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NUCLEOSIDE TRANSPORT IN GUINEA PIG MYOCYTES

COMPARISON OF THE AFFINITIES AND TRANSPORT VELOCITIES FOR ADENOSINE AND 2-CHLOROADENOSINE

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Abstract—The affinities of adenosine and 2-chloroadenosine for the nucleoside transport system of guinea pig myocytes were evaluated indirectly by studying the inhibition of the binding of [^{3}H]nitrobenzylthioinosine and directly by measuring the influx of [^{3}H]radiolabeled substrates. Maximal transport velocities of the two nucleosides were also obtained. [^{3}H]Nitrobenzylthioinosine bound to a single class of high-affinity sites (K_D of 0.8 nM) which possessed a maximal binding capacity (B_{max}) of 870,000 sites/cell. Adenosine, 2-chloroadenosine or the nucleoside transport inhibitor, dipyridamole, competitively inhibited the site-specific binding of [^{3}H]nitrobenzylthioinosine with K_i values of 318 μM, 22 μM and 75 nM respectively. Both [^{3}H]adenosine and [^{3}H]2-chloroadenosine entered myocytes in a saturable and inhibitible manner. Observed transport kinetic constants (K_m and V_{max}) were 146 μM and 24.2 pmoles/10^6 cells/sec, respectively, for adenosine and 36 μM and 11.7 pmoles/10^6 cells/sec, respectively for 2-chloroadenosine. Affinities of adenosine, 2-chloroadenosine, nitrobenzylthioinosine and dipyridamole for the nucleoside transport system derived from binding and influx methodologies were equivalent which confirms that [^{3}H]nitrobenzylthioinosine binding sites are closely associated with the nucleoside transporter.

Adenosine and some of its analogues possess a wide variety of cardiac actions such as coronary vasodilation, blockade of A-V nodal conduction, and depression of heart rate and force of contraction [1–3]. These actions are mediated via a number of extracellular adenosine receptors [4] and are rapidly terminated, possibly by the uptake of adenosine into surrounding cells by a membrane-located nucleoside transport system [5]. Inhibition of the nucleoside transport process has been proposed as a mechanism of drug-induced potentiation of the effects of adenosine. For example, the vasoactivity of the so-called coronary vasodilator drugs, dipyridamole or dilazep, is attributed to the potentiation of adenosine-induced vasodilation [6, 7]. Such agents also potentiate other actions of adenosine including those on cardiac muscle [8] and nonvascular smooth muscle [9].

The analogue 2-chloroadenosine is a potent agonist at adenosine receptors [4], but, as its actions are not potentiated by nucleoside transport inhibitors [8–11], it has been assumed that it is not a substrate for the nucleoside transport system [12, 13]. However, there is indirect evidence that 2-chloroadenosine does indeed interact with the nucleoside transporter. First, it is a competitive inhibitor (K_i value of 24 μM) of the transport of adenosine into L1210 cells [14]. Second, it is a competitive inhibitor (K_i value of 15–30 μM) of the binding of the potent and specific nucleoside transport inhibitor [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR) in guinea pig cortical membranes [15] and in rat and guinea pig cardiac membranes [16]. Unfortunately, these studies did not reveal whether 2-chloroadenosine is an inhibitor or a substrate for the nucleoside transport system. We have now compared the interaction of adenosine and 2-chloroadenosine with the nucleoside transport system in guinea pig myocytes. The use of dissociated myocytes permitted not only the evaluation of the affinities of adenosine and 2-chloroadenosine in competition assays with the high-affinity probe, [^{3}H]NBMPR, but also the direct measurement of the transport fluxes of adenosine and 2-chloroadenosine.

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MATERIALS AND METHODS

Preparation of guinea pig cardiac myocytes. Male Hartley guinea pigs (200–250 g) were killed by decapitation. Hearts were removed and placed in a carbogenated Joklik medium, pH 7.4, 37°, composed of minimum essential medium (Gibco Laboratories, Burlington, Ontario) supplemented with 1.2 mM $MgSO_4 \cdot 7H_2O$, 1 mM D,L-carnitine, 23.8 mM $NaHCO_3$, 0.1 mM adenosine, 60 mM taurine and 50 mM glucose. The hearts were perfused retrogradely via their aortas for 30–40 min with a collagenase enzyme mixture containing 300 units/ml Sigma collagenase type V, 150 units/ml Sigma collagenase type IA, and 0.1% bovine serum albumin, prepared in Joklik medium. After removing the hearts from the perfusion apparatus, the ventricles were agitated in a medium containing 85 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4 \cdot 7H_2O$, 60 mM taurine, 50 mM glucose, 5 mM pyruvate, 5 mM ATP and 5% polyvinylpyrolidone. Following mechanical dissociation, the ventricular myocytes were washed twice and resuspended in assay medium containing 137 mM NaCl, 6.3 mM $Na_2HPO_4 \cdot 7H_2O$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6H_2O$,

