant; k_{-1}, dissociation rate constant; NECA, 5′-(*N*-ethylcarboxamido)adenosine; PMSF, phenylmethylsulfonyl fluoride; (*R*)-PIA, N^6-(*R*)-(phenylisopropyl) adenosine; (*S*)-PIA, N^6-(S)-(phenylisopropyl)adenosine; τ and τ^{-1}, relaxation time and reciprocal relaxation time, respectively; CHAPS, 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate.

brain A_1 adenosine receptors and rat cardiac muscarinic and striatal D-1 dopamine receptors apparently exist tightly coupled to G protein(s).

Results of the present studies suggest that molecular properties of porcine atrial adenosine receptors are not grossly altered in digitonin/cholate. Adenosine receptor-G protein interactions are also well preserved in this detergent system. Thus, the solubilized cardiac A_1 adenosine receptor provides a potential model for the study of receptor-G protein interactions in detergent solution.

Experimental Procedures

Materials. Digitonin and sodium cholate were obtained from Sigma Chemical Co. (St. Louis, MO). [^{125}I]HPIA (1930 Ci/mmol) and all other compounds were purchased from sources previously described (18).

Membrane preparation and receptor solubilization. Porcine atrial membranes were prepared and solubilized as previously described for the atrial muscarinic receptor (30), with minor modifications. Briefly, digitonin and sodium cholate were added to a porcine atrial membrane P3 preparation to give a final concentration of 0.4%, w/v, digitonin, 0.08%, w/v, sodium cholate, and 4.95 mg of protein/ml. This mixture was then centrifuged at 100,000 × *g* for 1 hr at 4° in a Beckman L8-55M Ultracentrifuge. The supernatant (El) was discarded and the pellet was resuspended to give a protein concentration of 10 mg/ml, assuming a 30% protein loss during the first extraction. Digitonin and sodium cholate were added to this membrane suspension to give a final concentration of 0.8%, w/v, digitonin, 0.16%, w/v, sodium cholate, and 8 mg of protein/ml. After a 10-min incubation at 22°, the membrane-detergent mixture was then diluted 2-fold and centrifuged as above. The supernatant from this second extraction contained solubilized cardiac A_1 adenosine receptors in approximately 50% yield (in [^{125}I]HPIA sites) and was used for all experiments described here. Before the second solubilization step, membranes were incubated with 5 mM $MgCl_2$ and 1 mM DTT on ice for 30 min. Preincubation with Mg^{2+} and DTT improved both the yield and stability of the solubilized cardiac A_1 adenosine receptor. Protease inhibitors present during all solubilization procedures were PMSF (0.1 mM), Na_2EDTA (1 mM), egg white trypsin inhibitor (100 μg/ml), leupeptin (0.7 μg/ml), and bacitracin (0.01%, w/v). Solubilized receptor preparations were used immediately or were stored on ice. Storage on ice for up to 7 days did not adversely affect receptor specific activity. Immediately before radioligand binding studies, solubilized receptor preparations (~1 mg of protein/ml) were warmed to 22° and incubated with 5 units/ml adenosine deaminase for 30 min. Adenosine deaminase-treated extracts were then used directly in radioligand binding experiments (the concentration of adenosine deaminase was approximately 4 units/ml in assay tubes).

Radioligand binding experiments. All equilibrium radioligand binding experiments were carried out for 120 min at 37° in a volume of 95μl containing 20 mM imidazole (pH 7.4), 4 mM $MgCl_2$, 0.3%, w/v, digitonin, 0.06%, w/v, sodium cholate, and 30–70 μg of protein. Nonspecific binding was defined as that occurring in the presence of 100 μM 2-CADO, 3 mM theophylline, or 1 mM GTP (which gave identical values) and amounted to approximately 10% of total [^{125}I]HPIA binding at a concentration equal to its K_D. Bound [^{125}I]HPIA was separated from free radioligand by rapid filtration (Brandel Cell Harvester M-24R; Brandel Scientific, Gaithersburg, MD) over Schleicher and Schuell No. 32 glass fiber filters that had been presoaked in 0.5%, w/v, polyethyleneimine, similar to the method described by Bruns *et al.* (31). Kinetic experiments were carried out under identical conditions for varying time intervals and were terminated by rapid filtration. Radioactivity on the filters was quantified by use of a Beckman 4000 γ counter at a counting efficiency of 75%. Protein concentration was determined by the method of Lowry *et al.* (32).

Data analysis. Results of saturation and competition experiments were analyzed by use of Lundon I Saturation Analysis Software (Lundon Software, Cleveland, OH) and EBDA (Elsevier-Biosoft, Cambridge, UK), respectively. [^{125}I]HPIA association experiments were analyzed by linear transformation of binding data to a natural logarithm plot:

$$\ln([B_0 - B_t]/[B_0]) = -t/\tau \quad (1)$$

where B_0 and B_t are the amounts of [^{125}I]HPIA specifically bound at equilibrium any time t, respectively, and τ is the relaxation time for the single kinetic phase. Fitting kinetic data to this equation generates a plot with a slope corresponding to $-\tau^{-1}$. Values of τ^{-1} were plotted versus [^{125}I]HPIA concentration and fitted with the pseudo-first order rate equation for a simple bimolecular association reaction:

$$\tau^{-1} = k_{+1}[^{125}\text{I-}]\text{HPIA}] + k_{-1} \quad (2)$$

where k_{+1} and k_{-1} are the association and dissociation rate constants for [^{125}I]HPIA binding, respectively. [^{125}I]HPIA dissociation experiments were analyzed by fitting data to monophasic or biphasic decay equations:

$$B_t = B_0 e^{-t/\tau} \quad (3a)$$

$$B_t = B_1 e^{-t/\tau_1} + B_2 e^{-t/\tau_2} \quad (3b)$$

The values of $\tau_{1,2}$ are the relaxation times and $B_{1,2}$ the amplitudes of each kinetic phase. Reciprocal relaxation times for fast and slow phases of GTPγS-initiated [^{125}I]HPIA dissociation were plotted versus GTPγS concentration (see Fig. 6 B and C, respectively). Values of τ_1^{-1} and τ_2^{-1} appeared to increase in a hyperbolic manner as GTPγS concentration increased. Therefore, these data were analyzed according to Eq. 4 using an iterative curve-fitting routine.

