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INHIBITION OF THE TRANSPORT OF ADENOSINE, OTHER NUCLEOSIDES AND HYPOXANTHINE IN NOVIKOFF RAT HEPATOMA CELLS BY METHYLXANTHINES, PAPAVERINE, N^6-CYCLOHEXYLADENOSINE AND N^6-PHENYLISOPROPYLADENOSINE

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Abstract—Theophylline, caffeine, isobutylmethylxanthine, papaverine, N^6-cyclohexyladenosine, N^6-allyl-N^6-cyclohexyladenosine (ACHA) and N^6-L-phenylisopropyladenosine (L-PIA) inhibited the transport of adenosine, uridine and hypoxanthine in Novikoff rat hepatoma cells. The IC_{50} values for the inhibition of uridine transport ranged from 5 μM for ACHA to 3200 μM for caffeine and were inversely proportional to the lipid solubility of the inhibitors. L-PIA and papaverine inhibited uridine influx in a non-competitive manner, having a greater influence on the Michaelis–Menten constant than on maximum velocity, just as observed previously for the inhibition of nucleoside transport by dipyridamole and hypoxanthine. [^{3}H]L-PIA rapidly accumulated in Novikoff cells at 25° to about five times higher levels than present extracellularly. The initial rates of L-PIA uptake were directly proportional to its extracellular concentration between 0.01 and 240 μM and not affected by structurally related analogs, methylxanthines, papaverine, dipyridamole, or 2 mM uridine, but were dependent on temperature. We conclude that L-PIA inhibits nucleoside transport in these cells without being significantly transported by the carrier, that it equilibrates rapidly across the plasma membrane without carrier mediation consistent with its lipophilicity, and that it accumulates concentratively in cells due to partitioning into membrane lipids and binding to intracellular components.

Adenosine (Ado) has many physiological functions. Some of these, such as the inhibition of lipolysis in fat cells, coronary vasolidation, and inhibitory modulation of neuronal activity, are mediated by its binding to adenyl cyclase-coupled cell surface receptors [1–6]. Non-metabolizable, lipophilic analogs of Ado such as N^6-*R*(−)-1-methyl-2-phenylethyladenosine (generally referred to as N^6-L-phenylisopropyladenosine or L-PIA), N^6-cyclohexyladenosine (CHA) and 5′-*N*-ethylcarboxamidoadenosine (NECA) have been very useful in identifying different receptors and assessing the kinetics of binding [1–6].

At least two receptors have been implicated in these functions in various cell types. Binding to one receptor (R_a) enhances adenyl cyclase activity, whereas binding to a second receptor (R_i) inhibits its activity. Binding of the Ado agonists to both receptors is strongly inhibited by various methylxanthines which, in most cases, is correlated with an inhibition of the physiological effects of the agonists.

Binding of the agonists and its inhibition by the antagonists are generally assessed with partially purified cell membrane fractions. Little, however, is known as to the extent that the agonists and antagonists interact with the nucleoside transporter present in membranes of most mammalian cells (for example, various types of cultured cells possess about 10^5 carriers/cell; Refs. 7–9). Some lipophilic nucleoside analogs, such as nitrobenzylthioinosine (NBTI), bind to the nucleoside carrier with as high an affinity ($K_D \sim 1$ nM; Refs. 8–10) as the agonists to the Ado R_i receptor. On the other hand, theophylline has been found to inhibit the uptake of nucleosides and hypoxanthine (Hyp) by cultured mammalian cells [11–14]. Indirect evidence suggested that the inhibitory effects were mediated at the level of their transport into the cell. In the present study we have confirmed this conclusion by directly determining the effects of various methylxanthines on the transport of Ado, uridine (Urd) and Hyp in variants of Novikoff cells in which substrate phosphorylation was blocked by a deficiency in the appropriate phosphorylating enzymes. We also show that agonists of the Ado receptors are potent inhibitors of nucleoside and Hyp transport, compare their effects to those of papaverine and dipyridamole, and relate the potency of inhibition to the lipid solubility of these substances. We also have studied the interaction of radiolabeled L-PIA with these cells.

