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Kinetic Analysis of Ligand Binding to the Ehrlich Cell Nucleoside Transporter: Pharmacological Characterization of Allosteric Interactions With the [^{3}H]Nitrobenzylthioinosine Binding Site

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SUMMARY

Kinetic analysis of the binding of [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR) to Ehrlich ascites tumor cell plasma membranes was conducted in the presence and absence of a variety of nucleoside transport inhibitors and substrates. The association of [^{3}H]NBMPR with Ehrlich cell membranes occurred in two distinct phases, possibly reflecting functional conformation changes in the [^{3}H]NBMPR binding site/nucleoside transporter complex. Inhibitors of the equilibrium binding of [^{3}H]NBMPR, tested at submaximal inhibitory concentrations, generally decreased the rate of association of [^{3}H]NBMPR, but the magnitude of this effect varied significantly with the agent tested. Adenosine and diazepam had relatively minor effects on the association rate, whereas dipyridamole and mioflazine slowed the rate dramatically. Inhibitors of nucleoside transport also decreased the rate of dissociation of [^{3}H]NBMPR, with an order of potency significantly different from their relative potencies as inhibitors of the equilibrium binding of [^{3}H]NBMPR. Dilazep, dipyridamole, and mioflazine were effective inhibitors of both [^{3}H]NBMPR dissociation and equilibrium binding. The lidoflazine analogue R75231, on the other hand, had no effect on the rate of dissociation of [^{3}H]NBMPR at concentrations below 300 μM, even though it was one of the most potent inhibitors of [^{3}H]NBMPR binding tested (K_i < 100 nM). In contrast, a series of natural substrates for the nucleoside transport system enhanced the rate of dissociation of [^{3}H]NBMPR with an order of effectiveness that paralleled their relative affinities for the permeant site of the transporter. The most effective enhancers of [^{3}H]NBMPR dissociation, however, were the benzodiazepines diazepam, chlordiazepoxide, and triazolam. Comparable effects of adenosine and dipyridamole on [^{3}H]NBMPR dissociation rate were obtained upon solubilization of the membranes with octylglucoside, suggesting that this phenomenon was not due to changes in membrane fluidity. These results are compatible with the existence of specific ligand recognition sites on the nucleoside transport complex of Ehrlich cells that are pharmacologically distinct from, but allosterically linked to, the high affinity binding sites for [^{3}H]NBMPR. The marked effects on [^{3}H]NBMPR binding kinetics that result from ligand interactions with these sites must be considered in the design and analysis of all studies involving the use of [^{3}H]NBMPR as a high affinity probe for the nucleoside transport system.

NBMPR is a potent inhibitor of nucleoside transport and has achieved wide popularity as an investigational tool to prevent the cellular accumulation and subsequent metabolism of nucleosides (1–3). [^{3}H]NBMPR has been used extensively as a specific probe for proteins associated with the plasma membrane-located nucleoside transport system and has proven invaluable in the characterization of drug interactions with nucleoside transporters (2–5). Based on the results of equilibrium binding studies, [^{3}H]NBMPR epitomizes the ideal radioligand probe. [^{3}H]NBMPR binds reversibly with very high affinity (K_D < 1 nM) to a single class of apparently noncooperative sites (Hill coefficients not different from unity), in a wide variety of preparations (2–4). The supposition that this binding site is a component of the nucleoside transporter is based on the excellent correlation between the binding of [^{3}H]NBMPR and the inhibition of nucleoside flux (2, 3, 6–9) and on the fact that other inhibitors of nucleoside transport competitively inhibit the binding of [^{3}H]NBMPR at concentrations that parallel those required to inhibit nucleoside flux (10, 11). Nevertheless, a number of results have been reported in the literature that are not compatible with a simple, noncooperative binding site model and suggest a more complex interaction of inhibitors with the [^{3}H]NBMPR binding site/nucleoside transporter complex. For example, dipyridamole, a recognized inhibitor of nucleoside transport, inhibits [^{3}H]NBMPR binding with pseudo-Hill coefficients significantly less than unity in a variety of systems (10, 12–14). Conversely, a series of lidoflazine analogues exhibit pseudo-Hill coefficients significantly greater than unity for inhibition of [^{3}H]NBMPR binding to calf lung

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ABBREVIATIONS: NBMPR, 6-(4-nitrobenzylmercapto)purine ribonucleoside (nitrobenzylthioinosine); NBTGR, nitrobenzylthioguanosine; CV-1808, 2-phenylaminoadenosine; R75231, 2-(aminocarbonyl)-*N*-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis-(4-fluorophenyl)pentyl]-1-piperazineacetamide.

membranes (15). These results are indicative of either multiple binding sites or site cooperativity. Furthermore, the affinity of the nucleoside transport inhibitory site for [^{3}H]NBMPR and dipyridamole varies significantly among species and cell/membrane preparations (2–4, 12, 16, 17). The most compelling evidence for a complex interaction between nucleoside transport inhibitors and the [^{3}H]NBMPR binding site, however, is the capacity of various agents to modify the rate of dissociation of [^{3}H]NBMPR from its specific sites. Dipyridamole and dilazep inhibit the dissociation of [^{3}H]NBMPR, and a number of nucleosides have been shown to enhance [^{3}H]NBMPR dissociation (12, 18–22). Given the relatively high concentrations of inhibitors required to induce these effects, it has been suggested that they may be due to nonspecific membrane perturbations (2, 18, 19). However, an alternate explanation is the presence of another binding site, distinct from the NBMPR site, through which agents may modify [^{3}H]NBMPR binding site affinity (2, 20–22). As yet, the range of compounds that have been examined with sufficient rigor is inadequate to allow a distinction between these possibilities or to permit any conjectures regarding the mechanistic basis for this phenomenon. Given the widespread pharmacological use of NBMPR and other nucleoside transport inhibitors and the important role of the nucleoside transport system in regulating the extracellular levels of adenosine and, thereby, the degree of adenosine receptor activation (23), a thorough understanding of the interactions of inhibitors with the [^{3}H]NBMPR binding site/nucleoside transport complex is crucial.