$$\tau^{-1} = \frac{k_{-1}[\text{GTP}\gamma\text{S}]}{K_D + [\text{GTP}\gamma\text{S}]} \quad (4)$$

where τ^{-1}, k_{-1}, and K_D represent the reciprocal relaxation time, the rate constant for [^{125}I]HPIA dissociation, and the dissociation constant for GTPγS in each kinetic phase, respectively.

Results

Saturation analysis. [^{125}I]HPIA showed saturable binding to a homogeneous population of high affinity solubilized cardiac recognition sites with a B_{max} of 88 ± 4 fmol/mg of protein and a K_D of 1.4 ± 0.1 nM (Fig. 1A). A B_{max} of 88 fmol/mg of protein represents a 2.5-fold enrichment over that previously reported for the membrane-bound adenosine receptor of porcine atria (17, 18) (Table 1). In addition, solubilization in this mixed detergent system resulted in a greatly enhanced signal to noise ratio, inasmuch as 90% of total [^{125}I]HPIA binding was specific at a concentration equal to its K_D. In contrast, the corresponding percentage of [^{125}I]HPIA specific binding in porcine atrial membranes preparations is approximately 35% (Table 1).

Saturation binding data were linearly transformed and fitted by least squares regression analysis to generate a Scatchard-Rosenthal plot (Fig. 1B). This plot was monophasic over a 100-fold range of [^{125}I]HPIA concentrations, yielding a B_{max} of 91 ± 3 fmol/mg of protein and a K_D of 1.5 ± 0.2 nM.

[^{125}I]HPIA association and dissociation kinetics. Kinetic experiments were undertaken to determine the mechanism by which [^{125}I]HPIA binds to solubilized cardiac A_1 adenosine receptors. Under pseudo-first order reaction conditions, [^{125}I]HPIA association experiments were performed over a broad range of radioligand concentration. Values for τ^{-1} were obtained at each concentration of radioligand by fitting association data to Eq. 1 (Fig. 2A) and were then plotted versus [^{125}I]HPIA concentration (Fig. 2B). The latter plot was fitted to Eq. 2, giving a k_{+1} equal to (3.4 ± 0.1) × 10^7 M^{-1} min^{-1} and

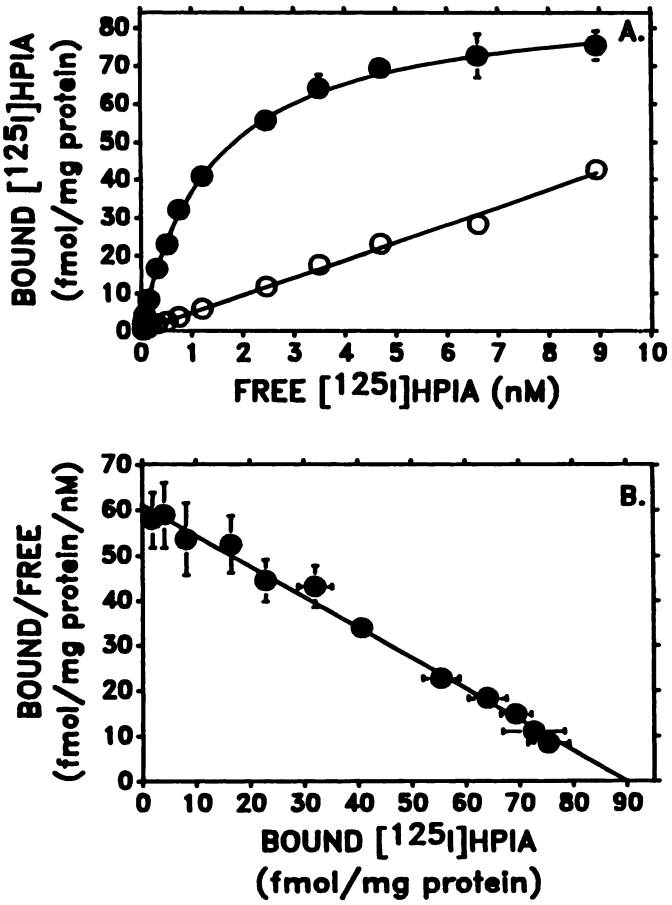

Fig. 1. A, [^{125}I]HPIA saturation isotherm in digitonin/cholate extracts of porcine atrial membranes. Specific binding (●) was defined as total binding (not shown) minus that occurring in the presence of 100 μM 2-CADO (○). Incubation conditions are described in Experimental Procedures. The theoretical *fit* shown was obtained using Lundon I Saturation Analysis Software, which yielded a B_{max} of 88 ± 4 fmol/mg of protein and a K_D of 1.4 ± 0.1 nM. At a ligand concentration equal to its K_D, approximately 90% of total [^{125}I]HPIA binding was specific. B, Transformation of saturation binding data shown in A to a Scatchard-Rosenthal replot. The *line* drawn represents a best fit as determined by linear regression (r = 0.98) and gives a B_{max} of 91 ± 3 fmol/mg of protein and a K_D of 1.5 ± 0.2 nM. All points represent the mean ± standard error of three independent experiments done in duplicate.

