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Characteristics of adenosine binding sites in atrial sarcolemmal membranes

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The studies reported here involve an exploration of the sites on atrial myocyte membranes with which adenosine interacts to produce its potent physiological effects in atrial muscle. Specific, high affinity binding of the stable adenosine analogs 2-chloro[^{3}H]adenosine (2-ClAdo) and [^{3}H]adenosine 5′-*N*-ethylcarboxamide (NECA) to atrial sarcolemmal membranes was measured in kinetic and equilibrium studies at 4°C and 35°C. Analysis of the [^{3}H]2-ClAdo binding isotherm indicated the presence of two classes of binding site with equilibrium K_{assoc} values estimated to be $5.7 \cdot 10^7$ M^{-1} and $2.7 \cdot 10^6$ M^{-1}. Displacement of bound [^{3}H]2-ClAdo by adenosine 5′-*N*-cyclopropylcarboxamide (NCPCA) and by several N^6-substituted adenosine analogs confirmed the presence of two classes of binding site. Analysis of the [^{3}H]NECA binding also revealed the presence of two types of binding site for this ligand. The methylxanthines isobutylmethylxanthine and theophylline displaced bound [^{3}H]2-ClAdo whereas adenosine uptake inhibitors and several other purines showed little activity. These atrial membrane binding sites exhibit many of the characteristics of the physiological adenosine receptors studied in intact atria. Furthermore, the [^{3}H]2-ClAdo binding sites were sensitive to treatment with proteolytic enzymes, suggesting that these sites exist on sarcolemmal membrane proteins.

Introduction

The purine nucleoside adenosine appears to be an important modulator of cardiovascular function under a variety of conditions such as ischemia, anoxia and strenuous exercise [1–4]. It decreases the force and rate of contraction of the atrial myocardium [5,6], attenuates the positive effects of catecholamines on both atrial and ventricular muscle [7–9], but does not appear to have a direct effect on ventricular muscle contractility [7,10]. Although adenosine acts on both the atrial and ventricular myocardium to protect the heart against oxygen deprivation and excessive catecholamine stimulation of metabolic and contractile activity [9,11], it is not yet known whether the sites with which adenosine interacts to produce its effects are the same in the two types of muscle.

Attempts to explore the nature of the adenosine receptors in atrial and ventricular muscle at the cellular or membrane level have been focused almost exclusively on the influence of adenosine on adenylate cyclase activity. There is virtually no information available about the characteristics of the membrane sites with which this purine interacts in atrial muscle to produce its very pronounced physiological effects. In the present study, we have examined the properties of the adenosine recognition sites in isolated atrial sarcolemmal membranes as the first step toward the complete biochemical characterization of cardiac receptors for adenosine. Radioligand binding assay proce-

Abbreviations: 2-ClAdo, 2-chloroadenosine; NECA, adenosine 5′-*N*-ethylcarboxamide; NCPCA, adenosine 5′-*N*-cyclopropylcarboxamide.

dures in which adenosine analogs were used to explore adenosine receptor sites in brain tissue have been used [12–15] with a modification of methods for preparing sarcolemmal membranes of relatively high purity [16–18] to study the atrial plasma membrane receptors for adenosine. Two metabolically stable ^{3}H-labeled adenosine analogs, 2-ClAdo and NECA were used as ligands, as both agents have been shown to decrease atrial contractility, presumably through interaction with receptors on the myocyte membranes [6]. The enrichment of 2-ClAdo-binding sites in bovine atrial sarcolemmal membranes was approximately equal to the enrichment of two other membrane entities, K^+-dependent phosphatase and Na^+-Ca^{2+} exchange. The atrial sarcolemmal membranes contain two classes of 2-ClAdo- and NECA-binding site. A preliminary account of these studies has been presented previously [19].

Materials and Methods

Preparation of sarcolemmal membranes. The atrial sarcolemmal membranes were prepared according to a combination of the procedures used to obtain plasma membranes from ventricular muscle [16–18]. In summary, fresh bovine heart atria (approx. 90 g) were homogenized in 8 vol. of cold 0.25 M sucrose/10 mM Tris-HCl with a Polytron PT 20 at a setting of 6 for three 10-s bursts. After filtration through polyethylene filters (1 241 μ and 925 μm pore sizes), the homogenate was mixed with 0.3 M KCl/25 mM sodium pyrophosphate (final concentrations) and centrifuged at 141 000 $\times g$ for 45 min. The pellets (P_1) were resuspended in homogenizing medium, treated with DNAase I as described [17], filtered through 437 μm mesh, and centrifuged at 141 000 $\times g$ for 45 min. The pellets (P_2) were resuspended in 160 mM KCl/10 mM Tris-HCl (pH 7.5), and filtered through 286 μm mesh, and 25 ml aliquots were layered on top of 13 ml of 30% (w/w) sucrose/10 mM Tris-HCl. Gradients were centrifuged at 120 000 $\times g$ for 90 min. The sarcolemmal membrane-enriched band at the interface of the 30% sucrose was collected, diluted with 160 mM KCl/10 mM Tris-HCl, and pelleted at 250 000 $\times g$ for 15 min in a Ti 60 rotor. The final membrane fraction was resuspended in 0.25 M sucrose (9–12 mg protein/ml), frozen in small aliquots in liquid N_2, and stored at −80°C for periods up to 6 weeks without loss of activity. The pellets (P_3) from the gradient step were also collected and tested for membrane markers and binding activity.

