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2-CHLOROADENOSINE, A PERMEANT FOR THE NUCLEOSIDE TRANSPORTER

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Abstract—Human erythrocytes were shown to possess a saturable uptake mechanism for 2-chloroadenosine (apparent K_m 23 μM, 22°). Uptake by this route was inhibited by nitrobenzylthioinosine, uridine and adenosine, but adenine had no effect. In addition, uridine caused the countertransport of 2-chloroadenosine and vice versa. 2-Chloroadenosine was also shown to be an apparent competitive inhibitor of uridine influx (apparent K_i value of 33 μM) and high-affinity nitrobenzylthioinosine binding (apparent K_i 0.18 mM). The apparent K_i value for inhibition of uridine influx was close to the apparent K_m value for 2-chloroadenosine uptake. Previous studies [Jarvis *et al.*, *Biochem. J.* **208**, 83 (1982)] have demonstrated that dog erythrocytes do not possess a saturable transport system for uridine and adenosine. Similarly, in the present study, the entry of 2-chloroadenosine into dog erythrocytes was slow and linear with concentration. Nitrobenzylthioinosine (NBMPR) had no effect on the uptake of 2-chloroadenosine into dog erythrocytes. These results demonstrate that 2-chloroadenosine enters human erythrocytes by the same nucleoside carrier as other nucleosides. It is suggested from these data that the previous explanation that the inability of nucleoside transport inhibitors to potentiate the pharmacological effects of 2-chloroadenosine was due to the failure of the nucleoside carrier to accept 2-chloroadenosine as a permeant may have to be reassessed.

In the past decade, a substantial amount of data has been collected suggesting a number of physiological roles for adenosine in various systems. For example, adenosine is believed to act as a neuroregulator and as a neurotransmitter in the central and peripheral nervous systems, to influence cardiac and renal function, and to regulate blood flow in heart, brain and skeletal muscle (for a recent review, see Ref. 1). Many of these actions of adenosine are probably mediated by specific receptors, and the action of adenosine with its receptor is terminated by the rapid removal of adenosine into surrounding cells by a nucleoside transport system [1]. Drugs which inhibit the transport of nucleosides across cell membranes have been demonstrated to potentiate the effects of adenosine especially with regard to the relaxation of smooth muscle [2–10]. However, relaxations caused by the potent adenosine analog, 2-chloroadenosine, which binds to adenosine receptors remained unaffected in the presence of nucleoside transport inhibitors, leading to the suggestion that 2-chloroadenosine is not a substrate for the nucleoside transport system [4, 6, 9]. As a result it is now generally assumed that 2-chloroadenosine is not a permeant for the adenosine transporter. Indeed, at a recent symposium on adenosine derivatives, a number of workers stated that it was known that 2-chloroadenosine was not taken up by tissues [11]. However, it has not formally been demonstrated that the nucleoside transporter is unable to accept 2-chloroadenosine as a substrate.

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Human erythrocytes possess a saturable, broad specificity, facilitated diffusion system for the transport of nucleosides across their cell membrane [12, 13]. The properties of this system are well defined and similar to those in many other animal cells (for reviews see Refs. 14–16) and, therefore, in this study the erythrocyte was used as a model system to investigate whether 2-chloroadenosine is a transported substrate for the nucleoside transporter. The interaction of 2-chloroadenosine with the nucleoside transporter was studied by indirect and direct methods, and the results from both experimental approaches are consistent with the view that this nucleoside is accepted by the carrier as a transported permeant.

MATERIALS AND METHODS

Erythrocytes. Blood from healthy human volunteers and dogs was collected into heparinized (Vacutainer) tubes and centrifuged at 1000 *g* for 10 min. The plasma and buffy coats were discarded, and the erythrocytes were washed three times with 20 vol. of a medium containing 140 mM NaCl, 5 mM KCl, 20 mM Tris–HCl (pH 7.4 at 22°), 2 mM $MgCl_2$ and 0.1 mM EDTA (disodium salt). Hemoglobin-free "ghosts" were prepared as described previously [17].