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MATERIALS AND METHODS

Cell culture. Sublines of Novikoff rat hepatoma cells deficient in Ado kinase (1–22; Ref. 15), uridine

kinase (1–14–7; Ref. 16) or Hyp phosphoribosyltransferase (1–9; Ref. 17) were propagated in suspension culture as described previously.

Nucleoside and Hyp transport. Substrate transport was measured as described previously [18–20]. Time courses of transmembrane equilibration of radiolabeled substrates were determined under zero-*trans* conditions by rapid kinetic techniques with suspensions of (1–3) × 10^7 cells/ml of a basal medium. An integrated rate equation describing the nucleoside carrier with directional symmetry and equality of movement of empty and nucleoside-loaded carrier was fitted to the data [19, 20]:

$$S_{2,t} = S_1 \left[1 - \exp\left(-\frac{tV + (1 + S_1/K)S_{2,t}}{K + 2S_1 + S_1^2/K} \right) \right] \quad (1)$$

where $S_{2,t}$ = concentration of intracellular substrate at time t; S_1 = extracellular concentration of substrate; K = Michaelis–Menten constant; and V = maximum velocity.

Octanol partition coefficient (K_{oct}). K_{oct} was determined for Ado, 2′-, 3′- and 5′-deoxyadenosine and L-PIA using radiolabeled substrates as described previously [21]. For unlabeled substances the procedure was modified as follows. Samples of 100 μM of test substrate in aqueous solution were shaken with an equal volume of octanol. After separation of the phases, each was lyophilized, and the residues were dissolved in 100 μM Tris–HCl, pH 7.4. The octanol phase solution was clarified by filtration through Whatman No. 1 paper. The absorbance of both solutions was measured at 257 nm (2′-dAdo), 265 nm (L-PIA), 270 nm [CHA, caffeine, theophylline, and 3-isobutyl-1-methylxanthine (IBMX)] or 276 nm [papaverine and (N^6-allyl-N^6-cyclohexyladenosine (ACHA)]. K_{oct} = absorbance (or radioactivity) of octanol phase/absorbance (or radioactivity) of aqueous phase. In the case of 2′-dAdo and L-PIA, similar values were obtained by the two methods using radiolabeled and unlabeled substances.

Materials. Materials were purchased as follows: [5-^{3}H]Urd and [2-^{3}H]Hyp from the Moravek Biochemicals Corp. (Brea, CA); [8-^{3}H]Ado from Schwarz/Mann (Spring Valley, NY); [Ade-2,8-^{3}H, ethyl-2-^{3}H]L-PIA (50 Ci/mmole) from the New England Nuclear Corp. (Boston, MA); unlabeled nucleosides, caffeine, theophylline and papaverine from the Sigma Chemical Co. (St. Louis, MO); IBMX from the Aldrich Chemical Co. (Milwaukee, WI); and NBTI from the Calbiochem (San Diego, CA). Dipyridamole (Persantin) was a gift from Geigy Pharmaceuticals (Ardsley, NY), and L-PIA and ACHA were supplied by Dr. O. H. Wilhelm, Boehringer-Mannheim GmbH (Mannheim, West Germany).

RESULTS AND DISCUSSION

Figure 1 illustrates initial time course of zero-*trans* uptake of Ado, Urd and Hyp (all at 160 μM) by Novikoff rat hepatoma cells deficient in Ado kinase,