The purity of isolated membranes was determined by monitoring the activity of K^+-stimulated *p*-nitrophenylphosphatase [16], and plasma membrane Na^+-Ca^{2+} exchange activity [20]. Mitochondrial contamination was monitored by the activity of succinate dehydrogenase [21]. Structural characteristics of the membrane fractions were examined by transmission electron microscopy [22].

Radioligand binding assays. Binding of [^{3}H]2-ClAdo to various subcellular fractions was measured initially by a filtration procedure [12–15], but most of these studies were conducted using a centrifugation method to separate the membranes from the medium. Aliquots of frozen membranes were thawed rapidly in the presence of 10 vol. of 20 mM potassium phosphate buffer (pH 7.4) at 37°C for 15 min, followed by treatment with adenosine deaminase (5 units/mg membrane protein) for 30 min at 34°C. Membranes were then precipitated at 39 000 $\times g$ for 45 min and resuspended in 20 mM potassium phosphate buffer (1–2 mg protein/ml). Incubation conditions for the binding assays were identical regardless of the separation technique and consisted of the following: 20 mM potassium phosphate buffer (pH 7.4), 100–150 μg membrane protein, indicated concentrations of [^{3}H]2-ClAdo or [^{3}H]NECA, and various agents tested in a final volume of 1 ml. Incubations were initiated with addition of the [^{3}H]2-ClAdo or [^{3}H]NECA and carried out for 120 min at 4°C unless indicated otherwise. In the filtration procedure incubations were terminated by filtration through Whatman GF/B filters followed by two 5 ml washes with potassium phosphate buffer. In the centrifugation procedure incubations were carried out in 4 ml polypropylene scintillation vials and the reactions terminated by centrifugation of the vials at 39 000 $\times g$ for 10 min at 4°C. The supernatant was aspirated, the surface of each pellet rinsed with 2.5 ml of buffer, and the pellets were dissolved in 0.1 ml of 1 M NaOH. All filters or pellets were counted using Beckman HP scintillation fluid. Specific binding activity was

determined by subtracting the radioactivity present in samples incubated in the presence of 100 μM N^6-(L-phenylisopropyl)adenosine, a potent adenosine agonist [23].

Other procedures. Protein concentrations were determined as in Ref. 24, and possible metabolism of [^{3}H]2-ClAdo during incubations was monitored by thin layer chromatography on Cellulose F plates with a developing solvent of isopropanol/ammonia/water (7 : 1 : 2). 1-cm squares were scraped into scintillation vials and the radioactivity counted in Triton X-100/toluene scintillation cocktail.

Binding data were analyzed by a curve-fitting procedure involving optimization of a weighted least-squares fit to a generalized Scatchard equation as described [25,26]. The weighting function used was the reciprocal of the variance of the amount bound [26]. The same program was used when the equation was that for non-independent sites with cooperativity described by Thompson and Klotz [27].

Materials. 2-Chloro[^{3}H]adenosine (10–11 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA, and [^{3}H]NECA (30 Ci/mmol) and $^{45}CaCl_2$ (10 Ci/g) from New England Nuclear, Boston, MA. The N^6-(L-phenylisopropyl)adenosine was purchased from Boehringer-Mannheim, Indianapolis, IN, and N^6-cyclohexyladenosine from Calbiochem-Behring, San Diego, CA. Adenosine 5′-*N*-cyclopropylcarboxamide was a gift from Dr. E.L. Woroch, Abbott Laboratories, North Chicago, IL, and N^6-(D-phenylisopropyl)adenosine from Dr. John Daly, N.I.H. All other chemicals used were reagent grade.

Results

Subcellular distribution of 2-ClAdo binding

Procedures used for isolation of sarcolemmal membranes from bovine atria yielded a membrane fraction substantially enriched in plasma membrane markers (Table I), and electron microscopic examination of this fraction revealed fairly uniform membrane vesicles without the presence of identifiable mitochondrial particles. Both the plasma membrane enzyme ouabain-sensitive, K^+-stimulated *p*-nitrophenylphosphatase and the Na^+-Ca^{2+} antiport system were enriched approximately 13-fold relative to the homogenate (Table I). The mitochondrial membrane enzyme succinate dehydrogenase was highly enriched in the pellet (P_3) from the sucrose gradient step with slight contamination in the membrane fraction (not shown).