Nucleoside transport. The influx of nucleosides at 22° was measured by mixing 0.2-ml portions of cell suspension (hematocrit 20%) with 0.2 ml of medium containing the appropriate concentration of radioactive nucleoside. In inhibition studies, test compounds and radioactive nucleoside were added simultaneously. At specified time intervals, cells were

collected from incubation mixtures by one of two methods. In the first, used in measuring the slow uptake fluxes in dog erythrocytes, 1-ml portions of ice-cold medium were added to incubation mixtures (after uptake intervals of 0.5 to 30 min), and cells were pelleted and then rapidly washed four times with 1-ml ice-cold portions of medium, using a microcentrifuge (10 sec, 12,000 *g*). Previous control experiments had established that this washing procedure removed extracellular labeled permeant without significant loss of radioactivity from cells with slow nucleoside uptake rates [18]. For more rapid fluxes (2–30 sec), nucleoside uptake was terminated by an "inhibitor-oil-stop" method. Portions of the incubation medium (0.2 ml) were added to 0.8 ml of ice-cold "stopper" medium [containing 10 μM nitrobenzylthioguanosine (NBTGR), a potent inhibitor of nucleoside transport] layered on top of ice-cold di-*n*-butyl phthalate contained in 1.5-ml microcentrifuge tubes. Tubes were immediately centrifuged (12,000 *g*, 10 sec), and the aqueous and di-*n*-butyl phthalate layers were removed by suction. Radioactivity associated with the cell pellet was determined using an LKB/Wallac 1217 scintillation counter with automatic quench correction and disintegrations per minute conversion [18]. Blank values (radioactivity that became associated with cells during an uptake interval of zero time) were obtained by processing cell samples exposed simultaneously to radioactively labeled nucleoside and 10 μM NBTGR at 0°. Transport rates were calculated after subtraction of the blank.

In other experiments, the ability of test compounds to cause the inward countertransport of radioactively labeled uridine and 2-chloroadenosine was measured as previously described [19].

Calculations. Where applicable, kinetic constants of influx (apparent K_m and V_{max}) and inhibition constants for nucleoside influx (apparent K_i) were determined by linear regression analysis of s/v versus s plots and $1/v$ versus i plots, respectively, where v is the initial uptake rate, s is the extracellular nucleoside concentration, and i is the extracellular inhibitor concentration.

Nitrobenzylthioinosine (NBMPR) binding. Equilibrium binding of [^{3}H]NBMPR to human erythrocyte "ghosts" was determined at 22° as previously described [17].

Chemicals. [G-^{3}H]NBMPR (16 Ci/mmole) and [8-^{3}H]2-chloroadenosine (18 Ci/mmole) were purchased from Moravek Biochemicals Inc. (Brea, CA), and [5,6-^{3}H]uridine (42 Ci/mmole) and [2,8-^{3}H] adenosine (48 Ci/mmole) were obtained from ICN (Irvine, CA). The radiochemical purity of these compounds was greater than 98% and was periodically checked by TLC. NBMPR and NBTGR were gifts from Dr. A. R. P. Paterson, Cancer Research Group (McEachern Laboratory), University of Alberta, Edmonton, Alberta, Canada. All other reagents were from Sigma.

RESULTS

In the first series of experiments, the ability of 2-chloroadenosine to interact with the nucleoside transporter was determined by indirect methods.

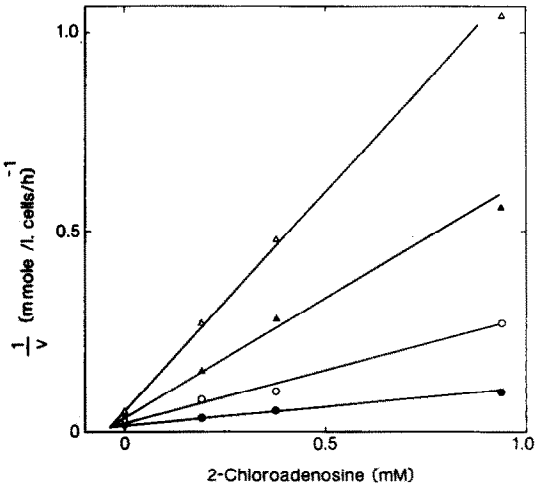

Fig. 1. Dixon plot of 2-chloroadenosine inhibition of uridine influx by human erythrocytes. The reciprocals of uridine uptake rates (22°, means of duplicate estimates) at concentrations of 1.06 (●), 0.25 (○), 0.13 (▲) and 0.066 (△) mM are plotted against the respective concentrations of inhibitor (2-chloroadenosine). Apparent K_i was 33 μM.