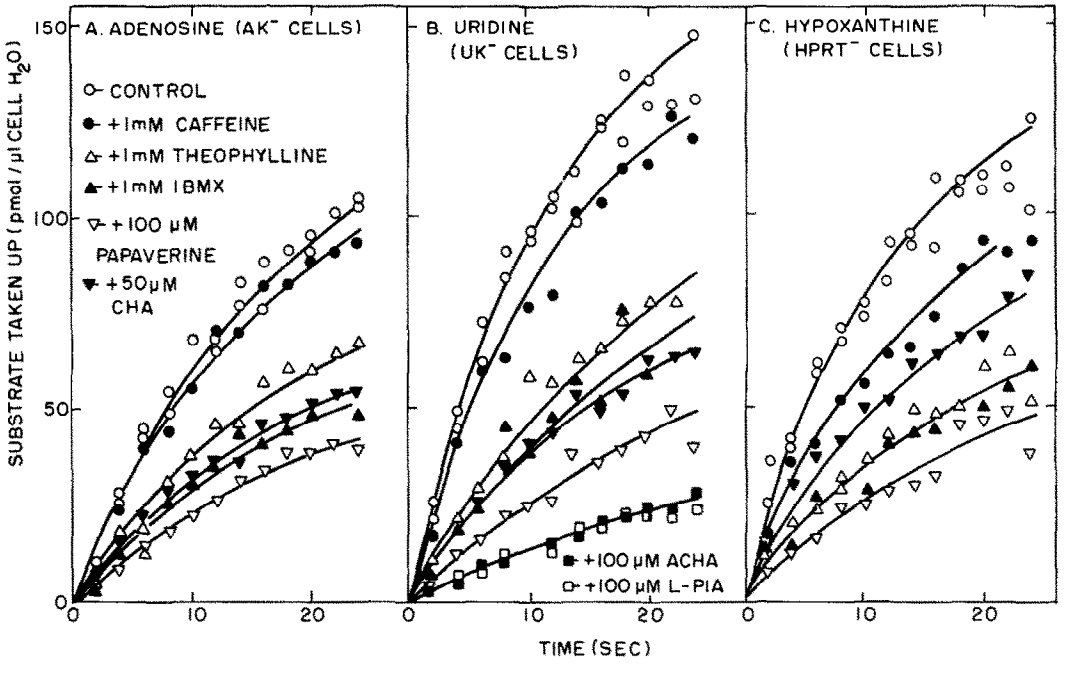

Fig. 1. Inhibition of transport of Ado (A), Urd (B) and Hyp (C) by methylxanthines, papaverine, CHA, ACHA and L-PIA. The initial time courses of uptake of 160 μM [^{3}H]Ado (637 cpm/μl), [^{3}H]Urd (802 cpm/μl) and [^{3}H]Hyp (373 cpm/μl) were determined by rapid kinetic techniques (see Materials and Methods) at 25° in Ado kinase-deficient (AK^-), Urd kinase-deficient (UK^-) or Hyp phosphoribosyltransferase-deficient ($HPRT^-$) Novikoff cells respectively. The inhibitors were added simultaneously with substrate to the indicated final concentrations.