The present study was undertaken to define and rationalize the complexities of ligand interactions with the [^{3}H]NBMPR binding site/nucleoside transporter. This involved a detailed assessment of the effects of a range of concentrations of a wide variety of nucleoside transport inhibitors and substrates on the kinetics of [^{3}H]NBMPR binding in a defined model system. A preliminary report of this study was presented at the International Symposium on Pharmacology of Purinergic Receptors: Implications for Drug Design (1990 IUPHAR Satellite Symposium) (24).

Experimental Procedures

Materials. [^{3}H]NBMPR (16 Ci/mmol) was from Moravek Biochemicals, Inc. (Brea, CA). Dilazep [*N,N'*-bis[3-(3,4,5-trimethoxybenzoyloxy)propyl]-homopiperazine] was generously provided by Asta Werke (Frankfurt, Germany), and mioflazine [3-(aminocarbonyl)-4-[4,4-bis(4-fluorophenyl)butyl]-*N*-(2,6-dichlorophenyl)-1-piperazineacetamide 2HC1], soluflazine [3-(aminocarbonyl)-4-(2,6-dichlorophenyl)-4-[(4-fluorophenyl)-4-(3-pyridinyl)-butyl]-*N*-(2,6-dichlorophenyl)-1-piperazine-acetamide 2HC1], and R75231 [2-(aminocarbonyl)-*N*-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis-(4-fluorophenyl)pentyl]-1-piperazineace-tamide] were gifts from Dr. H. Van Belle, Janssen Research Foundation (Beerse, Belgium). Diazepam, chlordiazepoxide, and triazolam (Hoffmann La Roche, Toronto, Canada) were obtained from Dr. J. T. Hamilton, University of Western Ontario. Asolectin (soybean phospholipids) was purchased from Associated Concentrates (Woodside, NY) and stored under N_2. Octylglucoside was purchased from Calbiochem Biochemicals Inc. (San Diego, CA) CV-1808 (2-phenylaminoadenosine), 2-chloroadenosine, and cyclopentyladenosine were purchased from Research Biochemicals, Inc. (Natick, MA) Other nucleosides, NBMPR, NBTGR, and dipyridamole [2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine] were supplied by Sigma (St. Louis, MO). All other compounds were of reagent grade.

Isolation of Ehrlich cell plasma membranes. Ehrlich ascites cells were grown as an intraperitoneal culture in mice (Swiss, male, ≈30 g). Cells were isolated and processed to obtain plasma membranes, as described previously (10). In brief, washed cells were swollen by suspension in 1 mM $ZnCl_2$, fragmented using a Polytron PT-10-20 homogenizer, and resuspended in 9.25% sucrose, with removal of nuclei by centrifugation (three times) for 1 min at 900 × *g*. Plasma membranes were then separated on a 30–60% (w/w) sucrose gradient and stored at −80° in 15% dimethylsulfoxide for up to 3 months. No loss in [^{3}H]NBMPR binding activity was observed over this time period.

Solubilization of Ehrlich cell plasma membranes. Plasma membranes were washed extensively, to remove dimethylsulfoxide, and then incubated with a combination of octylglucoside (1%, w/v) and asolectin (0.15%, w/v) in 50 mM Tris·HCl (pH 7.1) at 4° for 60 min. The mixtures were then centrifuged at 120,000 × *g* for 60 min, and the supernatant was retained for immediate use in radioligand binding assays. This procedure resulted in a 90% solubilization of [^{3}H]NBMPR binding activity from plasma membranes, with retention of many of the ligand-binding characteristics of the native membrane (10).

[^{3}H]NBMPR binding. Washed plasma membranes were incubated with [^{3}H]NBMPR, at 22° in 50 mM Tris·HCl buffer (pH 7.1), in the presence and absence of competing agents (1 ml final volume). The binding reaction was terminated by vacuum filtration through Whatman GF/B filters, followed by two washes with 4 ml of 10 mM Tris·HCl, pH 7.1 at 4°, using a modified Brandel cell harvester (M-24R). For dissociation rate studies, 10 μM NBTGR was included in combination with the test inhibitors, to ensure that reassociation was blocked completely. In association rate studies, membranes were not preincubated with inhibitor before addition of [^{3}H]NBMPR; preliminary studies indicated that similar results were obtained with or without preincubation.

To measure [^{3}H]NBMPR binding to solubilized membrane preparations, proteins were precipitated, before filtration, with a mixture of polyethylene glycol (10%, w/v, final concentration and γ-globulins (1.65 mg/ml final concentration), at 4°. After filtration, the precipitates were rinsed twice with buffer containing 8% (w/v) polyethylene glycol, at 4°. This precipitation procedure does not affect [^{3}H]NBMPR binding to intact membranes (10).