10 mM glucose and 5% polyvinylpyrolidone. Cell viability was judged by tryptan blue exclusion and rod-shaped appearance.

[^{3}H]*NBMPR binding assays.* Equilibrium binding assays (final volume 1 ml) were performed as previously described [17]. Briefly, assays were initiated by adding myocyte suspension (400 μl containing 30,000–60,000 cells) to assay mixtures containing appropriate concentrations of [^{3}H]NBMPR in the absence or presence of a specific inhibitor of nucleoside transport such as nitrobenzylthioguanosine (NBTGR, 10 μM) or dilazep (100 μM). Cells were incubated at 22° for 40 min which was sufficient time, as determined from time–course experiments (results not shown), to allow the binding reaction to attain equilibrium. The reaction was terminated by centrifugation (Eppendorf 5412 microcentrifuge for 15 sec). The surface of the cell pellets was washed once with ice-cold (1 ml) assay medium before processing for counting of [^{3}H]radioactivity by liquid scintillation spectrometry (Beckman LS6800).

Nucleoside transport measurements. All measurements of adenosine and 2-chloroadenosine fluxes were conducted at 22° in polypropylene microcentrifuge tubes (1.5 ml) as described previously [17]. Uptake intervals were initiated by the addition of a known concentration of either [^{3}H]adenosine or [^{3}H]2-chloroadenosine to myocyte suspensions (final concentration 200,000–500,000 cells per 0.45 ml assay) and subsequent rapid mixing. Uptake intervals were terminated by a "cold-inhibitor-stop" method by the addition of an ice-cold inhibitor solution containing 200 μM dilazep, 40 μM NBMPR and 1 mM substrate (adenosine or 2-chloroadenosine). Thereafter, the cells were immediately pelleted by centrifugation (15 sec in an Eppendorf 5412 microcentrifuge), washed once, digested with NCS tissue solubilizer (0.3 ml), and counted for [^{3}H]radioactivity by liquid scintillation spectrometry.

Rates of zero-*trans* influx of adenosine and 2-chloroadenosine were determined by incubating myocytes in [^{3}H]adenosine (10–400 μM) or [^{3}H]2-chloroadenosine (1–200 μM). Incubation intervals (15 sec), determined from time–courses of nucleoside uptake, were chosen in order that initial rates of permeant fluxes were measured. In addition, at the time intervals used, the intracellular concentration of the permeant did not exceed 20% of the corresponding extracellular concentration. Mediated nucleoside uptake was defined as the difference between total nucleoside uptake and the uptake of substrate in the presence of high concentrations of nucleoside transport inhibitors such as NBMPR (20 μM) or dilazep (100 μM). Transport constants (K_m and V_{max}) were calculated from linear regression analysis of v/s versus v plots of the mediated transport components.

Water space estimations. Internal water spaces of myocyte preparations were calculated as the difference between the total water space and sucrose space. These were determined by processing cell suspensions (as described above) with ^3H_2O and [^{14}C]sucrose respectively. The mean intracellular volume of the cells from guinea pig and rat heart was approximately 10 μl per million cells.

Radiochemicals and drugs. [G-^{3}H]Nitrobenzylthioinosine (sp. act. 37 Ci/mmole), [^{3}H]adenosine (sp. act. 17.8 Ci/mmole) and [^{3}H]2-chloroadenosine (sp. act. 9 Ci/mmole) were obtained from Moravek Biochemicals, California. Where necessary, these radiochemicals were purified to greater than 98% radiochemical purity by high performance liquid chromatography using a C_{18} μBondapak column eluted with methanol–water solutions. Dilazep was donated by Hoffman-La Roche. Nitrobenzylthioinosine and nitrobenzylthioguanosine were provided by Dr. A. R. P. Paterson, Cancer Research Unit (McEachern Laboratory), University of Alberta. Unlabeled adenosine, 2-chloroadenosine and dipyridamole were purchased from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