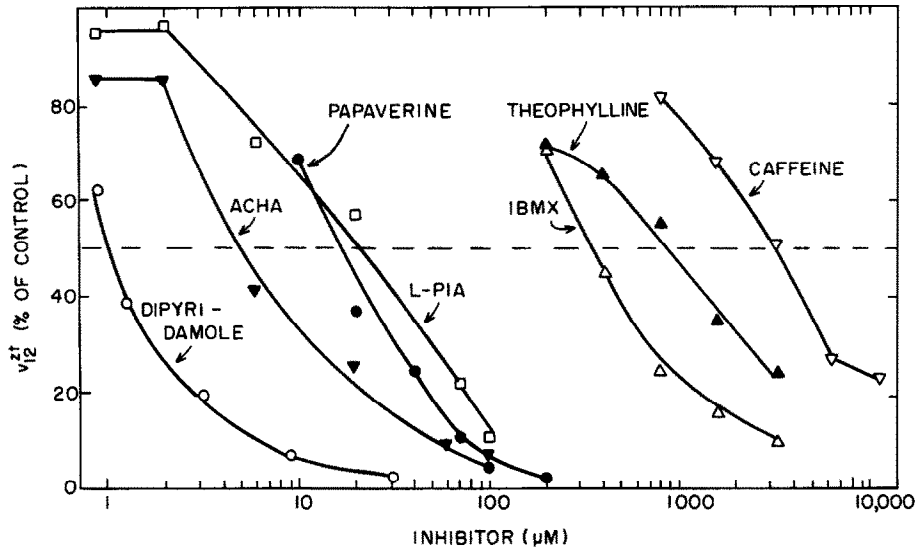

Fig. 2. Dose–response curves for the inhibition of Urd influx in Urd kinase-deficient Novkoff cells by methylxanthines, Ado analogs, papaverine and dipyridamole. The results are summaries of several experiments in which the influx of 160 μM [^{3}H]Urd was measured as illustrated in Fig. 1 in the absence and presence of various concentrations of the indicated inhibitors. Equation 1 was fitted to each uptake time course with K fixed at 250 μM [19] and the initial velocity (v_{12}^{zt}) was calculated by substituting the estimated value of V into $v_{12}^{zt} = S_1V/(K + S_1)$ which is the slope of the curve described by Equation 1 at $t = 0$. Control values for v_{12}^{zt} were all approximately 10 pmoles/μl cell water · sec.

Urd kinase and Hyp phosphoribosyltransferase, respectively, in the presence and absence of methylxanthines, CHA and papaverine. For Ado transport measurements, the cells were pretreated with 10 μM 2-deoxycoformycin (dCF), a concentration of dCF which was shown previously to practically completely inhibit the deamination of Ado in these cells [22]. Chromatographic analyses of the acid-soluble pools of labeled cells indicated that conversion of the nucleosides to nucleotides was insignificant during 2 min of incubation (data not shown; for general discussion, see Ref. 19). The results show that the methylxanthines, CHA and papaverine inhibited to about the same extent the transport of Ado and Urd which is mediated by the same broadly-specific nucleoside carrier as well as that of Hyp which is transported by a different carrier [17, 19, 20]. Because of the similar degree of inhibition observed for all three substrates, we selected one of them (Urd) for a more detailed analysis of the inhibition with the following results.

First, the data in Fig. 1B illustrate that ACHA and L-PIA also strongly inhibited nucleoside transport. Second, the various substances similarly inhibited Urd transport in Chinese hamster cells and mouse L cells as in Novikoff cells (data not shown). Figure 2 shows the dose–response patterns of the inhibitions of Urd transport in Urd kinase-deficient Novikoff cells by the various substances as well as, for comparison, by dipyridamole. The order of effectiveness of the inhibition by the three methylxanthines was IBMX > theophylline > caffeine, which is the same order of their effectiveness as antagonists of Ado binding to both the R_a and R_i Ado receptors [3, 4], although the IC_{50} values for nucleoside transport

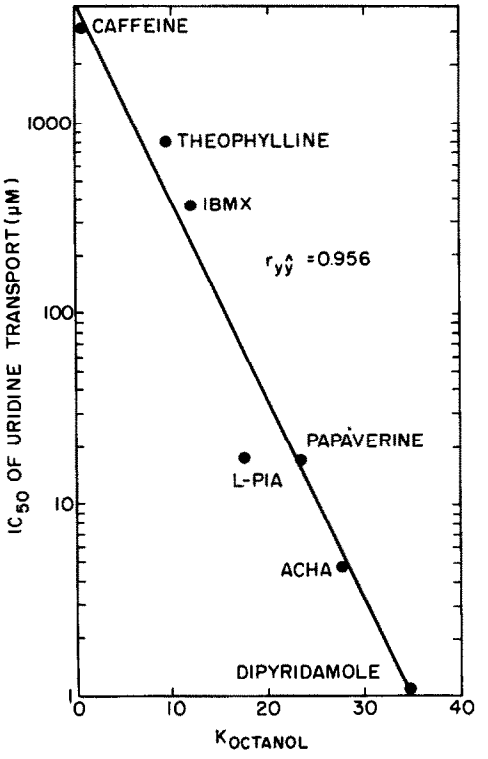

Fig. 3. Relationship between potency of inhibition of Urd transport and lipid solubility of various inhibitors. The concentrations causing 50% inhibition of Urd influx at 160 μM (IC_{50}) were estimated from the data in Fig. 2. K_{oct} was determined as described under Materials and Methods. The correlation coefficient (r_{yy}) of the regression line is shown.

