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STERIC REQUIREMENTS FOR BINDING OF ADENOSINE TO A MEMBRANE CARRIER IN CANINE HEART*

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SUMMARY

The steric requirements for the binding of adenosine to its putative membrane carrier in dog hearts were studied by testing the ability of adenosine analogs infused intracoronary to inhibit the uptake of [8-^{14}C]adenosine. The affinity of adenosine for the carrier appears to depend on the purinyl 6-amino group, the 2′- and 3′-hydroxyls, and the *anti* conformation at the glycosidic bond. There is very little bulk tolerance at the site of attachment of the sugar hydroxyls. The interaction of adenosine and its carrier may be an example of active site-directed specificity.

INTRODUCTION

There is considerable evidence that coronary vascular tone may be regulated primarily by the concentration of adenosine in the cardiac interstitial space[1,2]. This nucleoside, which is a potent coronary vasodilator, is present in oxygenated cardiac muscle, its concentration rising rapidly during myocardial ischemia[2,3]. Adenosine released into the cardiac interstitial space is rapidly taken up and phosphorylated by cardiac muscle cells[4,5], the rate being sufficiently rapid to constitute an important mechanism for regulating the adenosine concentration in the interstitial space[6]. Although the mechanism of adenosine uptake in mammalian myocardium has not been conclusively identified, the available evidence[6,7] strongly suggests that it is facilitated diffusion, the mechanism unequivocally identified in other types of cells[8–12]. Adenosine uptake in dog heart appears to follow Michaelis–Menten kinetics, having values for K_m [11.6±1.4 (S.E.) μM] and V [4.9±0.5 (S.E.) nmoles·min^{-1}·g left ventricle^{-1}] substantially different from those of either adenosine kinase (ATP: adenosine-5′-phosphotransferase, EC 2.7.1.20; K_m, 0.4 μM; V, 23 nmoles·min^{-1}·g left ventricle^{-1}) or adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4; K_m, 43 μM; V, 1.2 μmoles·min^{-1}·g left ventricle^{-1})[6]. Adenosine uptake is inhibited

* In conducting the research described in this report, the investigators adhered to the *Guide for Laboratory Animal Facilities and Care*, as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

by 5 μM dipyridamole and 10 μM 6-(*p*-nitrobenzylthio)guanosine, but neither compound inhibits either adenosine kinase or deaminase at these concentrations under the conditions described in ref. 6. Ouabain does not inhibit adenosine uptake. Although these findings tend to exclude uptake by simple diffusion or *via* a Na^+-dependent carrier, it has not been possible to demonstrate countertransport of adenosine, probably because this nucleoside is immediately metabolized upon entry into the cell, so that there is no intracellular pool available for exchange[6]. In guinea pig heart, adenosine uptake also follows Michaelis–Menten kinetics, is strongly temperature dependent, and is inhibited by dipyridamole[7]. The K_m and V of uptake for guinea pig heart, 1 μM and 4.5 nmoles$\cdot$min$^{-1}\cdot$g^{-1}, were different from the corresponding values in rat heart, 5 μM and 12 nmoles$\cdot$min$^{-1}\cdot$g^{-1}, respectively. The differences in uptake parameters between dog, guinea pig, and rat, as well as the failure of dipyridamole to inhibit adenosine uptake in rat heart[7] suggest that there are quantitative and perhaps also qualitative species differences in adenosine uptake.

Taube and Berlin[8] have found that purine and pyrimidine nucleosides are transported in rabbit polymorphonuclear leukocytes by a common carrier and that the pyrimidine moiety of the base and the sugar 3′-hydroxyl are the most critical structural features for binding to this carrier. Transport of nucleosides *via* a common carrier is not a general finding, however, for Plagemann[10] and Scholtissek[11] have presented evidence that in Novikoff hepatoma cells and chicken fibroblasts, respectively, there are multiple nucleoside carrier systems, each relatively specific for its own nucleoside.

Because of the species differences in the kinetics of adenosine uptake and the heterogeneity of uptake systems in other cell types, the structural requirements for binding of this nucleoside to the putative carrier in dog heart sarcolemma were determined and are the subject of this report. Selectively substituted adenosine analogs were tested for the ability, when infused intracoronary, to inhibit myocardial adenosine uptake. These analogs were either 6- and/or 8-substituted purine ribofuranosides or were adenine furanosides of sugars differing from ribose by single alterations at either the anomeric carbon or one of the epimeric carbons. Inhibition of adenosine uptake by one of these nucleosides was interpreted as evidence that the analog being tested possessed the essential features for binding to the carrier, whereas failure to inhibit adenosine uptake was interpreted as evidence that the site which had been selectively altered was not involved in the interaction of the nucleoside and the adenosine carrier.