a value of k_{-1} equal to (2.45 ± 0.40) × 10^{-2} min^{-1}. The linear dependence of τ^{-1} on ligand concentration suggests that [^{125}I]HPIA interacts with the solubilized cardiac A_1 adenosine receptor in simple bimolecular reaction under the reaction conditions employed (4 mM Mg^{++} and 37°). Values of k_{-1} and k_{+1} can be used to calculate an equilibrium dissociation constant of 0.73 nM, assuming a simple bimolecular binding mechanism. This value is in reasonable agreement with the dissociation constant obtained from equilibrium binding experiments (see above and Fig. 1). The value of k_{-1} was independently determined by addition of 2-CADO (final concentration of 100 μM in a volume that represented 2% of incubate volume) to an equilibrated mixture of [^{125}I]HPIA and solubilized porcine atrial adenosine receptors (Fig. 3). Dissociation of [^{125}I]HPIA initiated by 2-CADO was monophasic and yielded a k_{-1} of (1.6 ± 0.1) × 10^{-2} min^{-1} or a half-time for dissociation of approximately 43 min, in good agreement with the value determined from the analysis of association kinetics (Fig. 2B). All of the present kinetic data agree with those observed for [^{125}I]HPIA binding to the membrane-bound adenosine receptor of porcine atria (18), which were also consistent with a simple bimolecular binding mechanism.

TABLE 1

Comparison of the binding properties of [^{125}I-]HPIA to membrane-bound and solubilized porcine atrial adenosine receptors

	Membrane Bound[a]	Solubilized Receptor
Mechanism of binding	Simple bimolecular	Simple bimolecular
k_{+1} (M^{-1} min^{-1})	(1.9 ± 0.2) × 10^7	(3.36 ± 0.10) × 10^7
k_{-1} (extrapolated, min^{-1})	(4.5 ± 0.1) × 10^{-2}	(2.45 ± 0.40) × 10^{-2}
k_{-1} (measured, min^{-1})[b]	(1.6 ± 0.2) × 10^{-2}	(1.6 ± 0.1) × 10^{-2}
K_D (equilibrium)	2.5 ± 0.4 nM	1.4 ± 0.1 nM
K_D (kinetic)	2.4 nM	0.73 nM
B_{max} (fmol/mg of protein)	35 ± 3	88 ± 4
Specific binding at K_D	~35%	~90%
Agonist inhibition profile	CPA = (*R*)-PIA > NECA = (*S*)-PIA	CPA = (*R*)-PIA > NECA > (*S*)-PIA
Guanine nucleotide inhibition	70–80%	100%
Guanine nucleotide inhibition profile	Gpp(NH)p > GTPγS > GDP = GTP	GTPγS > Gpp(NH)p > GTP > GDP

[a] From Ref. 19.
[b] k_{-1} was measured by addition of 2-CADO (final concentration = 100 μM) to an equilibrated mixture of porcine atrial membranes or solubilized receptor and [^{125}I-]HPIA.

Guanine nucleotide titration experiments. As a means of investigating guanine nucleotide modulation of [^{125}I]HPIA binding to solubilized cardiac A_1 adenosine receptors, titration experiments for a series of guanine nucleotides were performed (Fig. 4). The purpose of these experiments was to determine whether the binding of this agonist radioligand demonstrates sensitivity to guanine nucleotides, as had been reported for solubilized brain adenosine receptors and was suggested by the high affinity nature of [^{125}I]HPIA binding (see above). The rank order potency for guanine nucleotides negatively modulating specific binding of [^{125}I]HPIA was as follows: GTPγS > Gpp(NH)p > GTP > GDP. 5′-GMP, in concentrations up to 1 mM, was ineffective as a negative modulator of [^{125}I]HPIA binding (data not shown). All active guanine nucleotides inhibited 100% of [^{125}I]HPIA binding to solubilized cardiac A_1 adenosine receptors. This finding is in contrast to experiments using the membrane-bound adenosine receptor of porcine atria, in which all guanine nucleotides (except GTPγS) maximally inhibited 70–80% of [^{125}I]HPIA binding (18). These titration experiments provide evidence that [^{125}I]HPIA, solubilized cardiac A_1 adenosine receptors, and guanine nucleotide binding protein(s) interact to form a ternary complex that possesses high affinity for adenosine receptor agonists. The sensitivity of high affinity agonist binding to negative modulation by guanine nucleotides is consistent with a negative heterotropic binding interaction (33).

Kinetics of guanine nucleotide-induced [^{125}I]HPIA dissociation. The kinetic nature of guanine nucleotide destabilization of high affinity agonist binding was studied by following the binding of [^{125}I]HPIA after addition of saturating concentrations of guanine nucleotides to an equilibrated mixture of solubilized receptors and radioligand. The purpose of these experiments was to determine the kinetic characteristics of [^{125}I]HPIA dissociation from the guanine nucleotide-sensitive binding component. In contrast to monophasic kinetics observed when dissociation was initiated by 2-CADO (Fig. 3), [^{125}I]HPIA dissociation induced by guanine nucleotides appeared biphasic (Fig. 5). The magnitude of dissociation rate