Table 1. Kinetic analysis of the inhibition of Urd influx by papaverine and L-PIA*

[I] (μM)	Papaverine		L-PIA	
	K (μM)	V (μM/sec)	K (μM)	V (μM/sec)
0	253 ± 24	24.2 ± 0.7	124 ± 13	10.0 ± 0.3
20	446 ± 33	15.7 ± 0.5	177 ± 13	7.0 ± 0.2
40	539 ± 76	10.4 ± 0.8	203 ± 17	5.6 ± 0.1
80	910 ± 182	10.3 ± 1.3	288 ± 20	4.6 ± 0.1
$K_{i,\text{slope}}$ (μM)	10.9 ± 1.1		19.4 ± 1.9	(16.3 ± 4.0)
$K_{i,\text{intercept}}$ (μM)	37.6 ± 5.2		56.3 ± 4.2	(40.0 ± 6.0)
$r_{y\hat{y}}$	0.999		0.999	(0.992)

* The results are from two independent experiments, one with each inhibitor. In each experiment Urd influx was measured by the rapid kinetic technique (see Fig. 1) at five Urd concentrations (60–960 μM) in the absence and presence of the indicated concentrations of papaverine or L-PIA. K and V were computed for each inhibitor concentration by fitting Equation 1 to the data pooled for the five Urd concentrations. The values are summarised ± S.E. of estimate. Initial transport velocities were calculated for each Urd concentration as the slope of the uptake curve at $t = 0$: $v_{12}^{t_1} = S_1V/(K + S_1)$. Then the following equation: $v_{12}^{t_1} = (VS_1)/[K(1 + I/K_{i,\text{slope}}) + S_1(1 + I/K_{i,\text{intercept}})]$ was fitted to the pooled $v_{12}^{t_1}$ values for each inhibitor. The best-fitting parameters for $K_{i,\text{slope}}$ and $K_{i,\text{intercept}}$ are given ± S.E. of estimate ($r_{y\hat{y}}$ = correlation coefficient). The values in parentheses are for an additional experiment with L-PIA which was conducted in the same manner as described.