Figure 1 shows the effect of 2-chloroadenosine (0.19 to 0.95 mM) on uridine influx (0.06 to 1.0 mM) at 22° by human erythrocytes. The results demonstrate that 2-chloroadenosine was an effective inhibitor of uridine influx with an apparent K_i value of 33 μM. Analysis of the data by the alternative Lineweaver–Burk procedure gave a plot consistent with competitive inhibition.

NBMPR is a potent and specific inhibitor of nucleoside transport, inhibition resulting from the reversible binding of NBMPR to high-affinity sites on the nucleoside transporter [16, 17, 20]. In mammalian erythrocytes, these NBMPR binding sites appear to be present only on functional nucleoside transport elements [17, 21]. The ability of 2-chloroadenosine to inhibit high-affinity NBMPR binding to human erythrocyte membranes is shown in the data depicted in Fig. 2. NBMPR binding was reduced in the presence of 2-chloroadenosine in an apparent competitive manner with an apparent inhibition constant of 0.18 mM. The linearity of the re-plot of apparent K_d values versus 2-chloroadenosine concentration (inset to Fig. 2) provides further evidence of simple competitive inhibition of binding activity.

The data of Figs. 1 and 2 are consistent with the idea that 2-chloroadenosine interacts with the permeant site of the nucleoside transporter. However, such data provide no conclusive information on whether 2-chloroadenosine is a transported permeant. Both transported and non-transported permeants could be expected to inhibit uridine transport and NBMPR binding in a competitive manner. Therefore, to differentiate between a transported and a non-transported permeant, it became necessary to investigate directly the transport of isotopically labeled 2-chloroadenosine.

Figure 3 illustrates the time course of zero-*trans* entry of 2-chloroadenosine at 22° in the presence and absence of the nucleoside transport inhibitor, NBTGR (5 μM), by fresh human erythrocytes.

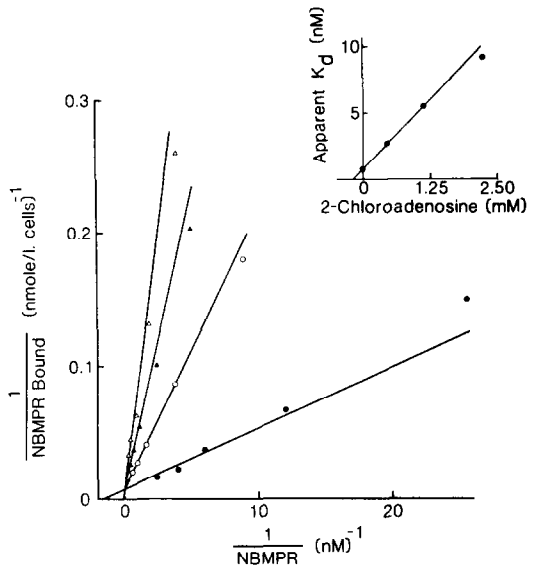

Fig. 2. Mass law analysis of 2-chloroadenosine inhibition of the binding of NBMPR to human erythrocytes. The reciprocals of [^{3}H]NBMPR bound to high-affinity sites in the presence of various concentrations of 2-chloroadenosine at 22° are plotted against the respective reciprocals of the free equilibrium concentrations of [^{3}H]NBMPR. Extracellular 2-chloroadenosine concentrations (mM) were 2.22 (△), 1.11 (▲), 0.44 (○) and 0 (●). The inset shows a plot of the apparent K_d values, as derived from the double-reciprocal plot, versus initial 2-chloroadenosine concentrations. Apparent K_i was 0.18 mM.