MATERIALS AND METHODS

Nucleosides* were obtained from the following commercial sources: Sigma Chemical Co. (adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 6-mercaptopurine riboside, 6-methylmercaptopurine riboside); Calbiochem Corp. (inosine); P-L Biochemicals (adenine arabinoside); Aldrich Chemical Co. (8-bromoadenosine); Zellstoffabrik Waldhof GmbH (Mannheim) (6-methylaminopurine riboside); Terra-Marine

* In order to avoid ambiguity, the structural formulae of the nucleosides studied are shown in Fig. 1. The trivial name adenine lyxoside refers to adenine-9-α-L-lyxofuranoside and psicofuranine refers to adenine-9-β-D-ribo-hex-2-ulofuranoside. The remainder of the compounds are all 9(H)-purinyl-β-D-pentofuranosides.

Bioresearch, Inc. (adenine lyxoside); New England Nuclear Corp. ([8-^{14}C]adenosine). A generous supply of psicofuranine was a gift of Dr G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich., and adenine xylofuranoside was obtained from Dr H. B. Wood Jr of the Cancer Chemotherapy National Service Center, Bethesda, Md. The following compounds were synthesized by the methods cited: 2′-*O*- and 3′-*O*-methyladenosine[13,14], 5′-deoxyadenosine[15], and 5′-chloro-5′-deoxyadenosine[16]. The purity of the nucleosides obtained commercially was checked by thin-layer chromatography on silica gel IB-F (J. T. Baker Co.) in two solvent systems: butanol–water–ammonia (84:16:5, v/v/v) and isopropanol–acetic acid–water (5:2:3, v/v/v). The compounds synthesized in this laboratory were similarly checked for purity and found chromatographically homogeneous and were further characterized by melting point, spectral maxima, and optical rotation, all of which closely agreed with literature values.

Inhibiting effects of nucleosides on adenosine uptake

Beagle dogs of either sex, weighing 9–15 kg were used. Preparation of the animals, insertion of various devices and measurements made were otherwise the same as previously described[6]. Briefly, the experimental preparation consisted of a pentobarbital-anesthetized, open-chest dog whose coronary artery was cannulated and perfused by blood from the left common carotid artery. Coronary flow and perfusion pressure were measured with an electromagnetic flowmeter and a strain gauge manometer attached to the perfusion line. Catheters inserted into the femoral artery and coronary sinus were used to obtain blood samples.

[^{14}C]Adenosine uptake rate, v, in moles·min^{-1}·g left ventricle^{-1}, was calculated as the difference between the rate of adenosine infusion and the product of coronary blood flow rate and coronary sinus radioactivity corrected for recirculation by the formula

$$v = \frac{C_i}{A} \cdot \frac{AV - (\mathrm{CBF} \cdot \mathrm{CVR})}{\mathrm{LVW}} \tag{1}$$

where C_i=adenosine concentration of infusate (moles/l), A=activity of infusate (dpm/ml), V=adenosine infusion rate (l/min), CBF=coronary blood flow rate (l/min), CVR=corrected coronary venous radioactivity (coronary sinus radioactivity *minus* arterial blood radioactivity in dpm/ml), and LVW=left ventricle weight (g). The concentration of adenosine in coronary plasma water during adenosine infusion, C, in moles/l, was calculated by the formula

$$C = \frac{C_i \cdot V}{\mathrm{CBF} \cdot (1 - \mathrm{Hct}) \cdot 0.92} \tag{2}$$

where Hct=hematocrit (ml/100 ml) and 0.92=plasma water content (l of water/l of plasma). The estimated K_m and V for adenosine uptake were 11.6±1.4 (S.E.) μM and 4.9±0.5 (S.E.) nmoles·min^{-1}·g left ventricle^{-1}, respectively.