Nonspecific binding of [^{3}H]NBMPR (<5% of total binding) was assessed by preincubation of membranes for at least 30 min with 10 μM NBTGR. Protein was measured by the method of Lowry *et al.* (25), using bovine serum albumin as the standard. Radioactive material retained on the filters was quantified using standard liquid scintillation counting techniques. All values reported were derived from computer-generated hyperbolic, sigmoid, or exponential association curves (GraphPAD InPlot, version 3.01) fitted to the specific (NBTGR-sensitive) binding data obtained directly (no mathematical transformation) from each experiment. K_i values for inhibition of [^{3}H]NBMPR binding were calculated from the relationship $K_i = IC_{50}/(1 + [L]/K_D)$, where IC_{50} was interpolated from best fit sigmoid curves of percentage of inhibition versus log inhibitor concentration, $[L]$ is the concentration of [^{3}H]NBMPR used (0.65 nM), and K_D is the equilibrium dissociation constant for [^{3}H]NBMPR binding to Ehrlich cell membranes (K_D = 0.11 nM) obtained by equilibrium binding analysis.

[^{3}H]Uridine influx. Cells were washed in isotonic saline, resuspended in Dulbecco's phosphate-buffered saline (pH 7.4), and then depleted of ATP by incubation with rotenone (20 ng/ml) and 2-deoxyglucose (2 mM). ATP depletion was necessary to prevent intracellular trapping of [^{3}H]uridine as its nucleotide metabolites. All assays were conducted at room temperature (≈22°) with 100 μM [^{3}H]uridine, using a 20-sec incubation time, as described previously (10). Cells were exposed to [^{3}H]uridine and nucleoside inhibitors concurrently. Preliminary experiments conducted in the presence and absence of erythro-9-(2-hydroxy-3-nonyl)adenine established that adenosine metabolism by adenosine deaminase was minimal under these assay conditions. Nonmediated uptake of [^{3}H]uridine was assessed by preincubation of cells with a combination of NBTGR(10 μM) and dipyridamole (10 μM). Assays were terminated by centrifugation of the cells through a layer

of silicone/mineral oil, and the cell pellet was digested with 1 N NaOH and analyzed for ^3H content by standard liquid scintillation counting techniques. K_i values for nucleoside inhibition of [^{3}H]uridine uptake were calculated from IC_{50} values in a manner analogous to that described above for inhibition of [^{3}H]NBMPR binding, except that in this case [L] represents the assay concentration of [^{3}H]uridine (100 μM) and K_D represents the K_m of [^{3}H]uridine for the Ehrlich cell nucleoside transporter (156 μM).[1] Note that these [^{3}H]uridine influx studies do not distinguish between the NBMPR-sensitive and NBMPR-resistant nucleoside transporters that are known to exist in Ehrlich cells (10). Such a distinction was not considered necessary in the present study, because the nucleosides tested have similar affinities for both forms of the transporter (2).[1]

Results

Association rate analysis. The time course of binding of [^{3}H]NBMPR to Ehrlich cell plasma membranes was established in detail using 0.65 nM [^{3}H]NBMPR (Fig. 1A). The rate of specific binding was best described as a two-component exponential association, where 79 ± 2% of the binding reaction was rapid, with a $t_{1/2}$ of 0.40 ± 0.02 min (phase 1), and the remaining 21% of the binding (phase 2) was much slower, with a $t_{1/2}$ of 2.68 ± 0.19 min (29 experiments). Similar experiments conducted using a range of concentrations of [^{3}H]NBMPR allowed the estimation, assuming pseudo-first-order kinetics, of association (k_{+1}) and dissociation (k_{-1}) rate constants of 1.06 ± 0.05 min^{-1} nM^{-1} and 0.25 ± 0.03 min^{-1}, respectively (linear regression, 47 data points) for the initial phase (phase 1) of [^{3}H]NBMPR binding (Fig. 1B). Although the ratio of phase 1 to phase 2 association remained relatively constant over the [^{3}H]NBMPR concentration range tested (0.1–1.2 nM), the intraexperimental variability observed in the phase 2 component made the calculation of independent rate constants for this component impractical. Consequently, the contribution of phase 2 to the overall association rate was estimated using the composite k_{ob} [((% phase 1 × k_{ob_1}) + (% phase 2 × k_{ob_2}))/100] determined at each [^{3}H]NBMPR concentration; composite k_{+1} and k_{-1} values were 0.81 ± 0.08 min^{-1} nM^{-1} and 0.15 ± 0.04 min^{-1}, respectively (linear regression, 33 data points).