NBTGR was a potent inhibitor of 2-chloroadenosine influx and reduced the rate of influx by 90%. In subsequent kinetic studies using human erythrocytes, initial 2-chloroadenosine uptake rates were determined using a 3- to 5-sec incubation period.

Figure 4 shows the concentration dependence of 2-chloroadenosine uptake by human erythrocytes at 22°, the data plotted as s/v versus s plots. Uptake

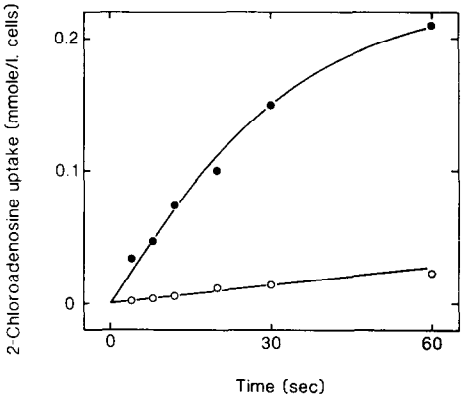

Fig. 3. Time course of 2-chloroadenosine uptake by human erythrocytes at 22°. Intervals of 2-chloroadenosine (1 mM) influx were initiated as described in Materials and Methods in the presence (○) or absence (●) of 5 μM NBTGR and terminated by the inhibitor-oil-stop method. For subsequent experiments, intervals of permeant flux (3–5 sec) were chosen in order that initial rates of transport were measured.

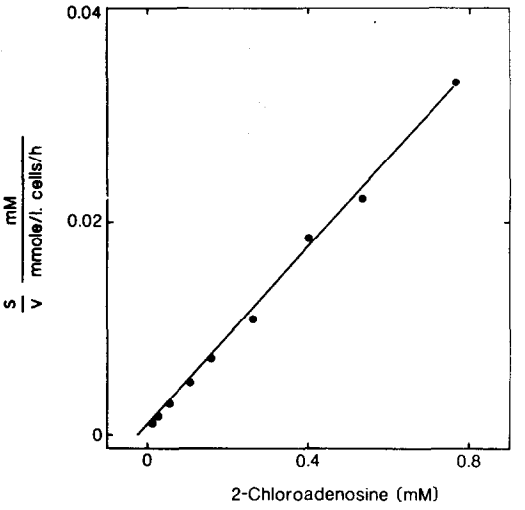

Fig. 4. Concentration dependence of 2-chloroadenosine uptake by human erythrocytes at 22°. Data are presented as s/v versus s plots where s is the extracellular 2-chloroadenosine concentration and v the initial rate of 2-chloroadenosine flux (mmoles/liter cells per hr). Linear least squares analyses of these data yielded the following kinetic constants: apparent K_m value of 23 μM, and V_{max} value of 24.1 mmoles/liter cells per hr.

of 2-chloroadenosine by human erythrocytes was saturable and conformed to simple Michaelis–Menten kinetics, giving an apparent K_m value of 23 μM with a V_{max} estimate of 24.1 mmoles/liter cells per hr. This apparent K_m value was almost identical to the apparent K_i value for 2-chloroadenosine inhibition of uridine influx (see Fig. 1), suggesting that uridine and 2-chloroadenosine are transported by the same system. The permeability of fresh human erythrocytes from four different subjects to saturating concentrations of uridine, adenosine and 2-chloroadenosine (2, 1 and 1 mM respectively) was compared at 22°. Human erythrocytes showed a 4-fold difference in transport rate for the three nucleosides (94.9 ± 1.4, 43.4 ± 4.2 and 24.2 ± 4.9 mmoles/liter cells per hr for uridine, adenosine and 2-chloroadenosine respectively).