The subcellular localization of the specific binding sites for 2-ClAdo is also shown in Table I. Specific binding activity showed a 9-fold purifica-

TABLE I

SUBCELLULAR DISTRIBUTION OF PROTEIN, ENZYME MARKERS, AND [^{3}H]2-ClAdo BINDING

Protein recovery and activity of enzymatic markers were monitored in each of five particulate fractions obtained during isolation of the atrial sarcolemmal membranes. 2-Chloroadenosine binding was measured in the presence of 10 nM [^{3}H]2-ClAdo for 120 min at 4°C by the centrifugation method. Enzymatic activities were determined as described under Methods, and the data are the means (±S.E.) from three membrane preparations.

Fraction	Protein (mg)	K^+-*p*-NPPase (μmol P_i/mg per h)	Purification	Na^+-Ca^{2+} exchange (nmol Ca^{2+}/mg per 15 s)	Purification	Amount bound (fmol 2-ClAdo/mg protein)	Purification
H	4990 ±337	0.3155 ±0.12	1.0	0.024 ±0.0005	1.0	24.6	1.0
P_1	1732 ±424	0.5060 ±0.35	1.6	0.047 ±0.014	1.96	52.9	2.2
P_2	1429 ±161	0.6226 ±0.09	2.0	0.048 ±0.013	1.96	65.5	2.7
P_3	991 ±181	0.330 ±0.062	1.1	0.053 ±0.006	2.2	69.0	2.8
MF	117 ±9.2	3.877 ±0.9	12.3	0.304 ±0.006	12.7	222.0	9.0

tion in the membrane-enriched fraction relative to the homogenate and paralleled the enrichment in K^+-activated phosphatase and Na^+-Ca^{2+} antiport activity.

Characteristics of [^{3}H]2-ClAdo binding to atrial sarcolemmal membranes

Isolated atrial sarcolemmal membranes were employed in all subsequent studies, and [^{3}H]2-ClAdo was used as the primary ligand for studying adenosine receptor sites, since it is not a substrate for adenosine deaminase or nucleoside uptake carriers [28]. Thin-layer chromatographic analysis of the desalted medium following incubation under standard conditions revealed more than 99% of the radioactivity to be present as 2-ClAdo. As a further precaution against measuring transport of the labelled ligand, most assays were conducted at 4°C. Binding increased linearly with the amount of membrane protein in the assay over the range 40–200 μg protein, and there was no difference between the binding to freshly prepared membranes and the binding to membranes frozen and stored in liquid N_2. The amount of specific [^{3}H]2-ClAdo binding to isolated membranes as measured by the centrifugation technique was a relatively small but consistent and saturable fraction of the total radioactivity bound to the membranes, constituting about 40–50% at the lowest ligand concentrations (Fig. 1).

The specific binding activity of the membranes was determined over a wide range of ligand concentrations (0.75 to 700 nM) as suggested elsewhere [29] to obtain the equilibrium binding constants (Fig. 2A). Computer analysis of the data by the generalized Scatchard equation revealed the presence of two classes of site (Fig. 2B). Data from each of five membrane preparations, with multiple

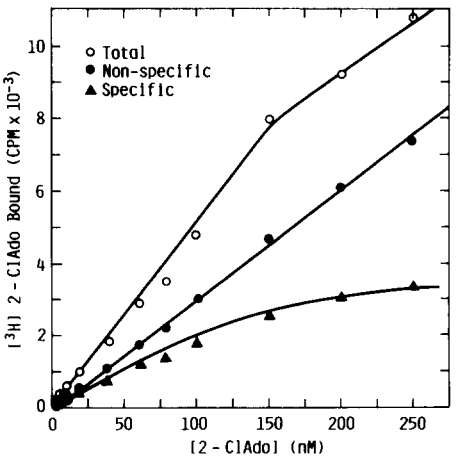

Fig. 1. Binding of [^{3}H]2-ClAdo to atrial membranes. The centrifugation procedure was used to measure the amount of [^{3}H]2-ClAdo bound to atrial membranes (100 μg protein) after 120 min incubation at 4°C. The nonspecific binding was determined in the presence of 100 μM N^6-(L)-phenylisopropyl)adenosine and was subtracted from the total binding to obtain the specific binding component. The data are means from triplicate determinations with two atrial membrane preparations, and each S.E. was less than 15% of the mean value.