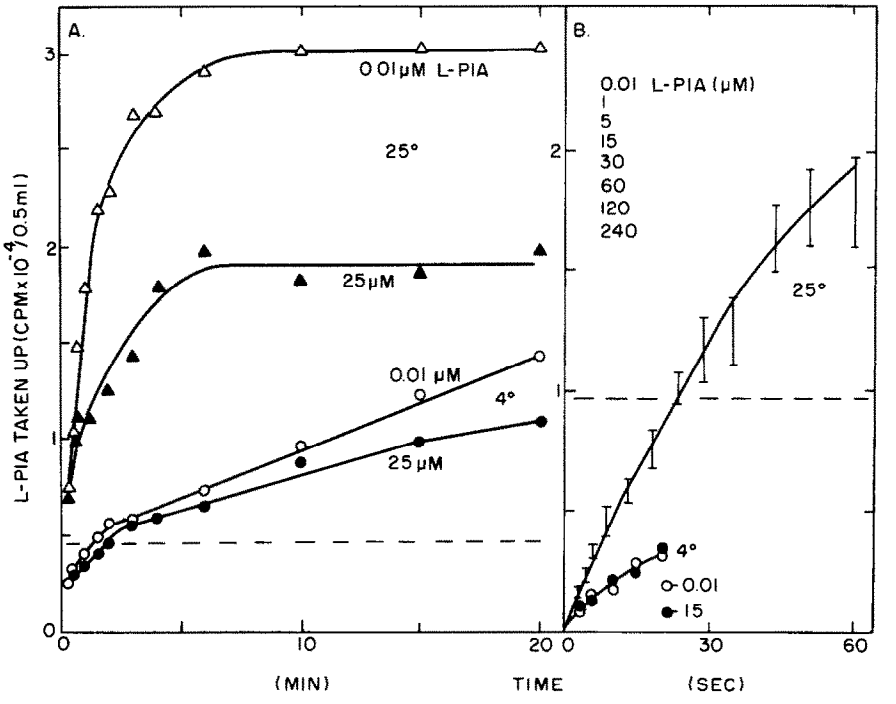

Fig. 4. Long-term (A) and short-term (B) uptake of [^{3}H]L-PIA by Novikoff cells as a function of concentration. The results in A and B are from two independent experiments. Suspensions of 1×10^7 cells/ml (A) or 2×10^7 cells/ml (B) were equilibrated at 4 or 25° as indicated. (A) Samples of the suspension were supplemented with 0.01 or 25 μM [^{3}H]L-PIA (350 cpm/μl, irrespective of concentration). At various times of incubation at the same temperatures the cells from duplicate 0.5-ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity. (B) The uptake of the indicated concentrations of [^{3}H]L-PIA (380 cpm/μl, irrespective of concentration) was measured by the rapid kinetic technique as described for nucleoside transport under Materials and Methods. All radioactivity values were corrected for substrate trapped in extracellular space of cell pellets. In B the radioactivity values for the different concentrations of L-PIA were the same within experimental errors; the vertical bars indicate the ranges of values observed. The broken lines indicate the intracellular concentrations of radioactivity equal to those in the medium.

inhibition are 2–3 logs higher than those for the inhibition of Ado receptor binding.

We found (Fig. 3) that overall the $\log_{10}$ of the IC_{50} of inhibition of Urd transport by the various inhibitors analyzed was roughly inversely proportional to the lipid solubility of these inhibitors as assessed by their octanol partition coefficient (K_{oct})

The mechanism of inhibition cannot be specified on the basis of these data. However, the results are consistent with the view expressed previously [19, 21] that the inhibition of transport by these highly lipophilic substances may involve, in part at least, their partitioning into membrane lipids and interaction with hydrophobic domains of the internal membrane carrier.

The kinetics of inhibition of Urd transport by the various substances are of little help in deciding on possible mechanisms of inhibition. Papaverine and L-PIA showed apparent linear, non-competitive inhibition of Urd transport (Table 1), just as previously observed for the inhibition of nucleoside transport by dipyridamole and Hyp [19]. It is of interest that both substances, whether structurally related (L-PIA) to the substrate or unrelated (papaverine and dipyridamole), exhibited the same pattern of inhibition. In addition, we have shown previously that even the natural substrates for the nucleoside carrier inhibit the transport of each other in an apparent non-competitive manner. The reasons for this are not clear. It is, however, of interest that all the inhibitors showed a stronger influence on K than on V ($K_{i,slope} < K_{i,intercept}$). In fact, in all cases the ratio of $K_{i,slope}/K_{i,intercept}$ has been between 0.2 and 0.4. Whether and to what extent lipophilic substances might inhibit transport processes by simple intercalation into the lipid bilayer is not known. In this respect, it is of interest that recent experiments have shown that papaverine causes a decrease in membrane fluidity in Ehrlich ascites cells [23]. Such a decrease in membrane fluidity could impede "movement" of the carrier and could also explain the inhibition by these substances of the non-mediated permeation through membranes of hydrophilic substances such as L-glucose and cytosine [21].