Aqueous solutions of the nucleosides to be tested (5–30 mM) were infused into the coronary perfusion line at rates of 0.1–1.0 ml/min. Whenever possible, the test nucleosides were infused at rates which caused inhibition of adenosine uptake. This aim was not always achieved. The solubility of 8-bromoadenosine, uridine, adenine

arabinoside, and adenine xyloside limited the coronary plasma concentrations which could be achieved by infusion at 1 ml/min, the highest rate at which distilled water, the solvent, could be infused without causing coronary vasodilation. The infusion of test substance was continued for several minutes in order to observe any effect on coronary blood flow rate, after which coronary blood flow rate was adjusted to control levels for the remainder of the experiment. After 5 min of infusion of the test substance, adenosine uptake studies were performed as previously described[6], adenosine being infused at five increasing rates.

The inhibitory effects of the test nucleosides were evaluated by calculating a K_i for each experimental point in an individual animal by the formula

$$K_i = \frac{[I]\,(1-i)}{i\left(1+\frac{[S]}{K_m}\right)}, \text{ where } i = 1 - \left(\frac{v_i}{v}\right). \tag{3}$$

In this formula, K_m is the apparent Michaelis constant for adenosine uptake, $[I]$ and $[S]$ are the concentrations of inhibitor and substrate, and v_i and v are the velocities of uptake in the presence and absence of inhibitor, respectively. The values for each animal were averaged, and these average values were used to calculate a mean and standard error for all the animals in that group. The use of this formula implies that inhibition is of the competitive type; this was confirmed for those compounds showing significant inhibition by double reciprocal plots (Fig. 2).

Because the value of K_i is strongly dependent on the value of i, estimates of the inhibition constant tend to be inaccurate when inhibition is weak, *i.e.*, when the difference between v and v_i is small. As this was frequently the case in this study, the degree of inhibition was evaluated independently by comparing v_i at an adenosine concentration of 5 μM, estimated by interpolation as shown in Fig. 2, with the rate of adenosine uptake expected in the absence of inhibitors. This particular adenosine concentration was chosen because adenosine concentrations within 0.5 μM of this value were common to all experiments, thus minimizing the error of interpolation. The uptake rate thus estimated, v_i, was compared with a similarly derived value for adenosine uptake in the absence of a second nucleoside by means of the Student's t test for unpaired samples.

RESULTS

Effect of nucleosides on adenosine uptake

Data on the effects of other nucleosides on adenosine uptake are given in Table I. Generally the two methods used to evaluate the inhibitory effect of a nucleoside agreed. As shown in the table, those compounds which significantly inhibited uptake at an adenosine concentration of 5 μM also had relatively low values for K_i. Substitution of a methyl group for one of the 6-amino hydrogens of adenosine caused only a relatively small loss of affinity, whereas substitution of other types of chemical groups in the purinyl-6 position caused a substantial loss of affinity. Even though 8-bromoadenosine has both a 6-amino group and is a riboside, it had the least affinity

TABLE I

EFFECT OF NUCLEOSIDES ON CARDIAC ADENOSINE UPTAKE

The uptake of adenosine infused into the coronary artery was estimated as described in the text. Rates of adenosine uptake (mean ± S.E.) in the presence of inhibitor, v_i, which are significantly lower than the rate of adenosine uptake in the absence of a second nucleoside ($P < 0.05$) are marked by an asterisk. Data on adenosine uptake are included for comparison, with the value for K_m listed in the column under K_i.

Compound	*Number of dogs*	*CPW Ado** (μM)	*Adenosine uptake* (v_i) ($nmoles \cdot min^{-1} \cdot g^{-1}$)	K_i (μM)
Purine ribosides				
Adenosine	4	—	1.43 ± 0.08	11.6** ± 1.4
6-Methylaminopurine riboside	4	60	0.81 ± 0.18*	54 ± 8.0
6-Methylmercaptopurine riboside	4	450	0.99 ± 0.19*	682 ± 85
6-Mercaptopurine riboside	4	401	0.78 ± 0.05*	329 ± 80
Inosine	4	417	0.96 ± 0.11*	583 ± 65
8-Bromoadenosine	4	285	1.17 ± 0.16	1280 ± 990
Pyrimidine ribosides				
Cytidine	4	492	1.47 ± 0.11	∞
Uridine	2	347	1.50 ± 0.18	∞
Adenine nucleosides				
2′-Deoxyadenosine	4	1029	0.66 ± 0.11	609 ± 53
Adenine arabinoside	4	301	1.42 ± 0.40	21 200 ± 25 600***
2′-*O*-Methyladenosine	4	462	1.29 ± 0.14	2924 ± 1 750
Psicofuranine	4	403	1.30 ± 0.12	2600 ± 350
3′-Deoxyadenosine	4	426	1.00 ± 0.09*	676 ± 60
Adenine xylofuranoside	4	385	1.45 ± 0.15	∞
3′-*O*-Methyladenosine	4	552	1.50 ± 0.10	∞
Adenine lyxoside	4	670	0.93 ± 0.22	670 ± 175
5′-Deoxyadenosine	4	233	0.71 ± 0.18*	159 ± 20
5′-Chloro-5′-deoxyadenosine	4	309	1.12 ± 0.09	745 ± 675

* Adenosine concentration in coronary plasma water.