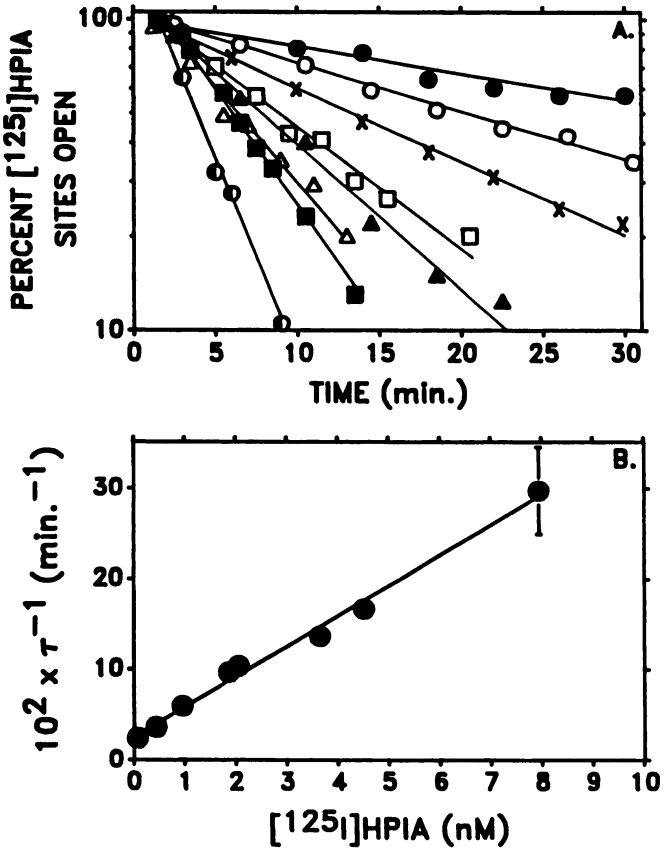

Fig. 2. A, [^{125}I]HPIA association experiments with the solubilized porcine atrial adenosine receptor. Experiments were carried out with a receptor concentration of 15–40 pM and indicated [^{125}I]HPIA concentrations (see below). Binding data were linearly transformed to a ln (percent [^{125}I]HPIA sites open) versus time plot and fitted to Eq. 1 (*lines* drawn are based on these theoretical fits). Slopes of these lines correspond to $-\tau^{-1}$ for that concentration of [^{125}I]HPIA. This analysis yielded the following values for τ^{-1} (min^{-1}): 0.077 nM (●), (2.4 ± 0.1) × 10^{-2}; 0.44 nM (○), (3.6 ± 0.1) × 10^{-2}; 0.955 nM (×), (5.9 ± 0.3) × 10^{-2}; 1.86 nM (□), (9.6 ± 0.4) × 10^{-2}; 2.05 nM (▲), (1.03 ± 0.1) × 10^{-1}; 3.65 nM (△), (1.36 ± 0.1) × 10^{-1}; 4.51 nM (■), (1.66 ± 0.06) × 10^{-1}; 7.94 nM (◐), (2.97 ± 0.5) × 10^{-1}. All experiments were conducted over the course of 36 hr in the same solubilized receptor preparation. B, τ^{-1} as a function of [^{125}I]HPIA concentration in the solubilized receptor preparation. The *line* drawn represents that obtained by fitting data to Eq. 2, which yields a k_{+1} of (3.4 ± 0.1) × 10^7 M^{-1} min^{-1} and a k_{-1} of (2.45 ± 0.40) × 10^{-2} min^{-1}, giving a kinetically derived K_D of 0.73 nM.

constants (0.1–0.2 min^{-1}) and amplitude (50–60% in the fast phase, 40–50% in the slow phase) of each kinetic phase was relatively constant for all guanine nucleotides tested. As a means of addressing molecular mechanisms involved in the generation of biphasic [^{125}I]HPIA dissociation kinetics, dissociation rate experiments were conducted over a large range of GTPγS concentration (Fig. 6A). Biphasic dissociation kinetics were observed over the entire range of GTPγS concentration tested (10 nM to 30 μM). These data were fitted with Eq. 3b to determine the values of the reciprocal relaxation times (τ_1^{-1}, τ_2^{-1}) and amplitudes (B_1, B_2) of each kinetic phase as a function of GTPγS concentration. A plot of the reciprocal relaxation times for fast and slow kinetic phases versus GTPγS concentration appeared hyperbolic (Fig. 6, B and C, respectively). Fitting these data with Eq. 4 generated the following parameter estimates: fast phase, k_{-1} = (1.3 ± 0.1) × 10^{-1} min^{-1}, K_D for

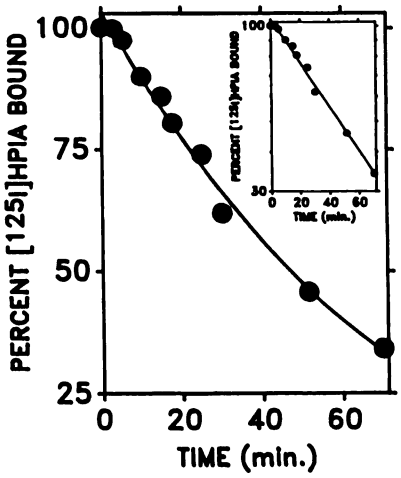

Fig. 3. [^{125}I]HPIA dissociation initiated by addition of 100 μM 2-CADO to an equilibrated mixture of [^{125}I]HPIA and solubilized cardiac A_1 adenosine receptors. Receptor and [^{125}I]HPIA concentration were approximately 50 and 750 pM, respectively. Data were fitted to Eq. 3a (*line* drawn is based on that fit), which gave a dissociation rate constant of (1.6 ± 0.1) × 10^{-2} min^{-1}. *Inset*, semilogarithmic transformation of dissociation data, which was fitted by linear regression and generated an identical estimate for k_{-1}. This figure depicts an experiment representative of three such experiments.

GTPγS = 86 ± 20 nM; slow phase, k_{-1} = (7.6 ± 0.3) × 10^{-2} min^{-1}, K_D for GTPγS = 481 ± 76 nM.

Pharmacological specificity of solubilized cardiac adenosine receptors. To address pharmacological specificity of solubilized cardiac adenosine receptors, the rank order potency of several adenosine receptor agonists and antagonists as inhibitors of [^{125}I]HPIA binding was determined. The adenosine A_1 receptor-selective agonists CPA and (*R*)-PIA were the most potent inhibitors of [^{125}I]HPIA binding whereas an A_2-selective ligand, CV-1808, was approximately 3 orders of magnitude less potent than these A_1-active ligands (Fig. 7 and Table 2). (*R*)-PIA was approximately 42-fold more potent as an inhibitor of [^{125}I]HPIA binding than its less active diastereomer, (*S*)-PIA. Indirect Hill slopes for all agonist and antagonist inhibitors of [^{125}I]HPIA binding did not differ from unity, providing evidence that this concentration of [^{125}I]HPIA (~750 pM) labels a homogeneous receptor population. With the exception of (*S*)-PIA, absolute potencies of all inhibitors of [^{125}I]HPIA binding are reasonably similar for studies employing either solubilized or membrane-bound porcine atrial adenosine receptors (18). These titration experiments suggest that the A_1 receptor subtype selectivity of the cardiac adenosine receptor is well maintained in this detergent system.