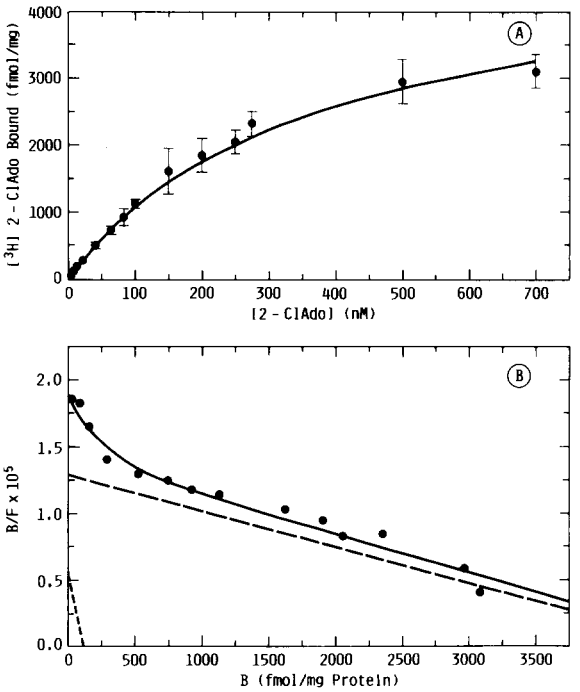

Fig. 2. Concentration dependence of specific [^{3}H]2-ClAdo binding to atrial sarcolemmal membranes. (A) Direct plot of binding activity measured by the centrifugation method after 120 min incubation at 4°C. The line represents the calculated binding isotherm obtained under the assumption of two independent classes of binding site. Data points are the means (±S.E.) from triplicate determinations with five atrial membrane preparations. (B) Scatchard plot of the data shown in (A). The solid line represents the curve obtained with the Scatchard equation when the following constants were used for the two independent sets of binding sites: $K_{assoc_1} = 5.7 \cdot 10^7$ M^{-1}, $B_{max_1} = 100$ fmol/mg protein, and $K_{assoc_2} = 2.7 \cdot 10^6$ M^{-1} and $B_{max_2} = 4800$ fmol/mg protein. The interrupted lines are the linear plot for each class of sites.

determinations per preparation, yielded similarly curvilinear Scatchard plots, and a composite of the means from those preparations is presented in Fig. 2. If we assumed 2-ClAdo binding to n classes of independent sites according to the Scatchard equation, minimization of the squares of the differences between the experimental data and the calculated binding isotherm was achieved when it was assumed that there are two classes of independent sites (see solid lines, Fig. 2A and 2B). The assumption of interactions among sites as described by Thompson and Klotz [27] did not improve the curve fitting significantly and thus did not appear warranted on the basis of available data. The Hill coefficient estimated from Hill plot analysis of the data shown in Fig. 2 was 0.97 ($r = 0.99$), indicating no cooperativity between binding sites. The estimated total binding capacity and respective association constants (K_{assoc}) for each class of sites were: 100 fmol/mg protein and $5.7 \cdot 10^7$ M^{-1} ($K_{dissoc} = 18$ nM) and 4800 fmol/mg protein and $2.7 \cdot 10^6$ M^{-1} ($K_{dissoc} = 372$ nM).

When the same ligand concentration range was used in binding assays conducted at 35°C, the estimated constants for [^{3}H]2-ClAdo binding to sarcolemmal membranes were: $K_{assoc} = 9.9 \cdot 10^7$ M^{-1} and B_{max} 75 fmol/mg for the high affinity sites, and $K_{assoc} = 3.0 \cdot 10^6$ M^{-1} and B_{max} 3700 fmol/mg for the low affinity sites. These estimates were obtained with averaged data from four experiments. Thus, the characteristics of [^{3}H]2-ClAdo binding to atrial sarcolemmal membranes determined at 35°C differed from those determined at 4°C primarily in terms of an increase in the affinity of the high-affinity sites and a small decrease in the B_{max} of both high- and low-affinity sites.

When [^{3}H]2-ClAdo binding to atrial membranes was measured at 4°C with the filtration technique, the presence of two classes of binding site was again observed. Data from three experiments with 2-ClAdo concentrations ranging from 1 to 250 nM were best fitted by assuming two classes of binding sites with the following K_{assoc}: $5.9 \cdot 10^7$ M^{-1} and $3.5 \cdot 10^6$ M^{-1} for the high- and low-affinity sites, respectively. The estimated B_{max} values were 10 fmol/mg protein and 1020 fmol/mg protein. We have consistently observed a lower binding capacity of the membranes when 2-ClAdo binding was measured by filtration both at 4°C and at 35°C. Sarcolemmal membranes are not retained very well by filters under ultrafiltration procedures [30], and such a loss of membranes may account for the lower binding observed with the filtration procedure.