** K_m (ref. 6).

*** The unusually large standard error for the estimate of K_i in the case of adenine arabinoside is due to the very small differences between v_i and the uninhibited rate of uptake.

for the carrier of any of the ribosides tested. The two pyrimidine nucleosides tested, uridine and cytidine, had no affinity for the carrier.

Alterations of the sugar moiety involving the 2′- and 3′-carbons were associated with substantial to essentially complete loss of the capacity to interact with the carrier. Both 2′- and 3′-deoxyadenosine had a moderate affinity for the carrier. The 2′- and 3′-epimers and *O*-methyl ethers had either little or none, reducing v_i insignificantly. The values of K_i calculated for these compounds do not show a significant difference between alterations at the 2′- or 3′-position. The affinity of psicofuranine, which has a bulky carbinol group *cis*-vicinal to the 3′-hydroxyl (which corresponds to the 2′-hydroxyl of adenosine) was substantially reduced, presumably because this

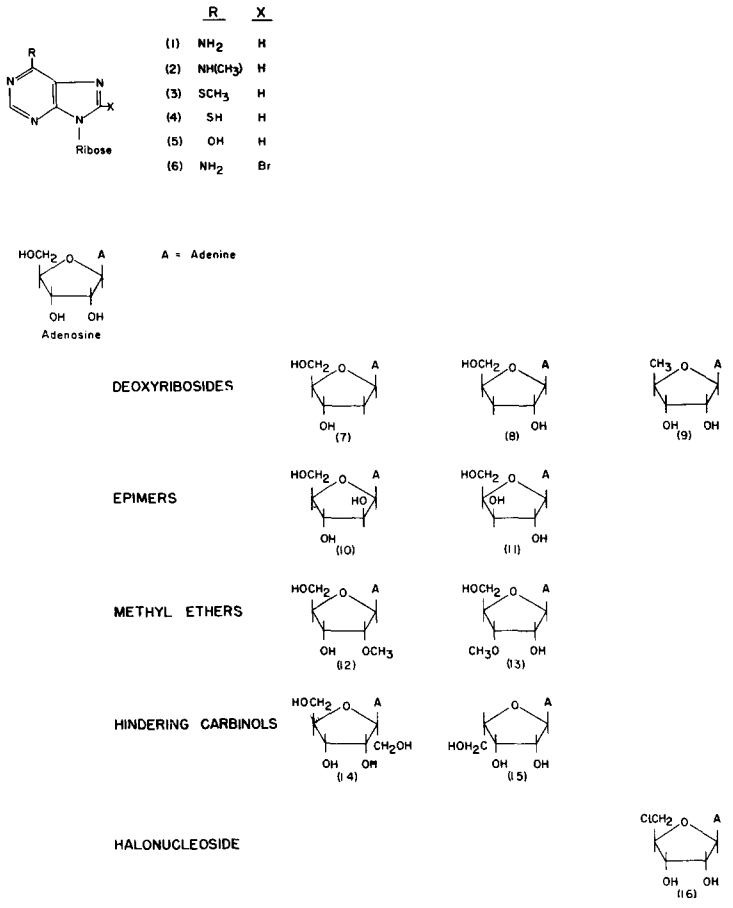

Fig. 1. Purine nucleosides examined for an effect on myocardial adenosine uptake. 1, Adenosine; 2, 6-methylaminopurine riboside; 3, 6-methylthiopurine riboside; 4, 6-mercaptopurine riboside; 5, inosine; 6, 8-bromoadenosine; 7, 2′-deoxyadenosine; 8, 3′-deoxyadenosine; 9, 5′-deoxyadenosine; 10, adenine arabinoside; 11, adenine xylofuranoside; 12, 2′-*O*-methyladenosine; 13, 3′-*O*-methyladenosine; 14, psicofuranine; 15, adenine lyxoside; and 16, 5′-chloro-5′-deoxyadenosine.