Binding of [^{3}H]*NBMPR.* The total amount of [^{3}H]NBMPR that became associated at equilibrium with guinea pig myocytes consisted of two components (Fig. 1), one of which was site-specific (inhibitible by nonisotopic NBMPR or other transport inhibitors) and saturable, whereas the other component (nonspecific binding) was proportional to the free [^{3}H]NBMPR concentration. Nonspecific binding was low (<5% of total at a K_D concentration) and was similar when estimated in the presence of either NBMPR (10 μM), dipyridamole (100 μM) or NBTGR (10 μM). Analysis of the inhibitible binding component by the method of Scatchard yielded a straight line plot (Fig. 1) which indicated that binding sites were of a single type. Also, Hill plots (data not shown) of the inhibitible binding component were linear and Hill coefficients (n_H) were not different from unity (1.00 ± 0.03, N = 6). The maximal binding capacity (B_{max} ± SE) and the apparent dissociation constant, K_D (95% confidence limits), of [^{3}H]NBMPR at these sites were 870,000 ± 36,000 sites/cell and 0.8 (0.6 to 1.0) nM. Endogenous adenosine may have been released from the myocytes during incubation. It is unlikely that concentrations were sufficient to influence [^{3}H]NBMPR binding because binding constants obtained from paired experiments in the presence or absence of adenosine deaminase were not significantly different.

The nucleosides, adenosine and 2-chloroadenosine, competitively inhibited the binding of [^{3}H]NBMPR to guinea pig cardiac myocytes with K_i values (95% confidence limits) of 318 (283–353) μM and 22 (4–42) μM, respectively (Fig. 2 and Table 1). It is conceivable that the apparent affinity of adenosine for these sites may have been reduced due to its metabolism by adenosine deaminase which may have leaked from the cells. However, in paired experiments, addition of the adenosine deaminase inhibitor, deoxycoformycin (100 nM), did not alter adenosine K_i values (316 μM control; 326 μM with deoxycoformycin). The nucleoside transport inhibitor, dipyridamole, competitively inhibited the site-specific binding of [^{3}H]NBMPR with a K_i value of 75 (44–132) nM. Binding was also competitively inhibited by other transport inhibitors such as dilazep and diazepam (data not shown).

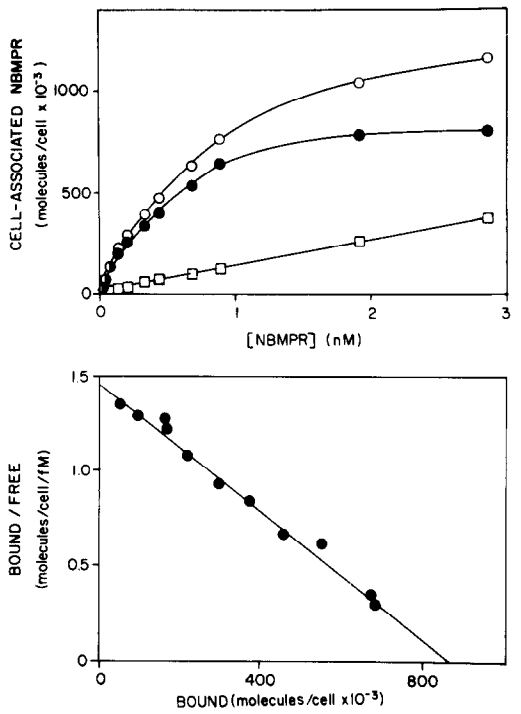

Fig. 1. Concentration dependence of the binding of [^{3}H]NBMPR to guinea pig cardiac myocytes (upper panel). Cells were incubated with graded concentrations of [^{3}H]NBMPR (abscissa) in the absence (total binding, ○) or presence (nonspecific binding, □) of 10 μM NBTGR for 40 min at 22°. Cell associated [^{3}H]NBMPR (ordinate) is expressed as molecules of NBMPR bound per cell. Specific binding (●), defined as total minus the nonspecific binding component, was saturable, whereas the nonspecific binding component was proportional to the [^{3}H]NBMPR concentration. Mass law analysis (lower panel) of the relationship between specific binding and equilibrium concentrations of free [^{3}H]NBMPR indicated that NBMPR bound to an apparent single class of sites. Ordinate: ratio of bound to free NBMPR (molecules/cell/fM); abscissa: site bound NBMPR (molecules/cell × 10^{-3}). Data in both panels represent values from a single experiment and are similar to six other experiments performed in duplicate. Average values for [^{3}H]NBMPR binding constants are reported in Table 1.