Differences between the two measurement techniques in detecting the amount of ligand bound at a given concentration can be seen in the kinetic plot of Fig. 3. The binding process was at equilibrium by 120 min of incubation at 4°C under both assay procedures, and this time period was selected for all 4°C binding measurements at equilibrium. Both types of assay shown in Fig. 3 were conducted under pseudo-first-order reaction conditions, but only filtration data were used to estimate association rate constants. The data were fitted by a multiple iteration program to both a single and a double exponential function. The latter function yielded one-half the error estimated with the single exponential function. Therefore, the two-kinetic-component model was considered more appropriate, and the estimated k_{obs} and

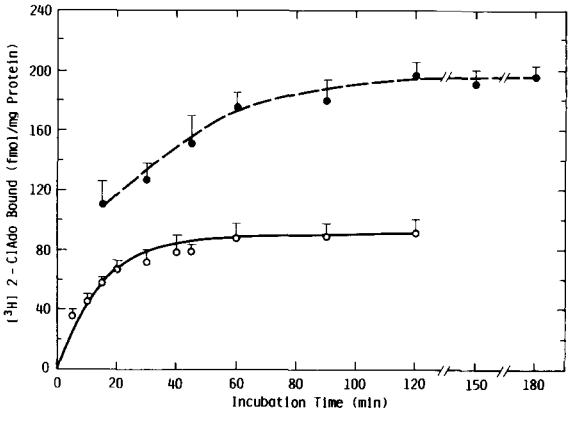

Fig. 3. Time-course of specific binding of [^{3}H]2-ClAdo to atrial sarcolemmal membranes. Incubations were carried out in the presence of 16 nM [^{3}H]2-ClAdo at 4°C and were terminated either by filtration (○) or by centrifugation (●). Each value is the mean (±S.E.) from three separate experiments with each procedure and triplicate determinations within each experiment. Data obtained from the filtration assays were used to estimate the rate constants under pseudo-first-order reaction conditions. The filtration data were fitted by a multiple iteration program, and the two kinetic component model provided the best fit. The estimated k_{obs} and equilibrium binding (B_{eq}) for each component were: $B_{eq1} = 66.3$ and $B_{eq2} = 23.3$ fmol/mg, and $k_{obs_1} = 0.086$ min^{-1} and $k_{obs_2} = 0.038$ min^{-1}.

equilibrium binding (B_{eq}) for each component are shown in Table II and Fig. 3, respectively. The [^{3}H]2-ClAdo bound to sarcolemmal membranes was dissociated by the addition of 100 μM 2-ClAdo with two clearly defined dissociation rates. Approximately 80% of the bound [^{3}H]2-ClAdo was dissociated by 60 min. An equivalent degree of dissociation could be brought about by a 10-fold dilution with 20 mM potassium phosphate buffer. All rate kinetics studies were conducted with 16 nM [^{3}H]2-ClAdo.

The k_{obs} for ligand association and the corresponding k_{dissoc} constants were used to calculate both the apparent asociation rate constants and the equilibrium association constants shown in Table II. The estimated values for the apparent rate and equilibrium constants when [^{3}H]2-ClAdo binding was measured at 35°C, are also included in Table II. These kinetic analyses indicated that measurement of ligand binding at 35°C causes a marked increase in the K_{assoc} of the high- and low-affinity sites. The K_{assoc} for the high-affinity sites estimated from the kinetic data obtained either at 4°C or 35°C was approx. 7.5-times greater than that determined from equilibrium binding studies.

Selectivity of 2-ClAdo binding sites for various adenosine analogs

Displacement of bound [^{3}H]2-ClAdo by NCPCA, adenosine, and a series of N^6-substituted adenosine derivatives is shown in Fig. 4. Each of the agents tested showed two apparent displacement processes. 2-Chloroadenosine was very effective in displacing itself, while the (+)-isomer of N^6-(phenylisopropyl)adenosine was very weak.

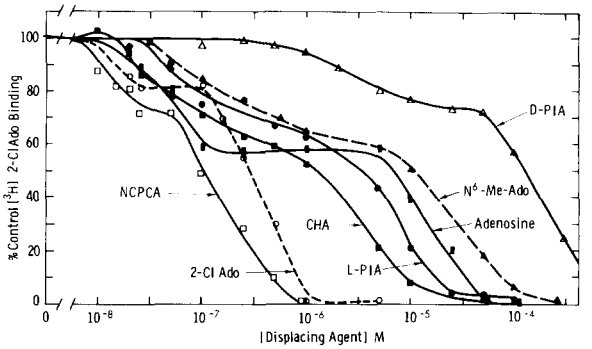

Fig. 4. Inhibition of specific [^{3}H]2-ClAdo binding to atrial sarcolemmal membranes by various adenosine agonists. Membranes (100–150 μg protein) were incubated with 16 nM [^{3}H]2-ClAdo and the indicated concentrations of the agonists at 4°C for 120 min. The specific binding was determined by the centrifugation procedure. Each value is the mean obtained from three experiments with triplicate determinations in each and each S.E. was less than 10% of the mean value. CHA, N^6-cyclohexyladenosine; N^6-MeAdo = N^6-methyladenosine.