Discussion

The mixed detergent system employed in these studies effectively solubilized porcine atrial adenosine receptors in good yield and provided sufficient stability to allow biochemical characterization. Solubilization of brain A_1 adenosine receptors has previously been reported to result in a either a marginal (21, 22) or a negative enrichment of adenosine receptor specific activity (19, 20, 23). Such findings suggest that these solubilization protocols were not optimized for selective solubilization of adenosine receptors and/or solubilized brain adenosine receptors were unstable in the detergents employed. It is, therefore, noteworthy that this double-extraction procedure employ-

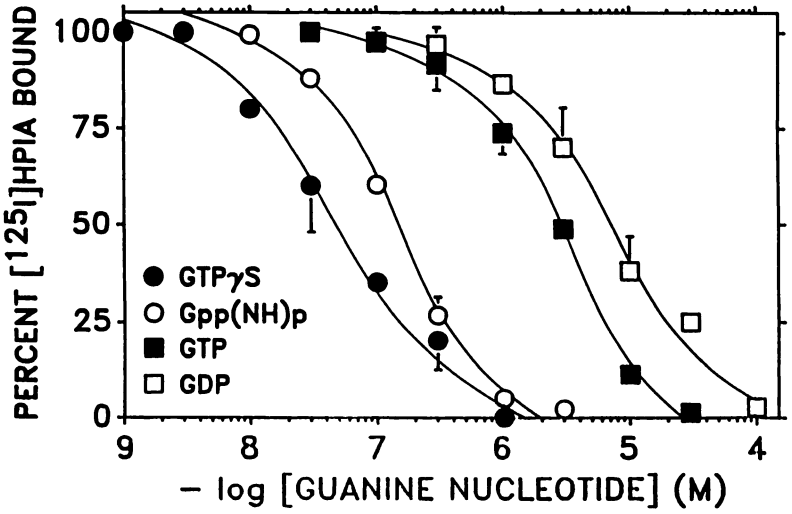

Fig. 4. Guanine nucleotide modulation of [^{125}I]HPIA binding in solubilized porcine atrial adenosine receptor preparations. Receptor and [^{125}I]HPIA concentrations were approximately 40 and 750 pM, respectively. Each *point* represents the mean ± standard error of 3–7 individual experiments. Titration *curves* drawn represent best fits as determined by EBDA software and give the following parameter estimates (IC_{50} ± SE, nM): GTPγS, 64.1 ± 12; Gpp(NH)p, 138 ± 13; GTP, 2600 ± 700; GDP, 8000 ± 6500.

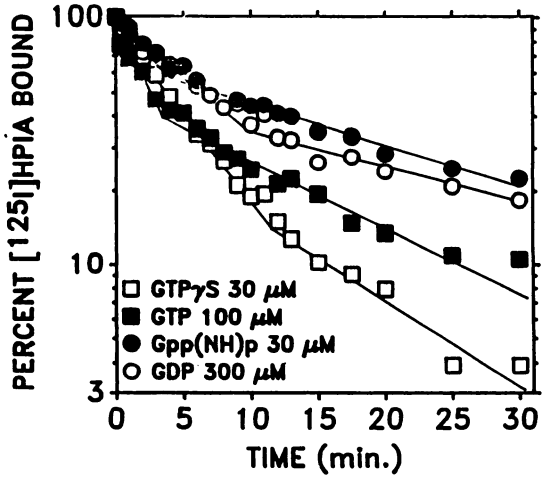

Fig. 5. Semilogarithmic plot of percent [^{125}I]HPIA bound versus time after addition of a saturating concentration of guanine nucleotide. Experimental conditions are as described in the legend to Fig. 4. Data were fitted to Eq. 3b (*lines* drawn are the results of those fits), which yielded the following parameter estimates (units of τ^{-1} are min^{-1}): GTPγS 67% fast, $(\tau_1)^{-1} = (15.0 \pm 0.3) \times 10^{-2}$, 33% slow, $(\tau_2)^{-1} = (7.6 \pm 1.0) \times 10^{-2}$; Gpp(NH)p, 37% fast, $(\tau_1)^{-1} = (11.7 \pm 0.7) \times 10^{-2}$, 63% slow, $(\tau_2)^{-1} = (3.7 \pm 0.2) \times 10^{-2}$; GTP, 53% fast, $(\tau_1)^{-1} = (19.5 \pm 2.1) \times 10^{-2}$, 47% slow, $(\tau_2)^{-1} = (5.9 \pm 0.4) \times 10^{-2}$; GDP, 54% fast, $(\tau_1)^{-1} = (9.0 \pm 0.5) \times 10^{-2}$, 46% slow, $(\tau_2)^{-1} = (3.2 \pm 0.4) \times 10^{-2}$.

ing a mixed detergent system solubilized cardiac adenosine receptors in good yield, resulted in an enrichment of adenosine receptor specific activity, and provided stability adequate for biochemical characterization. The solubilization protocol used here was originally optimized for selective solubilization of porcine atrial muscarinic receptors (30) and was modified only slightly for the present studies (Mg^{2+} and DTT preincubation). In addition to the enrichment of adenosine receptor specific activity, a dramatic increase in the [^{125}I]HPIA binding signal to noise ratio was observed upon solubilization. [^{125}I]HPIA, a marginally useful radioligand for the characterization of membrane-bound cardiac adenosine receptors (35% specific binding at K_D), appears to be an excellent agonist radioligand for the characterization of solubilized cardiac A_1 adenosine receptors (90% specific binding at K_D).