A series of nucleoside transport inhibitors and substrates were tested, at submaximal inhibitory concentrations, for their effects on the rate of association of [^{3}H]NBMPR with its specific binding sites (Figs. 2 and 3). It was not practical to measure inhibitor effects on the individual components of [^{3}H]NBMPR association, due to both the inherent experimental variability of phase 2 and the inability to distinguish between the inhibition of phase 1 association and the modification of the ratio of phase 1/phase 2. Therefore, analysis was done using the composite rate of association (see above). In general, agents that decreased the equilibrium binding of [^{3}H]NBMPR also inhibited the rate of association of [^{3}H]NBMPR, at analogous concentrations (fig. 2). NBTGR was the exception to this basic relationship; NBTGR was a very potent inhibitor of the equilibrium binding of [^{3}H]NBMPR, yet had no effect on the rate of association of [^{3}H]NBMPR (data not shown). IC_{50} values for inhibition of association (k_{ob}) were generally similar to the K_i values obtained for inhibition of equilibrium binding (Table 1); it is interesting to note, however, that the minor differences between these two parameters (see ratios in Table 1) followed a pattern compatible with the effects of these agents on [^{3}H]NBMPR dissociation (see below). To compare agents with different inhibitory potencies, the results from these studies were normalized by correlation of the half-time for [^{3}H]NBMPR association ($t_{1/2}$) in the presence of inhibitor with the percentage of inhibition of the equilibrium binding of [^{3}H]NBMPR (Fig. 3). A noncooperative competitive inhibitor would be expected to increase the $t_{1/2}$ in a manner directly proportional to the degree of inhibition of the equilibrium binding of [^{3}H]NBMPR. A linear relationship between $t_{1/2}$ and the inhibition of [^{3}H]NBMPR binding was observed for a range of concentrations of dilazep, R75231 (Fig. 3), and CV-1808 (data not shown). However, the other inhibitors tested did not affect [^{3}H]NBMPR association in a manner compatible with noncooperative competitive interactions. The effects of adenosine and diazepam on binding $t_{1/2}$ decreased with increasing inhibitor

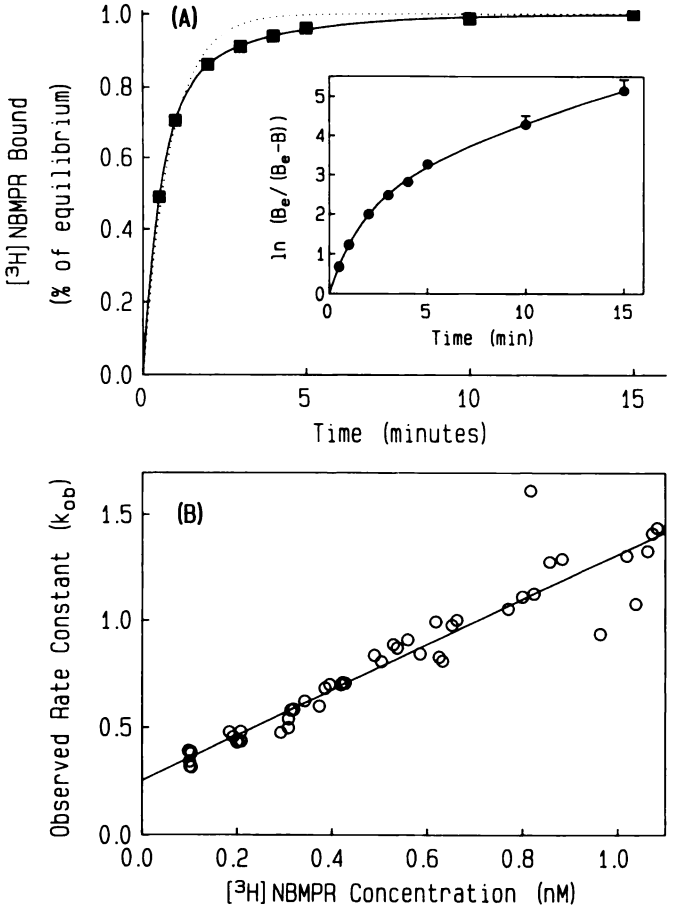

Fig. 1. Time course for association of [^{3}H]NBMPR with its specific binding sites in Ehrlich cell plasma membranes. A, Membranes were incubated with 0.65 nM [^{3}H]NBMPR for the indicated times, at 22° in 50 mM Tris·HCl buffer, pH 7.1, and the reaction was terminated by filtration through Whatman GF/B filters. *Solid and dotted lines*, best-fit curves to the data based on a two-site or one-site association model, respectively. *Inset*, pseudo-first-order plot of these data. B_e Amount bound at steady state; B, amount bound at each defined incubation time. The observed curvilinear plot indicates that this reaction was not a simple bimolecular one. Each *point* represents the mean ± standard error from 28 experiments. B, Time courses for the association of a range of concentrations of [^{3}H]NBMPR with Ehrlich cell membranes were constructed as described above. The observed rate constant (k_{ob}) was derived from the initial rate of association (major phase, representing 79% of the binding) and plotted against the concentration of [^{3}H]NBMPR used. The resulting linear plot (cumulative data from six independent experiments) was used to obtain estimates of the association (k_{+1}) and dissociation (k_{-1}) rate constants.

[1] J. R. Hammond, unpublished observations.