N^6-Cyclohexyladenosine, N^6-(L-phenylisopropyl)adenosine and N^6-methyladenosine exhibit agonist activity in several systems responsive to adenosine [6,23,28], and all of these compounds, including adenosine, were active in the displacement assays. In the studies of adenosine displacement of [^{3}H]2-ClAdo, the membranes were not pretreated with adenosine deaminase. The 5'-substituted carboxamide, NCPCA, an agent reported to be the strongest agonist in intact atria [6], was also the most active derivative in displacing [^{3}H]2-ClAdo.

The estimated concentration of each agent that produced 50% displacement of 2-ClAdo (IC_{50} val-

TABLE II

ESTIMATES OF RATE CONSTANTS FOR [^{3}H]2-ClAdo BINDING

All assays were performed by the filtration procedure described under Methods. The values represent the mean of estimated apparent rate constants from three experiments performed either at 4°C or 35°C. The rate constant for association (k_{assoc}) was estimated from the equation: $k_{assoc} = (k_{obs} - k_{dissoc})/[2\text{-ClAdo}]$.

Temperature of assays (°C)	Class of sites	k_{obs} association (min^{-1})	k_{dissoc} (min^{-1})	k_{assoc} ($M^{-1} \cdot min^{-1}$)	k_{assoc}/k_{dissoc} (M^{-1})
4	1	0.086	0.0105	$4.72 \cdot 10^6$	$4.49 \cdot 10^8$
	2	0.038	0.036	$1.06 \cdot 10^5$	$2.93 \cdot 10^6$
35	1	0.094	0.007	$5.54 \cdot 10^6$	$7.76 \cdot 10^8$
	2	0.06	0.055	$3.12 \cdot 10^5$	$5.68 \cdot 10^6$

ues) from the high- and low-affinity sites were determined by log probit analysis and are summarized in Table III, along with maximal percentage inhibition at 100 μM concentration. The pronounced two-phase displacement process exhibited by the adenosine agonists made it possible to calculate separate IC_{50} values for displacement from the high- and the lower-affinity binding sites. The small displacement produced by submicromolar concentrations of the adenosine receptor antagonists theophylline and isobutylmethylxanthine made it difficult to evaluate the efficacy of these agents in displacing bound [^{3}H]2-ClAdo from the high- and low-affinity sites independently. Thus, the single values for these agents shown in Table III were obtained by assuming displacement from a single class of sites. The purine nucleoside guanosine, the deaminated metabolite of adenosine, inosine, and the purine base, adenine, had very weak displacing activity as did the adenosine uptake inhibitors papaverine and nitrobenzylthioinosine (Table III).

Characteristics of [^{3}H]NECA binding sites in sarcolemmal membranes

Bound [^{3}H]2-ClAdo was displaced most effectively by NCPCA, and 2-ClAdo and NCPCA were both found to be very effective in decreasing atrial contractility [6]. Thus, a direct estimate of the binding constants of the sites that interact with

TABLE III

DISPLACEMENT OF [^{3}H]2-ClAdo BY ADENOSINE AND XANTHINE DERIVATIVES

IC_{50} values for displacement of bound [^{3}H]2-ClAdo from sarcolemmal membranes were determined by log probit analysis. Various concentrations of the agents were added to the assay 10 min prior to [^{3}H]2-ClAdo. Binding assays were conducted with 16 nM [^{3}H]2-ClAdo concentration with one exception as noted. Incubations were terminated by centrifugation. Data for each compound were obtained from 3–4 experiments with triplicate determinations in each. The S.E. for each agent was less than 10% of the mean values. N^6-CHA, N^6-cyclohexyladenosine; PIA, N^6-(phenylisopropyl)adenosine.

Agent	Estimated IC_{50} values		Maximal displacement at 100 μM (%)
	high affinity (nM)	low affinity (nM)	
Receptor agonists			
2-ClAdo	13 [a]	398	100
NCPCA	13	179	100
N^6-CHA	38	1550	100
Adenosine	53	20410	100
L-PIA	60	4100	100
N^6-MeAdo	96	17000	94
D-PIA	3180	320000	44
Receptor antagonists			
IMBX	–	600	100
Theophylline	–	2100	80
Uptake inhibitors			
Nitrobenzylthioinosine	–	10000	100
Papaverine	–	>100000	23
Other purines			
ATP	–	2240	100
Adenylylimidodiphosphate	–	7800	100
2′-Deoxyadenosine	–	8000	89
Adenine-9-3-arabinofuranoside	–	20000	100
Inosine	–	>100000	37
Guanosine	–	40000	60
Adenine	–	28000	72

[a] High-affinity displacement of 2-ClAdo measured with ^{3}H-labelled ligand at 4 nM concentration.