To further investigate the substrate specificity of the 2-chloroadenosine transport system, a number of approaches were used. In the first, various nucleosides and nucleobases were tested for their abilities to inhibit the influx of 2-chloroadenosine (Fig. 5). Adenosine was a more effective inhibitor than uridine (IC_{50} value of 0.18 and 0.60 mM, for adenosine and uridine respectively). In contrast, adenine had no significant effect on 2-chloroadenosine influx. In a separate experiment, uridine was shown to be an apparent competitive inhibitor of 2-chloroadenosine influx (apparent K_i 95 μM) (data not shown). This apparent K_i value is almost identical to previous estimates of the apparent K_m value for uridine influx by human erythrocytes [13], suggesting that uridine and 2-chloroadenosine are transported by the same system. Second, the same compounds were tested to see if they could effect the countertransport of 2-chloroadenosine (Fig. 6). Preincubation of human erythrocytes with 5 mM uridine, a non-metabolized nucleoside, or 5 mM 2-chloroadenosine resulted in a

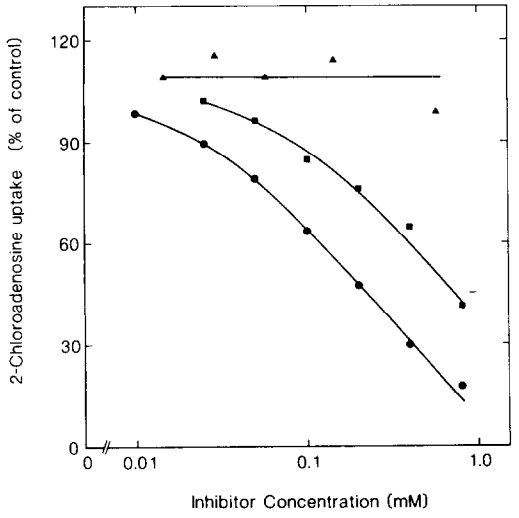

Fig. 5. Effects of adenosine, uridine and adenine on 2-chloroadenosine uptake by human erythrocytes. 2-Chloroadenosine uptake (50 μM, 22°) was measured in the presence of adenosine (●), uridine (■) and adenine (▲). Results are plotted as a percentage of control uptake values in the absence of inhibitor (17.6 mmoles/liter cells per hr). Values are the means of triplicate estimates.

transient accumulation of [^{3}H]2-chloroadenosine in which the maximum intracellular concentration of [^{3}H]2-chloroadenosine (0.55 mmole/liter cell water) was approximately 3-fold higher than the extracellular [^{3}H]2-chloroadenosine concentration (0.20 mmole/liter water). In contrast, the time courses of [^{3}H]2-chloroadenosine uptake by cells incubated

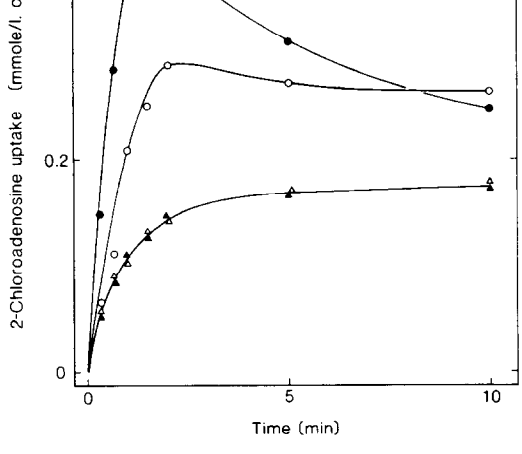

Fig. 6. Countertransport of 2-chloroadenosine after preloading human erythrocytes with 2-chloroadenosine or uridine or adenine. Human erythrocytes were "preloaded" with 5 mM 2-chloroadenosine (○), or 5 mM uridine (●), or 5 mM adenine (▲), or isotonic incubation medium (△); washed free of the extracellular test compound; and then resuspended in [^{3}H]2-chloroadenosine (final concentration 0.20 mM; 22°). The appearance of cell-associated [^{3}H]2-chloroadenosine was measured as a function of time.