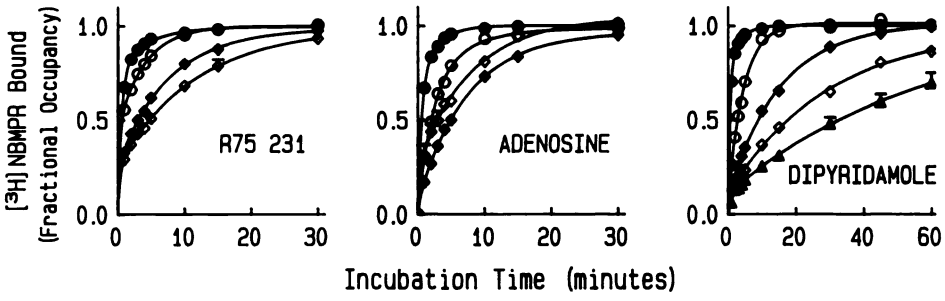

Fig. 2. Inhibition of the rate of association of [^{3}H]NBMPR with Ehrlich cell membranes by R75231, adenosine, and dipyridamole. Time courses for [^{3}H]NBMPR binding were constructed in the absence (●) and presence of the following concentrations of inhibitors: R75231, 3 (○), 30 (♦), and 300 (◇) nM; adenosine, 0.1 (○), 1 (♦), and 10 (◇) mM; dipyridamole, 0.3 (○), 3 (♦), 10 (◇), and 30 (▲) μM. These inhibitor concentrations were chosen to provide a 15–85% inhibition of the equilibrium binding of [^{3}H]NBMPR. *Ordinate of each plot*, fraction of the steady state binding of [^{3}H]NBMPR attained in the presence of the indicated concentrations of inhibitors at the incubation times shown (*abscissa*). Each *point* represents the mean ± standard error from five experiments. Note that 10 mM adenosine was not as effective as 1.0 mM adenosine in inhibiting the rate of association of [^{3}H]NBMPR.

concentration, leading to a hyperbolic relationship between $t_{1/2}$ and binding inhibition (Fig. 3). In contrast, the effects of dipyridamole and mioflazine on $t_{1/2}$ increased with increasing inhibitor concentration, such that an exponential relationship was observed (Fig. 3). Indeed, in the presence of 10 μM dipyridamole, incubation times in excess of 1 hr were required for [^{3}H]NBMPR binding equilibrium to be attained (Fig. 2).

Dissociation rate analysis. The rate of dissociation of [^{3}H]NBMPR from its specific sites was determined directly by incubating membranes with 0.65 nM [^{3}H]NBMPR for 45 min and then initiating dissociation by the addition of a supramaximal inhibitory concentration of NBTGR (10 μM). The dissociation of equilibrium bound [^{3}H]NBMPR from Ehrlich cell membranes was monophasic (Fig. 4), indicative of a simple noncooperative reaction between [^{3}H]NBMPR and its binding sites, with a k_{-1} value of 0.080 ± 0.002 min^{-1} (28 experiments). This value is significantly lower than the k_{-1} values derived from association rate studies (see above). A similar rate of dissociation of [^{3}H]NBMPR was obtained using intact Ehrlich cells (0.09 min^{-1}) (three experiments). However, when analogous studies were conducted using membranes solubilized with 1.0% octylglucoside, a significantly (Student's t test, $p < 0.05$) higher k_{-1} value of 0.157 ± 0.007 min^{-1} (six experiments) was obtained (Fig. 4).

As has been noted in other biological models (12, 18, 19, 21), high concentrations of adenosine and dipyridamole enhanced and inhibited, respectively, the rate of NBTGR-induced dissociation of [^{3}H]NBMPR from Ehrlich cell plasma membranes. In the present study, dipyridamole and adenosine also modified, in a similar manner, the rates of dissociation of [^{3}H]NBMPR from both octylglucoside-solubilized Ehrlich cell membranes and intact Ehrlich cells (Fig. 4, Table 2). Furthermore, although the NBTGR-induced rate of dissociation of [^{3}H]NBMPR from octylglucoside-solubilized preparations was significantly ($p < 0.05$) greater than that observed for intact plasma membranes, the relative effects of adenosine, dipyridamole, and NBTGR were similar for each preparation (Table 2).

The initial studies described above used concentrations of adenosine and dipyridamole that were close to their limits of solubility, but significant effects were also observed at much lower inhibitor concentrations and with a wide variety of different agents. Fig. 5 compares the inhibitory effects of a range of concentrations of dilazep, dipyridamole, and mioflazine on the rate of dissociation and the equilibrium binding of [^{3}H]NBMPR; the respective inhibition constants are shown in Table 3. There was considerable overlap in the range of concentrations of dipyridamole required to inhibit [^{3}H]NBMPR equilibrium binding and dissociation; for example, a concentration of dipyridamole that inhibited equilibrium binding by 75% inhibited the rate of [^{3}H]NBMPR dissociation by 25%. Mioflazine was similar to dipyridamole, in that the concentration-effect curves for the inhibition of [^{3}H]NBMPR dissociation and the inhibition of equilibrium binding overlapped extensively (Fig. 5). Mioflazine, however, did not suppress the dissociation rate of [^{3}H]NBMPR (maximum inhibition = 58%) to the same degree as did dipyridamole (maximum inhibition = 90%). In contrast, R75231, an analogue of lidoflazine similar to mioflazine, was a very poor inhibitor of [^{3}H]NBMPR dissociation even though it was 6-fold more potent than mioflazine as an inhibitor of the equilibrium binding of [^{3}H]NBMPR (Table 3).