group interfered with the ability of the 3′-hydroxyl to interact with the binding site. By contrast, adenine lyxoside, in which the 5′-carbinol is *cis*-vicinal to the 3′-hydroxyl, inhibited adenosine uptake relatively well. Although v_i was not significantly lower than control ($P \approx 0.06$), the K_i value, based on a greater number of experimental points, was significantly lower than the K_i of psicofuranine. Reduction of the 5′-hydroxyl produced the least interference with binding of all the sugar alterations, whereas substitution of chloride for the 5′-hydroxyl appeared to cause a moderate loss of activity.

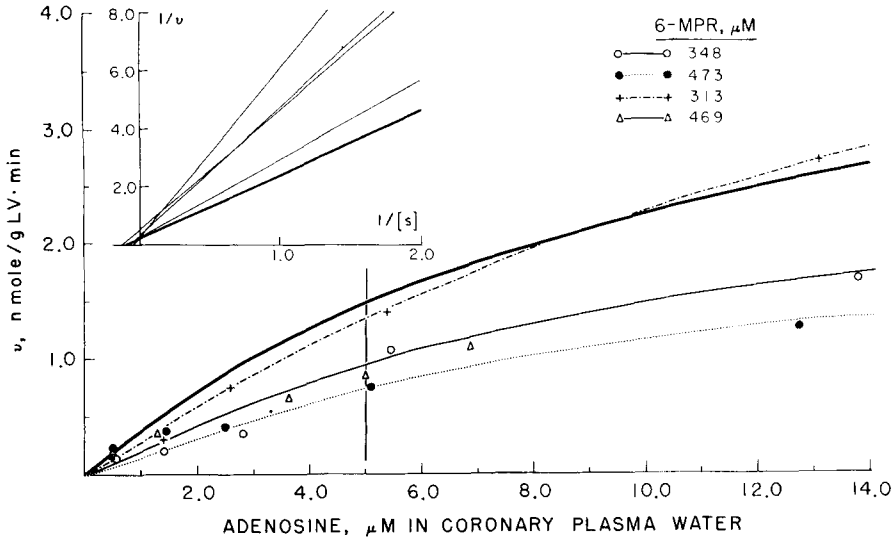

Fig. 2. Effects of intracoronary infusions of 6-mercaptopurine riboside (6-MPR) on the rate of myocardial uptake of adenosine. Each curve represents the results from one dog, except that in the experiments described by (○) and (△) the results were closely similar and so are represented by a single line. Also illustrated is the method of estimating the rate of adenosine uptake at a concentration of 5 μM in coronary plasma water for use in the independent method of evaluating the degree of inhibition produced by a test nucleoside (see text for details). Insert: Least squares regression lines for $1/v$ *vs* $1/[S]$ for each of these experiments. Individual experimental points are omitted for clarity. The correlation coefficients averaged +0.967 (range +0.933 to +0.992). The average value of V in the presence of inhibitor was 3.4 ± 0.4 (S.E.) nmole·min^{-1}·g left ventricle (LV)$^{-1}$, which was not significantly different from V in the absence of an inhibitor[6]. For comparison the average of data derived from a separate group of dogs not treated with a test nucleoside is depicted as a heavy line in each figure.

DISCUSSION

These results suggest that if adenosine uptake by dog heart does in fact occur *via* a carrier-mediated system, the binding of adenosine to the putative adenosine carrier of the cardiac sarcolemma requires the purine 6-amino group and the *erythro* configuration of the sugar moiety.

Substitution of a methyl group for one of the 6-amino hydrogens led to only a slight loss of affinity. If, however, the amino group was replaced by a thio, keto, or methylthio group, the affinity was lowered considerably. This study does not identify which property of the amino group is important for binding to the membrane carrier. Neither of the pyrimidine nucleosides tested had any demonstrable effect on adenosine uptake, suggesting that purine and pyrimidine nucleosides do not share a common carrier in dog heart, as they do in rabbit leukocytes[8].

The *erythro* configuration of the 2′- and 3′-hydroxyls, *i.e.* their orientation below the plane of the furanose ring, is an important determinant of binding to the putative adenosine carrier. This study suggests that both hydroxyls are equally important, as the effect of the same type of alterations at either C-2′ or C-3′ are quantitatively similar. The values of K_i given in the table reflect statistically insignificant differences in the rate of uptake rather than differences in affinity for the carrier.