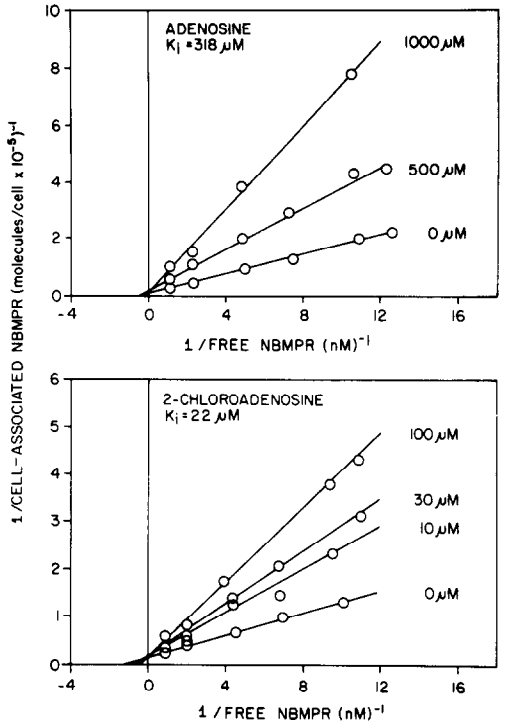

Fig. 2. Double-reciprocal plots of the inhibition of the site-specific binding of [^{3}H]NBMPR to guinea pig cardiac myocytes by adenosine (upper panel) or 2-chloroadenosine (lower panel). Two or three concentrations of each nucleoside were tested for their inhibition of the site-specific binding of [^{3}H]NBMPR at graded initial concentrations. Reciprocals of the final equilibrium concentrations (abscissa), represented by initial concentrations corrected for ligand depletion, were employed in calculation of K_i values. Inhibition by adenosine or 2-chloroadenosine was apparently competitive as plots of the reciprocals of site-bound NBMPR (ordinate) and the corresponding equilibrium concentration of NBMPR intersected on the ordinate. Plots in both panels represent data obtained from single experiments performed in duplicate. Average values of the inhibition constants are reported in Table 1.

Transport of [^{3}H]*adenosine and* [^{3}H]*2-chloroadenosine.* Time–courses of adenosine uptake were linear up to 90 sec for 1 μM adenosine, and up to 30 sec for 100 μM adenosine (Fig. 3). In addition, time–courses of 2-chloroadenosine uptake were linear up to 30 sec for both 1 and 100 μM concentrations. Substrate which became cell-associated in the presence of nucleoside transport inhibitor (the non-mediated component most likely representing passive diffusion) was linear and proportional to incubation time for intervals up to 1–2 min; thereafter its rate declined. The radioactivity derived from [^{3}H]adenosine (1 and 100 μM) concentrated approximately 6-fold and 2-fold, respectively, in the myocytes after 4 min of uptake. In contrast, cell-associated radioactivity derived from [^{3}H]2-chloroadenosine did not exceed significantly a tissue/medium ratio of unity during the time intervals (4 min) used in this study.

Zero-*trans* influx of adenosine and 2-chloroadenosine into guinea pig ventricular myocytes was found to be saturable (Fig. 4), and the transport of both permeants was inhibited by nucleoside transport inhibitors such as NBMPR or dilazep. The K_m (95% confidence limits) and V_{max} (mean ± SE) for adenosine influx were 146 (101–210) μM and 24.2 ± 1.4 pmoles/10^6 cells/sec. [^{3}H]2-Chloroadenosine had a higher affinity for the transport process with a K_m value of 36.2 (33.6 to 39.2) μM. However, it possessed a lower maximal velocity (11.7 ± 0.1 pmoles/10^6 cells/sec). NBMPR and dipyridamole inhibited the transport of [^{3}H]adenosine in a concentration-dependent manner (Fig. 5). NBMPR and dipyridamole also inhibited the transport of 2-chloroadenosine; their K_i values were similar to those found for their inhibition of adenosine transport (Table 1). In addition, over 90% of the transport activity was inhibited by NBMPR at concentrations of less than 20 nM. This contrasts with nucleoside transport in some other cell types, especially those of the rat, which exhibit a transport