A wide variety of purine and pyrimidine nucleosides enhanced the rate of dissociation of [^{3}H]NBMPR from its specific binding sites in Ehrlich cell membranes. Their relative potencies were estimated from the slopes of linear plots of nucleoside concentration versus percentage of enhancement of [^{3}H]NBMPR dissociation rate (see Fig. 6); EC_{20} values (concentration that enhances dissociation rate by 20%) interpolated from such plots are shown in Table 4. Nucleoside substrates for the transporter including 2-chloroadenosine (26), enhanced the rate of [^{3}H]NBMPR dissociation with an order of effectiveness similar to that found for their inhibition of both the equilibrium binding of [^{3}H]NBMPR and the cellular uptake of [^{3}H]uridine (2′-deoxyadenosine > 2-chloroadenosine > adenosine = thymidine > uridine > 2′-deoxycytidine > cytidine) (Table 4). However, if one compared nucleoside potencies relative to that of adenosine (see Fig. 7), it was clear that the enhancement of [^{3}H]NBMPR dissociation rate more closely reflected the inhibition of [^{3}H]uridine uptake (slope of correlation = 0.96) than it did the inhibition of the equilibrium binding of [^{3}H]NBMPR (slope of correlation = 1.76). On the other hand, cyclopentyladenosine and CV-1808, nucleoside analogues that are not considered to be substrates for the transporter, were 100 times more effective than adenosine as inhibitors of the equilibrium binding of [^{3}H]NBMPR and [^{3}H]uridine uptake (Table 4) but were only 6 times more potent than adenosine as enhancers of

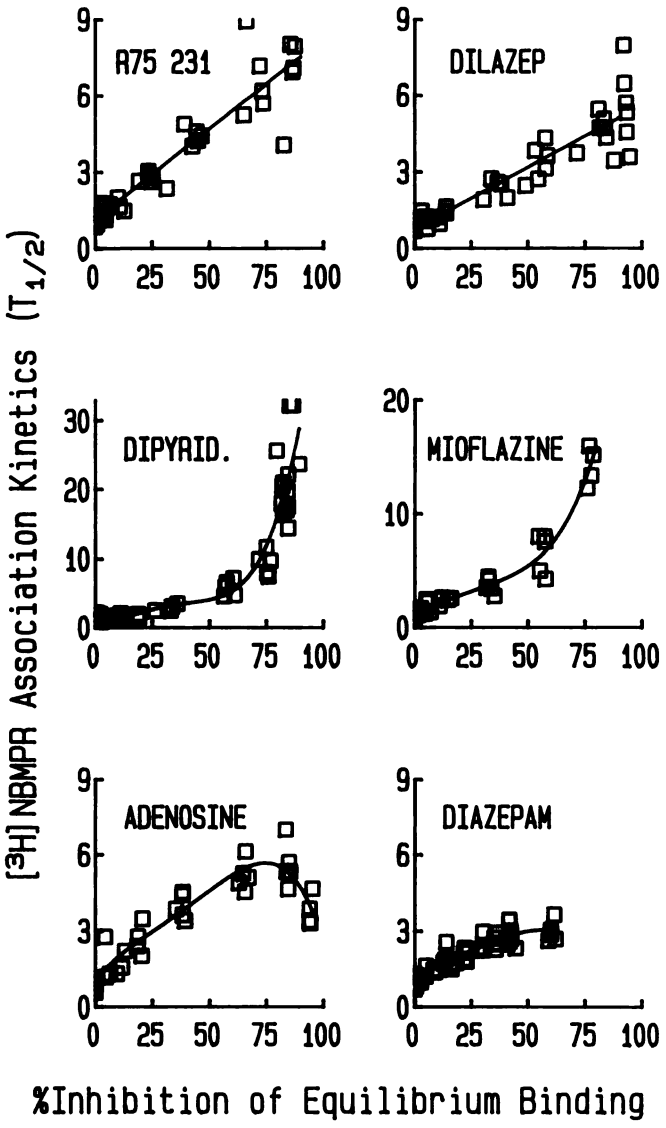

Fig. 3. Effect of inhibitors of [^{3}H]NBMPR binding on the rate of association of [^{3}H]NBMPR with Ehrlich cell membranes. Time courses for [^{3}H]NBMPR binding were constructed in the presence and absence of a range of concentrations of the indicated agents, to obtain the half-time for the [^{3}H]NBMPR binding reaction ($t_{1/2}$) as well as the steady state (equilibrium) level of binding in the presence of each concentration of inhibitor. To allow comparison of inhibitors with widely differing potencies, results were normalized by plotting of the association $t_{1/2}$ (*ordinate*) against the percentage of inhibition of the equilibrium binding (*abscissa*) observed for each concentration of inhibitor. Plots show the cumulative data from at least five independent experiments.

the rate of [^{3}H]NBMPR dissociation. The benzodiazepines diazepam, chlordiazepoxide, and triazolam were the most effective compounds tested for enhancing the rate of dissociation of [^{3}H]NBMPR (Table 4). ATP and the adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine had no effect on the rate of dissociation of [^{3}H]NBMPR, nor did these agents inhibit the equilibrium binding of [^{3}H]NBMPR to Ehrlich cell plasma membranes.

Equilibrium binding analysis. Equilibrium binding studies with [^{3}H]NBMPR (0.01–2 nM) indicated an apparent single class of noncooperative binding sites for [^{3}H]NBMPR in Ehrlich cell plasma membranes (linear Scatchard analyses), yielding a K_D value of 0.11 ± 0.02 nM (seven experiments). This is almost identical to the K_D value for [^{3}H]NBMPR of 0.10 nM calculated from the kinetic studies described above (NBTGR-induced k_{-1} + composite k_{+1}) and is comparable to that reported previously by this laboratory (10).