The data presented so far also do not allow any conclusions as to whether any of the inhibitors, including the Ado analogs L-PIA and ACHA, are transported by the nucleoside carrier. This question is not readily answerable because of the high lipid solubility of these inhibitors. On the basis of results with other lipophilic substances, it can be predicted that these inhibitors should equilibrate across the plasma membrane by simple diffusion alone within less than 1 min [24]. Indeed we found that at 25° [^{3}H] L-PIA at a concentration of 10 nM accumulated very rapidly in Novikoff cells to levels about three times above those present in the medium (Fig. 4A) (representing about 20% of the total L-PIA added). Chromatographic analyses of extracts of labeled cells indicated that all cell-associated radioactivity comigrated with authentic L-PIA (data not shown). A steady-state of L-PIA association with cells was reached within 10 min at 25°, but the maximum amount accumulated relative to the extracellular concentration was less at 25 μM than at 10 nM L-PIA (Fig. 4A), indicating the involvement of some "saturable" component in long-term uptake. In contrast, the initial rate of uptake of L-PIA by the cells showed no saturability at extracellular L-PIA concentrations between 10 nM and 240 μM (Fig. 4B). Higher concentrations of L-PIA could not be used because insufficient material was available. The initial rate of uptake of L-PIA at a concentration of 240 μM at 25° was about 9 pmoles/μl cell water · sec, which is similar to that observed for transported nucleosides at this concentration [19] but similar, too, to that expected for simple diffusion of a compound with the lipophilicity of L-PIA.

The initial rate of uptake of L-PIA was strongly temperature dependent (Fig. 4B), but, at 5 nM L-PIA, was not affected significantly by 0.5 μM NBTI, 40 μM dipyridamole, 100 μM papaverine, 250 μM IBMX, 500 μM theophylline, 100 μM ACHA or 100 μM CHA, all of which strongly inhibit nucleoside transport at the test concentrations, or by 2 mM Urd (data not shown). Only the long-term concentrative accumulation of L-PIA was reduced by all these substances.

On the other hand, the lipophilic analogs L-PIA, CHA and ACHA inhibited the transport of Urd two to three times more strongly than the structurally related hydrophilic nucleosides Ado, 2′- 3′- and 5′-deoxyadenosine (K_{oct} = 0.123, 0.219, 0.315, 0.273, respectively), which are known substrates for the nucleoside transporter [19]. Similarly, Thampy and Barnes recently reported [25] that L-PIA and CHA inhibit Ado uptake in cultured glial cells from chick embryo brain to a greater extent than various natural nucleosides, but less than NBTI or dipyridamole.

Taken together we interpret these results as follows: L-PIA, and probably CHA and ACHA, rapidly equilibrate without mediation across the plasma membrane. They inhibit nucleoside transport in Novikoff cells without being efficiently transported themselves. The inhibition of nucleoside transport by these Ado analogs or by the methylxanthines is probably mainly due to interaction of the inhibitors with the carrier *per se*, but this has not been proven unequivocally, and the notion that these inhibitors are bound to the nucleoside recognition site on the carrier is even more tenuous. The concentrative accumulation of L-PIA in cells probably reflects both its partition into membrane lipids and its binding to intracellular components. Of the two, partitioning into membrane lipids seems to be the minor component. On the basis of the K_{oct} of L-PIA and a total lipid content of Novikoff cells of 250 μg/10^7 cells [21], we estimate that, in the experiment illustrated in Fig. 4A, not more than 1000 cpm accumulated by the cells (in each sample at steady state) can be accounted for by partitioning of [^{3}H]L-PIA into membrane lipids. The remaining 24,000 cpm accumulated concentratively at 10 nM L-PIA is assumed to have been bound to intracellular components. Binding to these components seems to be partially saturable (about 40%) between 5 and 15 nM L-PIA, but not very specific. It is inhibited by diverse structurally unrelated compounds, but the reasons for this effect and the nature of the binding component are not known. That the binding of L-PIA requires membrane permeation and is not to extracellular receptors is indicated by the strong effect of temperature on

uptake. These various observations need to be considered in assessing the interaction of Ado agonists and antagonists with Ado receptors on whole cells or in crude membrane fractions.

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