Table 1. Comparison of the affinities of 2-chloroadenosine, adenosine, nitrobenzylthioinosine (NBMPR) and dipyridamole for the nucleoside transport system of guinea pig cardiac myocytes derived from [^{3}H]NBMPR binding studies and from transport studies utilizing [^{3}H]substrates

Drug	Binding	Transport
2-Chloroadenosine	22 (4–42) μM	36 (34–39) μM†
Adenosine	318 (283–353) μM	146 (101–210) μM†
NBMPR	0.8 (0.6–1.0) nM*	2.8 (1.1–7.6) nM
Dipyridamole	75 (44–132) nM	78 (60–101) nM

Values are geometric means (with 95% confidence limits) of three to six separate experiments performed in duplicate.

*,† Drug affinities for the nucleoside transporter are represented by $^*K_D$, $^\dagger K_m$ or K_i values as appropriate.

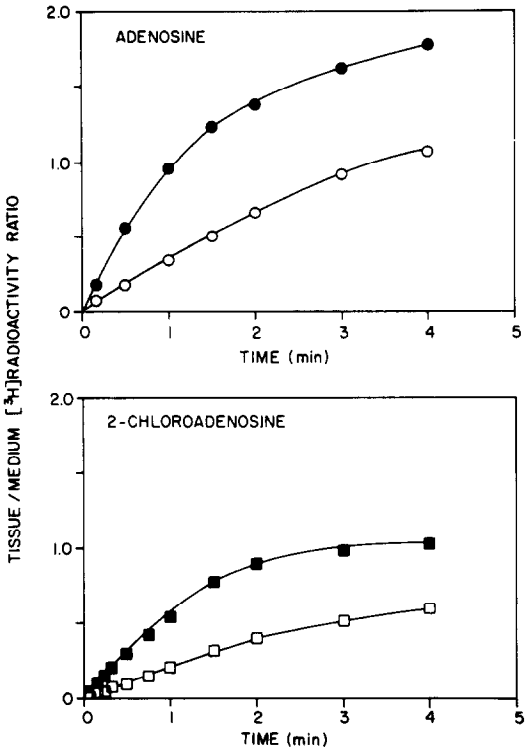

Fig. 3. Time–course of the uptake of [^{3}H]adenosine, 100 μM (upper panel), or [^{3}H]2-chloroadenosine, 100 μM (lower panel), into guinea pig cardiac myocytes at 22°. Cell-associated [^{3}H]radioactivity (ordinate) derived from [^{3}H]labeled substrate following a series of uptake intervals (abscissa) is expressed as a ratio to that present in the extracellular medium so that a tissue/medium ratio of unity represented equilibrium between intra- and extracellular tritium concentrations. Plots of total uptake (solid symbols) and the uptake which occurred in the presence of high concentrations of transport inhibitors (non-mediated component, open symbols) are included. For both substrates, reliable estimates of initial rates of uptake, representative of transport rates, could only be obtained for uptake intervals of less than 30 sec. These time–courses are for single concentrations of substrate (100 μM). Similar time–course experiments were performed with substrate concentrations over the range used in the determination of transport constants to ensure that reliable estimates of initial rates of uptake were obtained.