The effects of inhibitors on the equilibrium binding of [^{3}H]NBMPR have been alluded to in conjunction with the results of the dissociation rate studies (see above). The potencies of a variety of agents for inhibition of the equilibrium binding of [^{3}H]NBMPR are shown in Tables 1, 3, and 4. When a range of concentrations of inhibitor were tested against a single concentration of [^{3}H]NBMPR (0.65 nM), using an incubation time in excess of 2 hr, pseudo-Hill coefficients not different from unity were obtained for all inhibitors tested, including dipyridamole and mioflazine. However, if the more customary (see below) incubation times of less than 1 hr were used (Fig. 5, Table 3), pseudo-Hill coefficients significantly (Students *t* test, $p < 0.05$) less than unity were obtained for dipyridamole (0.76 ± 0.04; seven experiments), whereas mioflazine and R75231 had pseudo-Hill coefficients significantly greater than unity (1.58 ± 0.14 and 1.56 ± 0.16, respectively; five experiments). It is important to note that virtually all previous studies on the inhibition of the binding of [^{3}H]NBMPR have used incubation times of 1 hr or less. Although incubation time affected the slope of the inhibition curves for dipyridamole and mioflazine, the K_i values derived from such curves were not significantly different (Students *t* test, $p < 0.05$; compare values in Tables 1 and 3). However, the potency of R75231 appeared to increase 10-fold upon extension of the incubation time to 2 hr; the reasons for this discrepancy are under further investigation. This difference notwithstanding, R75231 was clearly one of the more potent inhibitors of [^{3}H]NBMPR binding tested in this study, yet it was relatively ineffective in modifying the rate of dissociation of [^{3}H]NBMPR.

Discussion

These data are consistent with a model in which the affinity of the [^{3}H]NBMPR binding site is modulated allosterically by ligand interactions with pharmacologically distinct site(s) on the nucleoside transport complex. The strong correlation between the enhancing effects of nucleosides on [^{3}H]NBMPR dissociation and their inhibitory effects on [^{3}H]uridine uptake suggests that this modulation may entail interactions with the permeant site of the transporter.

Detailed analysis of the rate of association of [^{3}H]NBMPR with Ehrlich cell membranes revealed a distinct two-phase process. A similar biphasic association rate profile for [^{3}H]NBMPR binding to sheep erythrocytes was apparent in a study by Jarvis and Young (see Fig. 4 in Ref. 6) but was not analyzed in detail by these investigators. Such results are indicative of either multiple forms of binding sites or different binding site pools. If multiple distinct binding sites were responsible, one would expect curvilinear Scatchard plots and/or multiphasic [^{3}H]NBMPR dissociation rate profiles, neither of which were observed in the present study. The possibility that the more slowly associating component may reflect the time required for diffusion of [^{3}H]NBMPR to sites located inside plasma membrane vesicles is also unlikely, due to the high lipophilicity of NBMPR (21). An alternate explanation is that [^{3}H]NBMPR binds with high affinity only to a specific conformation of the nucleoside transporter. The fast component of [^{3}H]NBMPR association (phase 1) may represent binding to transporters

TABLE 1

Comparison of the effects of nucleoside transport inhibitors on the rate of association of [^{3}H]NBMPR with their effects on the equilibrium binding (2 hr) of [^{3}H]NBMPR to Ehrlich cell membranes

The rate of association of [^{3}H]NBMPR (composite k_{ob}; see Results) was determined in the presence and absence of a range of concentrations of the indicated compounds. IC_{50} values for inhibition of association were derived from sigmoid curves fitted to plots of inhibitor concentration versus k_{ob}. IC_{50} values for the inhibition of the equilibrium binding of [^{3}H]NBMPR were obtained in the same experiments by fitting of sigmoid curves to plots of percentage of control binding versus inhibitor concentration, obtained after allowing at least 2 hr for binding equilibration to occur; K_i values were derived from the relationship $K_i = IC_{50}/(1 + [L]/K_D)$, as described in the text. Each value represents the mean ± standard error from at least five independent experiments. The ratios obtained for dipyridamole and dilazep are significantly greater than those for mioflazine and R75231, which are significantly greater than the ratios obtained for adenosine, diazepam, and CV-1808 (analysis of variance, $p < 0.05$).

Inhibitor	Rate of association, IC_{50}	Equilibrium binding, K_i	Ratio of IC_{50}/K_i
	M	*M*	
Adenosine	$2.98 \pm 0.30 \times 10^{-5}$	$7.54 \pm 0.03 \times 10^{-5}$	0.39 ± 0.04
Diazepam	$7.97 \pm 0.70 \times 10^{-6}$	$1.97 \pm 0.01 \times 10^{-5}$	0.40 ± 0.03
CV-1808	$1.95 \pm 0.23 \times 10^{-7}$	$7.43 \pm 0.23 \times 10^{-7}$	0.26 ± 0.04
Dipyridamole	$9.00 \pm 0.77 \times 10^{-8}$	$7.67 \pm 0.17 \times 10^{-8}$	1.17 ± 0.13
Dilazep	$3.62 \pm 0.51 \times 10^{-9}$	$2.71 \pm 0.12 \times 10^{-9}$	1.33 ± 0.26
Mioflazine	$2.63 \pm 0.21 \times 10^{-7}$	$3.36 \pm 0.03 \times 10^{-7}$	0.78 ± 0.07
R75231	$4.34 \pm 0.02 \times 10^{-9}$	$5.23 \pm 0.12 \times 10^{-9}$	0.83 ± 0.02