Thus, both 2′- and 3′-deoxyadenosine inhibited adenosine uptake, having K_i values 53 and 58 times the K_m for adenosine. However, the corresponding epimers had virtually no affinity for the carrier. It is unlikely that this is due simply to the loss of the contribution of the epimeric hydroxyl to binding, for one would then expect a K_i similar to that of the corresponding deoxyriboside. Rather, this may indicate that there is no bulk tolerance at that portion of the binding site at which the *cis*-diol attaches. The low affinities of psicofuranine and the 2′-*O*- and 3′-*O*-methyl ethers is additional evidence for dimensionally restricted access to the binding site. The relatively high affinity of adenine lyxoside (K_i of 670 μM) suggests that there may be somewhat more room to accomodate bulk in the vicinity of C-4′. The affinity of 5′-deoxyadenosine for the adenosine carrier was greater than that of any other adenine nucleoside, and the affinity of 5′-chloro-5′-deoxyadenosine was only moderately decreased despite the marked chemical differences between a hydroxyl group and a chlorine atom. These findings suggest that the 5′-hydroxyl is relatively less important than the secondary hydroxyls for binding. Unfortunately, additional C-5′-substituted adenine nucleosides were not available for a more complete evaluation.

The capacity of adenosine to interact with a membrane carrier may depend on the conformation of the nucleoside molecule as well as on the presence and configuration of its various functional groups. The shape of a purine nucleoside molecule in solution differs greatly from the shape and rigidity implied in the usual two-dimensional chemical diagram. For example, the base and ribose moieties are not coplanar but are oriented nearly perpendicular to each other. In addition, rotation about exocyclic bonds, particularly the N-9–C-1′ glycosidic bond, as well as puckering of the furanose ring could change the spatial orientation of the functional groups which bind to the carrier. The present study establishes that at least one of these conformational effects may be a determinant of the binding of adenosine to the putative membrane carrier.

Rotation of the purinyl moiety about the axis of the glycosidic bond results in either a *syn* or an *anti* conformation of the nucleoside molecule (Fig. 3). Apparently both forms of the adenosine molecule can exist in solution, as the energy barriers to rotation are not large[17], molecules in the *anti* conformation appearing to predominate[18]. The substitution of a bulky bromine atom on C-8 restricts rotation

Fig. 3. Effect of rotation about the glycosidic bond, a conformational effect which may influence the shape of a nucleoside molecule in solution and, thereby, its biological activity. Adenosine, at the left, is believed to exist mainly in the *anti* conformation. The bulky bromine atom in the 8-position of 8-bromoadenosine, right, restricts rotation about the glycosidic bond by interacting with C-2′, so that the favored conformation is *syn*.

mainly to the *syn* range[19], with a resultant loss of biological activity[20,21]. In the present study, 8-bromoadenosine was found to have a low affinity for the adenosine carrier. This lack of biological activity is interpreted as a conformational effect, for the molecule has all of the other chemical and steric features, namely the 6-amino group and the *erythro* configuration of the 2′- and 3′-hydroxyls, which appear to be important in the interaction of adenosine with its carrier.

The importance of the 2′- and 3′-hydroxyls of adenosine for binding to its membrane carrier in dog heart may represent an example of active site-directed specificity of the adenosine carrier. Berlin[22] has proposed that a principal requirement for binding to a carrier is "... that part of the substrate immediately adjacent to and including the functional group that will subsequently undergo enzymatic attack (this part of the substrate is referred to as its active site)". According to this concept, there is a degree of complementarity between the requirements for binding of a transported species to its carrier and its subsequent binding to intracellular enzymes. The metabolism of adenosine in the cardiac cell is critically dependent on the 5′-hydroxyl. Once adenosine enters the myocardial cell, it may either be enzymatically deaminated or phosphorylated, and it is now quite clear that the latter is the major metabolic pathway[4–6]. Adenosine kinase phosphorylates adenosine in the 5′-position (indeed, mammalian kinases capable of phosphorylating nucleosides in the 2′- or 3′-position are unknown), and further, the 5′-hydroxyl of adenosine is a critical requirement for binding to adenosine deaminase[23]. Thus, the importance of the contiguous 2′- and 3′-hydroxyls of ribose for binding to the carrier seems to be consistent with the principle of active site-directed specificity.

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