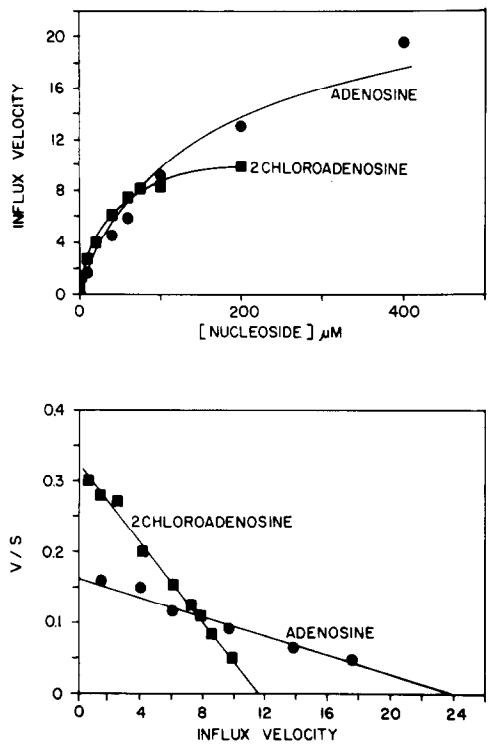

Fig. 4. Concentration dependence (upper panel) of the transport of [^{3}H]adenosine (●) and [^{3}H]2-chloroadenosine (■) into guinea pig cardiac myocytes at 22°. Influx velocities (pmoles/10^6 cells/sec) represent the initial rates of uptake (ordinate) of each substrate concentration (abscissa, μM) and were calculated as the difference between the total uptake and the uptake which occurred in the presence of high concentrations of nucleoside transport inhibitors (non-mediated component). Kinetic constants (K_m and V_{max}) were calculated from linear regression analysis of v/s (ordinate, pmoles/10^6 cells/sec/μM) versus v (abscissa, pmoles/10^6 cells/sec) plots (lower panel). The lines in each panel represent computer-generated values determined from the average kinetic constants of three separate experiments performed in duplicate. The points on each line are the mean values obtained from a representative experiment for each substrate.

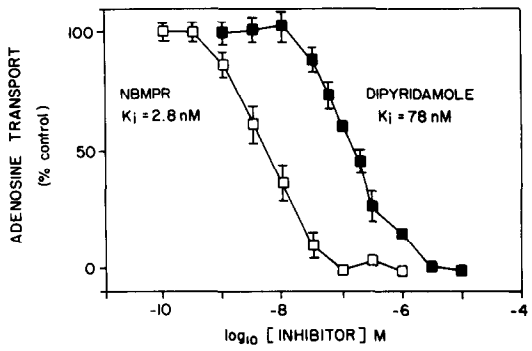

Fig. 5. Nucleoside transport inhibitor-induced inhibition of the transport of [^{3}H]adenosine by guinea pig cardiac myocytes. Adenosine transport (ordinate) represents the initial rate of inhibitible uptake of 100 μM adenosine in the presence of graded concentrations (abscissa) of NBMPR (□) or dipyridamole (■) which is expressed as a percentage of control. Inhibitible uptake was calculated as the difference between total uptake and the uptake which occurred in the presence of high concentrations of nucleoside transport inhibitors (non-mediated component). Inhibition constants were calculated from IC_{50} values from the relationship $K_i = IC_{50}/(1 + [S]/K_m)$ as described in Materials and Methods. Points represent mean values (±SE) from six separate experiments in which assay mixtures were in duplicate.

component that is only inhibitible by high concentrations (>10 μM) of NBMPR.

DISCUSSION

Adenosine and 2-chloroadenosine are both substrates for the nucleoside transport system in guinea pig myocytes. That these compounds interact with the nucleoside transporter was demonstrated both indirectly by analysis of the inhibition of the site-specific binding of the transporter ligand [^{3}H]NBMPR and directly by measuring initial rates of nucleoside influx. Only a single component of adenosine or 2-chloroadenosine transport was observed. Also, as NBMPR and dipyridamole inhibited the transport of both substrates with similar potencies, it appears that both nucleosides are transported by the same process.

NBMPR has been used widely as a probe for the nucleoside transport system in a variety of cultured cell types and membrane preparations [5, 18–21]. Based on several criteria, including orders of potency of adenosine and analogues and adenosine receptor antagonists, regional distributions, etc., it is clear that NBMPR sites are distinct from those for adenosine receptors [17, 22]. In guinea pig cardiac membranes NBMPR binds with high affinity [16], and it is inhibited by several recognized nucleoside transport inhibitors and by nucleosides. Based on extensive evidence from binding and transport studies in erythrocytes [17, 23–25], it was assumed that NBMPR also bound to transport inhibitory elements of the nucleoside transporter in heart. However, this study permitted the direct comparison of drug affinities for the NBMPR binding site with drug affinities for the nucleoside transport process. Good correlations exist between drug affinities determined by the two methods (Table 1), and this confirms that NBMPR sites in heart are closely associated with the membrane-located transporter. 2-Chloroadenosine has a higher affinity than adenosine for NBMPR sites in guinea pig myocytes. This represents a significant interaction, but it does not indicate whether 2-chloroadenosine is a substrate or inhibitor of the transporter. Definitive evidence that both adenosine and 2-chloroadenosine are substrates for the cardiac nucleoside transporter was obtained by measuring nucleoside fluxes directly.