Saturation data were clearly monophasic over the 100-fold range of [^{125}I]HPIA concentrations used. These data would appear to suggest that cardiac adenosine receptors, solubilized in this detergent system and assayed in the presence of high Mg^{2+} and 37°, exist as a homogeneous population displaying high affinity for agonists.

Kinetic experiments were performed to address the mechanism of [^{125}I]HPIA binding to solubilized cardiac A_1 adenosine receptors. Data from association experiments are consistent with [^{125}I]HPIA binding to the solubilized cardiac A_1 adenosine receptor in a simple bimolecular reaction of the type:

$$L + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} LR \qquad (5)$$

which is described by the pseudo-first order rate equation presented above (Eq. 2). This mechanism of binding is consistent with that observed for [^{125}I]HPIA interacting with membrane-bound cardiac adenosine receptors (18). Under these experimental conditions, we found no evidence for a ligand-dependent conformational change in receptor that would create a high affinity complex, such as [*LR*] interacting with a G protein(s). Rather, as postulated for its interaction with membrane-bound cardiac adenosine receptors, [^{125}I]HPIA appears to interact via a simple bimolecular reaction with solubilized cardiac A_1 adenosine receptors that exist precoupled to a G protein(s).

Brain adenosine receptors solubilized in sodium cholate (19, 23), digitonin (21), or CHAPS (22) maintain the ability to interact with G proteins, as evidenced by negative modulation of agonist binding by guanine nucleotides. Guanine nucleotides were 2 orders of magnitude more potent modulators of [^{125}I]HPIA binding to solubilized than to membrane-bound porcine atrial adenosine receptors and all active guanine nucleotides inhibited 100% of [^{125}I]HPIA binding. In contrast, guanine nucleotides inhibited only 75–80% of [^{125}I]HPIA binding to membrane-bound porcine atrial adenosine receptors (18). The inability of guanine nucleotides to inhibit 100% of [^{125}I]HPIA binding to membrane-bound porcine atrial adenosine receptors is likely to be the result of diffusional barriers in the membrane creating a subpopulation of high affinity agonist binding sites that are inaccessible and, thus, insensitive to negative modulation by polar guanine nucleotides. Solubilization of porcine atrial membranes with digitonin/cholate would appear to remove these diffusional barriers.

At saturating concentrations, all guanine nucleotides initiate a maximal rate of [^{125}I]HPIA dissociation of 0.1–0.2 min^{-1}.