previously with adenine were indistinguishable from those of cells with no exposure to adenine. It is interesting to note that the distribution ratio for 2-chloroadenosine (concentration in cell water: concentration in extracellular medium) reached values in excess of 1 for the control cells, indicating intracellular metabolism of 2-chloroadenosine. A recent study [21] has also reported that, contrary to previous suggestions, 2-chloroadenosine is phosphorylated by human B lymphoid cells and adenosine kinase participates, at least in part, in the reaction. Deamination of 2-chloroadenosine via adenosine deaminase did not occur. Similar countertransport experiments with [^{3}H]uridine also demonstrated that prior incubation of cells with either uridine or 2-chloroadenosine resulted in the countertransport of [^{3}H]uridine (data not shown). Finally, the uptake of 2-chloroadenosine by dog erythrocytes was examined. Previous studies [22] have demonstrated that these cells do not possess a saturable nucleoside transport system for either uridine or adenosine. Dog erythrocyte membranes also have no detectable high-affinity NBMPR binding sites [22]. In the present investigation, dog red blood cells gave a slow linear uptake of 2-chloroadenosine over the concentration range (0–4 mM) which was not inhibited significantly by 5 μM NBMPR (data not shown).

DISCUSSION

The experiments described in this paper were designed to test the widely held view that 2-chloroadenosine is not a transported permeant for the nucleoside transport system. Two complementary approaches were used. In the first, the interaction of non-radioactively labeled 2-chloroadenosine with the nucleoside carrier present in human erythrocytes was characterized. The second approach was to measure directly the transport of radioactively labeled 2-chloroadenosine by mammalian erythrocytes.

Human erythrocytes were shown to possess a saturable uptake mechanism for 2-chloroadenosine (apparent K_m for influx at 22° of approximately 20 μM). Uptake by this route was inhibited by micromolar concentrations of NBMPR. Nucleosides also inhibited the influx of 2-chloroadenosine but adenine had no effect. Adenosine was a more effective inhibitor than uridine, a result that is consistent with the known relative affinities of these two nucleosides for the nucleoside transporter. In addition, uridine caused the countertransport of 2-chloroadenosine and vice versa. These observations strongly suggest that uridine and 2-chloroadenosine are transported by the same mechanism. Further support for this conclusion comes from the finding that the entry of 2-chloroadenosine into dog erythrocytes was slow and linear with concentration. NBMPR had no effect on the uptake of 2-chloroadenosine by dog erythrocytes. Previous studies have shown that dog erythrocytes lack a functional nucleoside carrier [22]. The finding that 2-chloroadenosine was both a competitive inhibitor of uridine influx and high-affinity NBMPR binding is also consistent with the view that 2-chloroadenosine enters human erythrocytes by a broad specific nucleoside carrier. The apparent K_i

value for 2-chloroadenosine inhibition for NBMPR binding to human erythrocyte membranes was 0.18 mM, a value higher than the apparent K_i value for inhibition of uridine influx (approximately 33 μM, see Fig. 1). Previous experiments have also noted that the apparent K_i value for nucleoside inhibition of high-affinity NBMPR binding is higher than the apparent K_m for nucleoside influx but close to the apparent K_m of equilibrium exchange [23, 24]. There is, therefore, good evidence to support the view that 2-chloroadenosine enters human erythrocytes by the same nucleoside carrier as other nucleosides.

The applicability of the present findings to other cell types remains to be investigated. However, 2-chloroadenosine has been shown to be a competitive inhibitor of adenosine transport on L1210 cells (apparent K_i 24 μM) [25] and to block the binding of NBMPR to rat brain membranes [26]. These limited results would be consistent with the notion that 2-chloroadenosine is a transported substrate for the nucleoside carrier in these cells. Therefore, the previous explanation, that the inability of nucleoside transport inhibitors to potentiate the pharmacological effects of 2-chloroadenosine is because the adenosine transporter cannot accept 2-chloroadenosine as a permeant, may have to be reassessed [4, 6, 9]. In this regard, it should be noted that the net uptake of a substrate in the presence of transport inhibitors may be either decreased or increased depending on the kinetic constants for influx, efflux and inhibition and the substrate concentration [27, 28].

In conclusion, the data presented here are consistent with the notion that 2-chloroadenosine is a transported permeant for the nucleoside transporter in human erythrocytes.

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