It is well established that adenosine is a substrate for the nucleoside uptake process in cardiac tissue [16, 26–28]. However, it should be recognized that the cellular accumulation of radioactivity following incubation or perfusion of a tissue with radiolabeled permeant may be due to the combination of several processes in addition to that of membrane transport. Observed rates of cellular uptake (other than initial rates) may reflect entry by passive diffusion, metabolic trapping by conversion of adenosine to impermeable phosphates, and efflux of deaminated products such as inosine or hypoxanthine. Only initial rates of mediated nucleoside uptake measure transport [5]. Time–courses of adenosine uptake in myocytes indicated that equilibration between intra- and extracellular concentrations occurred rapidly (within 1 min). Consequently, in this study care was taken to ensure that initial rates of nucleoside uptake, representative of transport rates, were measured. This necessitated the use of short transport intervals (15 sec). At this time, the intracellular concentration of radiolabeled nucleoside did not exceed 20% of the corresponding extracellular concentration; consequently, rates which approximated initial rates of nucleoside uptake were measured. Both adenosine and 2-chloroadenosine entered cardiac cells by a saturable and inhibitible process. 2-Chloroadenosine had a higher affinity for the transport process, and the K_m value for transport (36 μM) is in close agreement with its K_i value (22 μM) derived from binding experiments. The affinity of adenosine for the transport process (146 μM) is in close agreement with recent initial rate studies of adenosine transport in various cell types [14, 29]. Other studies [27, 30] have reported higher affinities (1–10 μM) for adenosine, but these employed longer incubation intervals and no doubt measured the net effect of transport and subsequent intracellular metabolism by adenosine kinase (K_m of 0.5 to 5 μM). Adenosine kinase most likely was responsible for the apparent concentrating effect seen at the longer incubation intervals (greater than 60 sec) used in the present experiments. This resulted from the intracellular trapping of radiolabel due to the conversion of adenosine to impermeable adenine nucleotides. The role of adenosine kinase is confirmed by the demonstration that radiolabel derived from low concentrations of adenosine (1 μM) close to the K_m for adenosine kinase concentrated approximately 6-fold,

whereas higher concentration (100 μM), which would have saturated the enzyme, concentrated to a significantly lesser extent. Although it is unclear whether 2-chloroadenosine is a substrate for phosphorylation by cardiac adenosine kinase, we failed to show any concentrating effect with 1 μM or 100 μM 2-chloroadenosine. However, other workers [31] have shown that it is a substrate for adenosine kinase (and other kinases) in cultured human splenic B lymphoid cells.

The demonstration that 2-chloroadenosine and adenosine both enter heart cells by a saturable process, which is inhibited by agents such as NBMPR or dipyridamole, whereas only the actions of adenosine are potentiated by nucleoside transport inhibitors, would appear to raise doubts concerning the mechanism of transport inhibitor-induced potentiation of adenosine actions. However, using the transport kinetic constants obtained for these substrates and at the IC_{50} concentrations of 2-chloroadenosine and adenosine for the reduction of isometric tension of electrically driven guinea pig left atria (0.05 and 191 μM respectively [10], the velocity of inhibitable influx (transport) for these agonists can be calculated to be approximately 0.016 pmoles 2-chloroadenosine/10^6 cells/sec and 13.6 pmoles adenosine/10^6 cells/sec. This indicates that the velocity of adenosine transport would be approximately 850-fold greater than that of 2-chloroadenosine at their respective equi-effective concentrations in heart tissue. This difference may be further augmented at prolonged incubation intervals (greater than 60 sec) used in contractility studies, where total inhibitible entry of adenosine may be additionally enhanced due to the apparent concentrating effect due to metabolic conversion intracellularly to impermeable nucleotides by adenosine kinase or to inosine by adenosine deaminase. 2-Chloroadenosine is resistant to deamination and a poor substrate for adenosine kinase and so would be expected to undergo more limited removal than adenosine from the vicinity of its receptors. Thus, in experimental systems (e.g. heart) where 2-chloroadenosine possesses a higher potency than adenosine, the total removal of nucleoside from the vicinity of adenosine receptors by the combined effects of transport and subsequent metabolism is lower for 2-chloroadenosine than for adenosine. Therefore, it should not be unexpected that nucleoside transport inhibitors exert a greater potentiation of the effects of adenosine than those of 2-chloroadenosine. Interestingly, calculation of the influx velocities of adenosine and 2-chloroadenosine at comparable concentrations in the range zero to 70 μM reveal that the influx velocity of 2-chloroadenosine is slightly greater than that of adenosine.

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