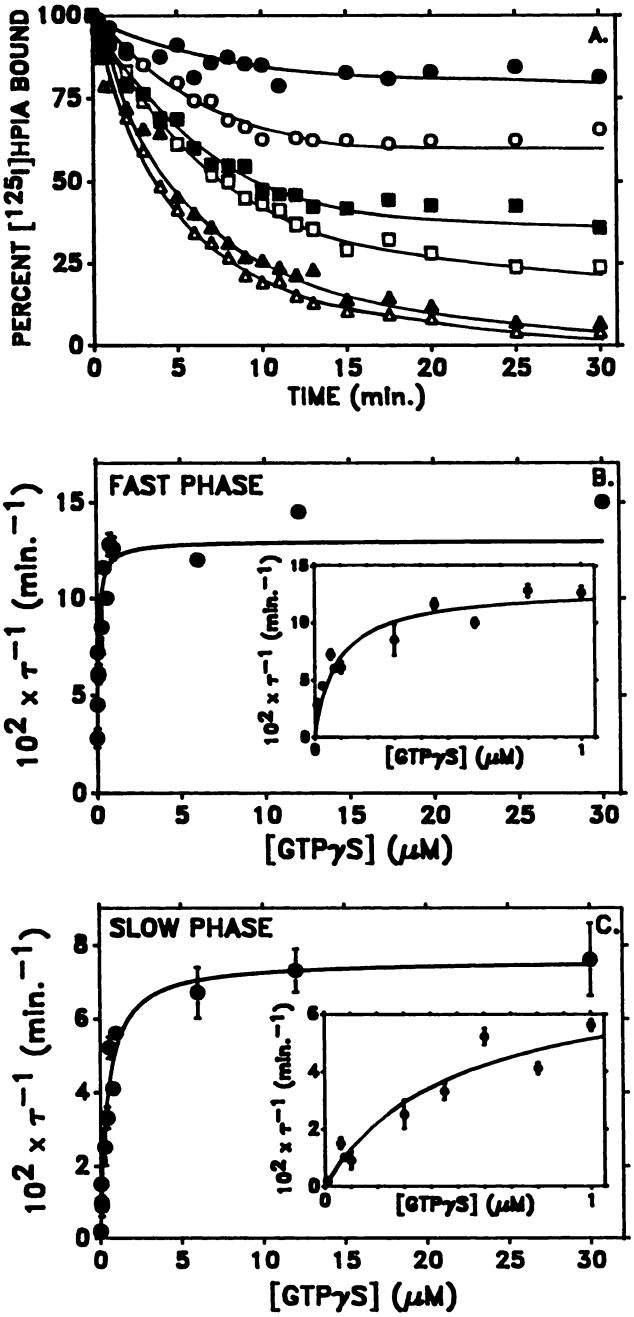

Fig. 6. Dissociation of specifically bound [^{125}I]HPIA from the solubilized porcine atrial adenosine receptor by GTPγS. [^{125}I]HPIA (0.7–0.8 nM) was allowed to equilibrate with a solubilized porcine atrial adenosine receptor preparation (50 pM and 215 nM in adenosine receptor and [^{35}S]GTPγS sites, respectively) for 120 min, at which time dissociation was initiated by addition of the indicated concentrations of GTPγS (see below). A, Fifteen concentrations of GTPγS were used to initiate [^{125}I]HPIA dissociation, of which six are shown here. Theoretical *curves* drawn are based on parameter estimates obtained by fitting data to Eq. 3b ●, 10 nM; ○, 30 nM; ■, 100 nM; □, 300 nM; ▲, 12 μM; △, 30 μM. B, Reciprocal relaxation times for the fast phase of GTPγS-initiated [^{125}I]HPIA dissociation versus GTPγS concentration. Data were fitted to Eq. 4, which gave a k_{-1} for the fast phase of GTPγS-initiated [^{125}I]HPIA dissociation of (1.3 ± 0.1) × 10^{-1} min^{-1} and a K_D for GTPγS of 86 ± 20 nM. *Inset*, magnification of *abscissa* in the range of 0–1 μM GTPγS concentration. Shown is the same theoretical fit described above. C, Reciprocal relaxation times for the slow phase of GTPγS-initiated [^{125}I]HPIA dissociation versus GTPγS concentration. Data were fitted as described in B, giving a k_{-1} for the slow phase of GTPγS-initiated [^{125}I]HPIA dissociation of (7.6 ± 0.3) × 10^{-2} min^{-1} and a K_D for GTPγS of 481 ± 76 nM. *Inset*, magnification of *abscissa* in the range of 0–1 μM GTPγS concentration (same theoretical fit).

This value is reasonably close to the rate constant for GDP dissociation from G_i in a reconstituted system (34). However, GDP release is unlikely to be the rate-limiting step in guanine nucleotide-induced [^{125}I]HPIA dissociation in the present studies. The system has been primed by a preincubation with [^{125}I]HPIA, which should decrease the affinity of G_i for GDP and, thus, allow for rapid binding of other guanine nucleotides. Indeed, the finding that GDP potently and rapidly modulated [^{125}I]HPIA binding provided direct evidence that preincubation with agonist resulted in dissociation of native GDP from the ternary complex.

Biphasic dissociation kinetics were observed for guanine nucleotide-initiated [^{125}I]HPIA dissociation. The magnitude of the reciprocal relaxation times for both fast and slow kinetic phases was hyperbolically dependent on GTPγS concentration. The simplest mechanism to explain this is the occurrence of two parallel reactions:

$$LRG_1 + N \overset{K_1}{\rightleftharpoons} LRG_1N \overset{k_{-1}}{\rightarrow} L + R + G_1N \qquad (6a)$$

$$LRG_2 + N \overset{K_2}{\rightleftharpoons} LRG_2N \overset{k_{-2}}{\rightarrow} L + R + G_2N \qquad (6b)$$

where *L*, *R*, *G*, and *N* represent [^{125}I]HPIA, solubilized cardiac adenosine receptor, G protein(s), and GTPγS, respectively, and LRG_1 and LRG_2 are independent, noninterconvertible populations of receptor-G protein complexes. $K_{1,2}$ and $k_{1,-2}$ represent the dissociation constant for GTPγS and the rate constant for GTPγS-initiated [^{125}I]HPIA dissociation for each parallel reaction, respectively. This model implies that biphasic [^{125}I]HPIA dissociation involves the interaction of guanine nucleotides with a heterologous population of *LRG* complexes differing in *G* rather than *R*. The basis for this hypothesis is that [^{125}I]HPIA apparently labels a homogeneous population of solubilized receptors, as evidenced by monophasic Scatchard-Rosenthal, Hill (data not shown), association rate, and 2-CADO-induced dissociation rate plots. Therefore, biphasic guanine nucleotide-induced [^{125}I]HPIA dissociation appears to arise from G protein, rather than receptor, heterogeneity. Heart contains considerable amounts of both G_i and G_o (35–40). Nathanson and co-workers (41), using quantitative immunoblotting, have reported that the level of $G_{o\alpha}$ in adult rat atria is approximately 60% that of $G_{i\alpha}$. One potential explanation for the appearance of two independent noninterconvertible populations of receptor-G protein complexes is interaction of cardiac adenosine receptors with both of these G proteins. Other G protein-coupled receptors, such as cloned M2 muscarinic receptors, have been shown to interact with two distinct G proteins related to inositol phosphate metabolism and inhibition of adenylyl cyclase activity (42). Functional consequences of adenosine receptor activation in heart that are known to be mediated via an interaction with G proteins include the activation of an inward rectifying K^+ channel (6) and inhibition of adenylyl cyclase activity (7–10),[1] both of which could be explained by the coupling of cardiac adenosine receptors to G_i. Brown and co-workers (43) have reported the existence of G_o-modulated K^+ channels in brain; however, the functional significance of G_o in heart and the possibility of adenosine receptor-G_o coupling remain unknown.

One must also consider the possibility that artifacts of detergent solubilization may have given rise to the appearance of two populations of receptor-G protein complexes. Detergent