NCPCA was obtained by measuring the binding of [^{3}H]NECA to atrial sarcolemmal membranes. NECA, and its close structural analog, NCPCA, have equivalent activity on atrial contractility [6]. Binding of [^{3}H]NECA to atrial membranes at equilibrium was measured by the centrifugation procedure described above and, again, two classes of binding site were observed (Fig. 5). The apparent binding constants (see legend) were almost identical to the IC_{50} values for NCPCA displacement of 2-ClAdo. In addition, the estimated K_{assoc} and density of [^{3}H]NECA binding sites in the sarcolemmal membranes were approximately equal to those determined for the 2-ClAdo binding sites, suggesting that both ligands interact with the same group of sites on atrial sarcolemmal membranes.

Effects of proteinase treatment on 2-ClAdo binding sites

Exposure of the isolated sarcolemmal membranes to the proteolytic enzymes trypsin and pronase produced a decrease in [^{3}H]2-ClAdo binding. Pretreatment of the membranes for 20 min at 35°C with trypsin and pronase in ratios of membrane protein to enzyme of 10:1 produced 51% inhibition of binding of 4 nM [^{3}H]2-ClAdo to both trypsin and pronase-treated membranes. These observations provide a preliminary indication that the atrial sarcolemmal membrane 2-ClAdo binding sites are protein in nature.

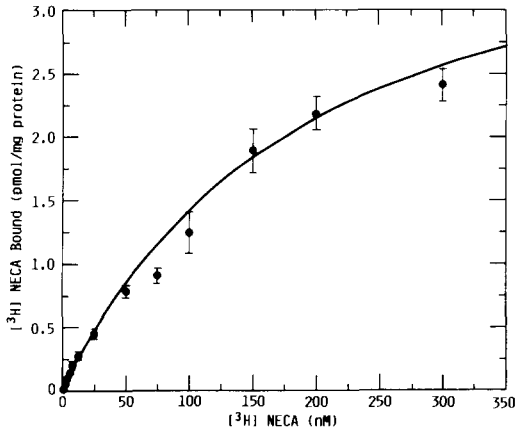

Fig. 5. Binding of [^{3}H]NECA to atrial membranes. The centrifugation procedure was used to measure the amount of [^{3}H]NECA bound to atrial membranes (100–150 μg protein) following 30 min incubation at 35°C. Nonspecific binding was determined in the presence of 100 μM N^6-(L-phenylisopropyl)adenosine. The data are means (±S.E.) from quadruplicate determinations with two atrial membrane preparations. Analysis of the data by the generalized Scatchard equation yielded the following constants for the high- and low-affinity sites, respectively: $K_{assoc_1} = 7.9 \cdot 10^7$ M^{-1} and $K_{assoc_2} = 5.1 \cdot 10^6\ M^{-1}$; $B_{max_1} = 50$ fmol/mg protein and $B_{max_2} = 4100$ fmol/mg protein.

Discussion

The studies reported here indicate the presence of specific high-affinity binding sites for the adenosine analogs [^{3}H]2-ClAdo and [^{3}H]NECA in a preparation highly enriched in atrial sarcolemmal membranes. 2-Chloroadenosine was selected for the initial studies because it interacts with adenosine receptors associated with both inhibition and activation of adenylate cyclase in other tissues, but does not interact with the 'P' site believed to be part of the catalytic component of the enzyme [23]. The binding of the carboxamide derivative [^{3}H]NECA was also examined, since the strongest displacing agent for 2-ClAdo was its close structural analog NCPCA. The use of N^6-(L-phenylisopropyl)adenosine to determine the nonspecific binding revealed that the specific [^{3}H]2-ClAdo binding sites constituted a relatively small component of the total sites with which 2-ClAdo and NECA interact. Nevertheless, the specific [^{3}H]2-ClAdo and [^{3}H]NECA binding was saturable and exhibited many of the characteristics expected of the adenosine receptor-ligand interaction.

The binding isotherm revealed the presence of two classes of sites, regardless of which ligand was tested, whether the assay was conducted at 4 or at 35°C, and whether the filtration or centrifugaton technique was used. The existence of multiple classes of binding site for 2-ClAdo, N^6-cyclohexyladenosine, NECA, and adenosine in brain neuronal and microvessel membranes has been reported by several investigators [14,31,32,33]. The two classes of binding site detected in the present studies may represent ligand binding to two different sites on a single macromolecule, to two different macromolecules in sarcolemmal membranes, or possibly to sites located on different types of membranes in the preparation used.