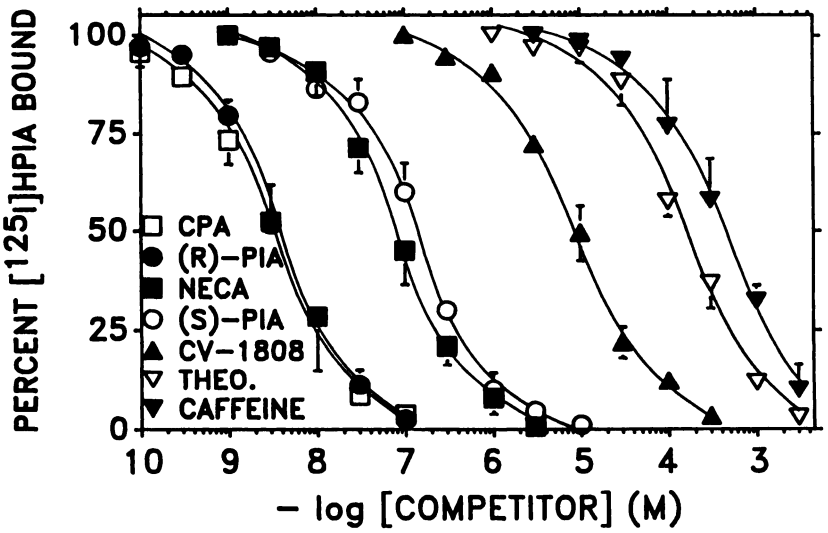

Fig. 7. Adenosine receptor agonists and antagonists inhibiting the binding of [^{125}I]HPIA in solubilized porcine atrial adenosine receptor preparations. Receptor and [^{125}I]HPIA concentrations were fixed at approximately 40 and 750 pM, respectively. Each *point* represents the mean ± standard error of 3–5 experiments. *Lines* drawn represent best fits as determined by EBDA software. Parameter estimates used in curve fitting are given in Table 1 *Theo*, theophylline.

TABLE 2

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

Receptor and [^{125}I]HPIA concentrations were approximately 40 and 750 pM, respectively. Parameter estimates were obtained using EBDA and represent the mean ± standard error of 3–5 experiments.

Compound	Apparent K_D	Slope Factor
	nM	
CPA	2.1 ± 0.8	0.98 ± 0.06
(*R*)-PIA	2.2 ± 0.5	1.01 ± 0.005
NECA	49 ± 11	1.05 ± 0.06
(*S*)-PIA	94 ± 20	0.95 ± 0.06
CV-1808	5,640 ± 1,940	1.00 ± 0.1
Theophylline	106,000 ± 9,000	1.08 ± 0.04
Caffeine	264,000 ± 95,000	1.00 ± 0.04

solubilization most certainly alters the microenvironment in which the cardiac adenosine receptor exists. Effectors, with which the receptor is not associated in the membrane (potentially G_0), will gain access to the receptor provided these effectors and the receptor cosolubilize. Alternatively, the presence of detergent may have altered guanine nucleotide-initiated [^{125}I]HPIA dissociation kinetics. This hypothesis is supported by the finding that GTP-initiated [^{125}I]HPIA dissociation from membrane-bound porcine atrial adenosine receptors is monophasic and occurs with a rate constant of approximately 1.3 min^{-1} (18), 1 order of magnitude greater than was observed in detergent solution. However, the present results are in accordance with the demonstration that guanine nucleotide-induced dissociation of the agonist radioligand [^{3}H]oxotremorine-M was slower in solubilized than in membrane-bound cardiac muscarinic receptor preparations (24). Questions involving the validity of the present kinetic measurements may be addressed in the absence of detergent by using purified components of the ternary complex in a reconstituted system.

Several potential reaction mechanisms are inconsistent with the kinetic behavior of GTPγS-initiated [^{125}I]HPIA dissociation. A bifurcated reaction scheme such as:

$$\begin{array}{ccccccc} LRG & + & N & \rightleftharpoons & LRGN & \rightarrow & L + R + GN \\ & & & & \Downarrow\Uparrow & & \\ L & & + & & RGN & & \end{array} \quad (7)$$

would not explain the observed biphasic GTPγS-initiated [^{125}I]HPIA dissociation kinetics. Such a reaction mechanism would predict that, at increasing concentrations of N, the fast phase of [^{125}I]HPIA dissociation would dominate the observed kinetics. However, consistent with the hypothesis of two parallel dissociation reactions described above, the amplitude of the slow phase increased with increasing nucleotide concentration. Rebinding of agonist is also unlikely to account for biphasic guanine nucleotide-initiated [^{125}I]HPIA dissociation kinetics. The results of [^{125}I]HPIA dissociation experiments in which 100 μM 2-CADO was added simultaneously with guanine nucleotide (GDP) were not qualitatively or quantitatively different from dissociation experiments done in the absence of 2-CADO (data not shown). This finding suggests that, at least in the instance of GDP-initiated [^{125}I]HPIA dissociation, rebinding of agonist does not contribute to the observed biphasic dissociation kinetics.

Adenosine receptor agonists inhibited [^{125}I]HPIA binding in a manner consistent with an interaction at A_1 adenosine receptors (44). The relative and absolute potencies of adenosine receptor agonists inhibiting [^{125}I]HPIA binding to solubilized cardiac adenosine receptors were nearly identical to experiments employing membrane-bound receptors (18). This finding indicates that the pharmacological specificity of solubilized cardiac A_1 adenosine receptors is preserved in this detergent system. Interestingly, both the relative and absolute potency of (*S*)-PIA in the present experiments were decreased when compared with studies employing membrane-bound receptors (18). NECA and (*S*)-PIA were essentially equipotent inhibitors of [^{125}I]HPIA binding to membrane-bound porcine atrial adenosine receptors, whereas a 2-fold difference in apparent K_D values exists in solubilized receptor experiments. Moreover, the potency ratio for the diastereomers of PIA in the present experiments is 2-fold greater than that observed in experiments conducted with the membrane-bound receptors. These findings suggest that the enhanced potency of (*S*)-PIA in higher mammalian tissues (14, 17, 18) may not be a function of differences in receptor proteins per se but rather due to other membrane-related factors such as differing lipid composition.

In summary, a detergent system consisting of digitonin and sodium cholate effectively solubilized cardiac A_1 adenosine receptors in good yield, afforded reasonable stability, and preserved receptor-G protein interactions as well as pharmacolog-

ical specificity. This solubilization procedure appears to be useful for biochemical characterization of cardiac adenosine receptors and the interaction of agonist radioligands with receptor-G protein complexes in detergent solution. Further studies are required to delineate the molecular events involved in the generation of two independent, noninterconvertible populations of cardiac adenosine receptor-G protein complexes.

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