The effect of temperature on the binding constants was similar to that reported by Murphy and

Snyder [34] for N^6-(L-phenylisopropyl)adenosine binding to brain membranes. The higher K_{assoc} obtained for the high-affinity binding sites under the rate kinetic as opposed to the equilibrium conditions may be due to the fact that in the equilibrium binding studies, the large contribution of low-affinity sites makes it difficult to obtain a precise estimate of the K_{assoc} of the high-affinity sites. Since the rate kinetic studies were conducted with a fairly low [^{3}H]2-ClAdo concentration, the contribution of the high-affinity sites constituted a larger proportion of the total binding activity and, thus, the kinetics of binding to the higher-affinity sites may have been determined more accurately under these conditions.

One prominent characteristic of the atrial sarcolemmal binding sites detected either with [^{3}H]2-ClAdo or [^{3}H]NECA was the high density of the lower-affinity binding sites as compared with that of the high-affinity sites. In a very recent series of experiments, the binding of a ligand believed to be selective for the A_1 receptor subtype, [^{3}H]cyclohexyladenosine, was found to exhibit a binding isotherm nearly identical to those obtained with 2-ClAdo and NECA. The estimated binding constants for the high- and low-affinity sites seen with this ligand were: $K_{assoc1} = 3.9 \cdot 10^7$ M^{-1}, $B_{max_1} = 200$ fmol/mg protein, and $K_{assoc2} = 6.97 \cdot 10^6$ M^{-1}, $B_{max2} = 4900$ fmol/mg protein. In this respect, adenosine receptors in atrial membranes do indeed differ from those in brain membranes, but they bear great similarity to the receptors described in several peripheral systems. For example, the densities of high-affinity sites in tissues such as brain microvessels, coronary arteries, and placenta range from 84 to 1200 fmol/mg protein and those of lower-affinity sites from 1100 to 11 700 fmol/mg protein [33,35,36]. Clearly, the data obtained with atrial sarcolemmal membranes are within these ranges, and this high density of sites may indeed be a distinguishing characteristic of adenosine receptors which exist in certain types of peripheral tissue, particularly muscle.

The relationship of the adenosine analog binding sites to the physiologic adenosine receptors was evaluated by examining the selectivity of the binding sites for agents with known physiologic activity in atrial tissue. Collis [6] reported the following relative order of potency for a series of adenosine analogs mediating a decrease in the force of contraction of guinea-pig atrial muscle: NCPCA ≈ NECA > N^6-cyclohexyladenosine > N^6-(L-phenylisopropyl)adenosine = 2-ClAdo > -adenosine > N^6-(D-phenylisopropyl)adenosine. The selectivity of the [^{3}H]2-ClAdo binding sites in atrial sarcolemmal membranes in the present study was quite similar to that of the physiologic adenosine receptors [6]. The order of potency for the various agonists shown in Table III differs from that determined by the physiologic response only in the higher relative potencies of 2-ClAdo and adenosine. With the exception of NCPCA, 2-ClAdo was the most effective agent in displacing [^{3}H]2-ClAdo from atrial sarcolemmal membranes. The higher potency of adenosine in the ligand displacement assays may be due to the presence of inactivating processes in the intact atrial tissue, since adenosine was as potent as N^6-cyclohexyladenosine when purine transport activity was blocked [6].

The methylxanthines displaced bound [^{3}H]2-ClAdo, with isobutylmethylxanthine being more potent than theophylline as has been observed in other tissues [12–15]. The weak displacing activity of ATP and adenylylimidodiphosphate is consistent with the observations of Collis and Pettinger [37] that both of these agents caused decreases in the force of contraction in guinea-pig atria. The potency of these nucleotides, however, was considerably less than that of 2-ClAdo [37]. The ribose-modified agents adenine-9-β-arabinofuranoside and 2′-deoxyadenosine were much less active than most of the purine-modified agents, suggesting that the binding was not occurring to P sites. The very limited displacement by papaverine and nitrobenzylthioinosine indicates that [^{3}H]2-ClAdo was not binding substantially to the adenosine uptake sites, and the lack of significant displacement by other purines such as guanosine and adenine further substantiates the specificity of the binding interactions.

In summary, this series of studies involved an exploration of the subcellular localization and the kinetic characteristics of adenosine binding sites in bovine atria. The results indicated that the plasma membrane fraction is enriched in adenosine binding activity, that 2-ClAdo is binding to membrane proteins, and that the 2-ClAdo binding sites in the

isolated atrial membranes exhbit many of the characteristics of the physiological receptors for adenosine in atrial myocytes. The displacement studies suggest that the sites labelled by [^{3}H]2-ClAdo in atrial sarcolemmal membranes may correspond to the A_2 type of physiologic adenosine receptors, as these A_2 receptors are optimally activated by NCPCA and NECA, followed by 2-ClAdo, adenosine, N^6-cyclohexyladenosine and N^6(L-phenylisopropyl)adenosine [23]. Studies are currently underway to solubilize the binding sites. Preliminary results indicate that sodium cholate treatment of the membranes does solubilize the binding sites and thus should enable us to proceed with the isolation and further molecular characterization of these membrane entities.

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