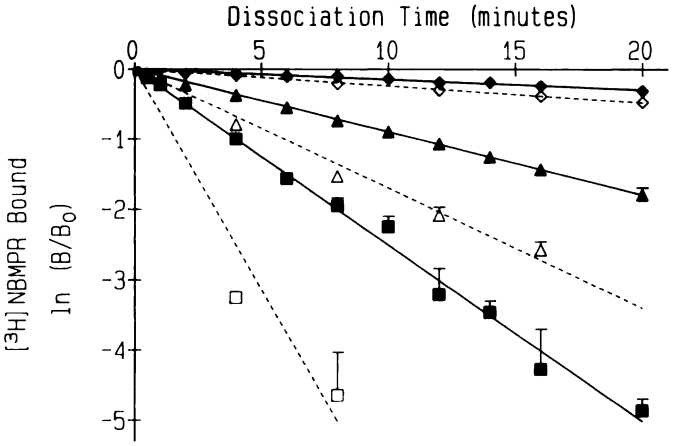

Fig. 4. Dissociation of [^{3}H]NBMPR from intact Ehrlich cell plasma membranes (*solid symbols*) and membranes solubilized with 1% octylglucoside/0.15% asolectin (*open symbols*). Preparations were incubated with 0.65 nM [^{3}H]NBMPR for 60 min at 22° in 50 mM Tris·HCl buffer, pH 7.1. Dissociation of [^{3}H]NBMPR was induced by the addition of 10 μM NBTGR alone (*triangles*) or in combination with 6 mM adenosine (*squares*) or 100 μM dipyridamole (*diamonds*). Data are plotted as $\ln(B/B_0)$ against time, where B = site-bound [^{3}H]NBMPR at each time after the addition of inhibitor and B_0 = site-bound [^{3}H]NBMPR at equilibrium (time 0). Assays were terminated as described in the text, and each *point* is the mean ± standard error from five experiments. The k_{-1} values derived from these experiments are shown in Table 2.

that are initially in the appropriate conformation; the slower association component (phase 2) may represent the rate at which the remaining transporters attain the appropriate conformation. Based on a model proposed by Jarvis and Young (27), it is tempting to speculate that the ratio of fast/slow [^{3}H]NBMPR association rates may be related directly to the ratio of outward/inward facing conformations of the nucleoside transporter.

The ability of [^{3}H]NBMPR to dissociate from its binding sites also appears to decrease with time. This was illustrated in the present study by the significant difference between the dissociation rate constant derived from the association rate studies (0.25 min^{-1}; see Fig. 1) and that obtained by NBTGR-induced displacement of equilibrium binding (0.08 min^{-1}; see Fig. 4). The former value represents the initial ligand dissociation characteristics, whereas the latter was obtained after a 45-min incubation of membranes with [^{3}H]NBMPR.

The capacities of dipyridamole and adenosine to modify the rate of dissociation of [^{3}H]NBMPR have been well documented (12, 18, 19, 21). The present study extends these observations to include a wide variety of other nucleoside transport inhibitors and substrates. In past studies, this phenomenon has sometimes been attributed to nonspecific membrane fluidity effects of high concentrations of these agents (2, 18, 19). This, however, is not a viable explanation for the present results, because similar effects were observed using membranes solubilized ("fluidized") in octylglucoside. Furthermore, not all agents that inhibited the equilibrium binding of [^{3}H]NBMPR inhibited the NBTGR-induced dissociation of [^{3}H]NBMPR. Most notable was R75231, which was more potent than dipyridamole and mioflazine as an inhibitor of [^{3}H]NBMPR binding but had no significant effect on the rate of dissociation of [^{3}H]NBMPR. Another lidoflazine analogue similar to R75231, R57974 [2-(aminocarbonyl)-4-[5,5-bis(4-fluorophenyl)pentyl]-

TABLE 2

Comparison of the rates of dissociation of [^{3}H]NBMPR from Ehrlich cell plasma membranes, octylglucoside-solubilized plasma membranes, and intact Ehrlich cells

Dissociation rate constants (k_{-1}) were calculated as the slopes of simple linear regression lines fitted to data plotted as shown in Fig. 4. Ratio represents the k_{-1} value obtained in the presence of dipyridamole or adenosine divided by the k_{-1} value obtained using NBTGR alone. Each value is the mean ± standard error from the number of experiments indicated in parentheses.

Preparation	NBTGR, k_{-1}	Dipyridamole/NBTGR		Adenosine/NBTGR	
		k_{-1}	Ratio	k_{-1}	Ratio
	min^{-1}	min^{-1}		min^{-1}	
Membranes	−0.090 ± 0.005 (6)	−0.014 ± 0.001 (6)	0.16 ± 0.01	−0.24 ± 0.01 (5)	2.6 ± 0.2
Soluble	−0.164 ± 0.007 (7)	−0.024 ± 0.001 (4)	0.17 ± 0.02	−0.31 ± 0.01 (3)	2.1 ± 0.1
Cells	−0.088 (2)	−0.016 (2)	0.18		

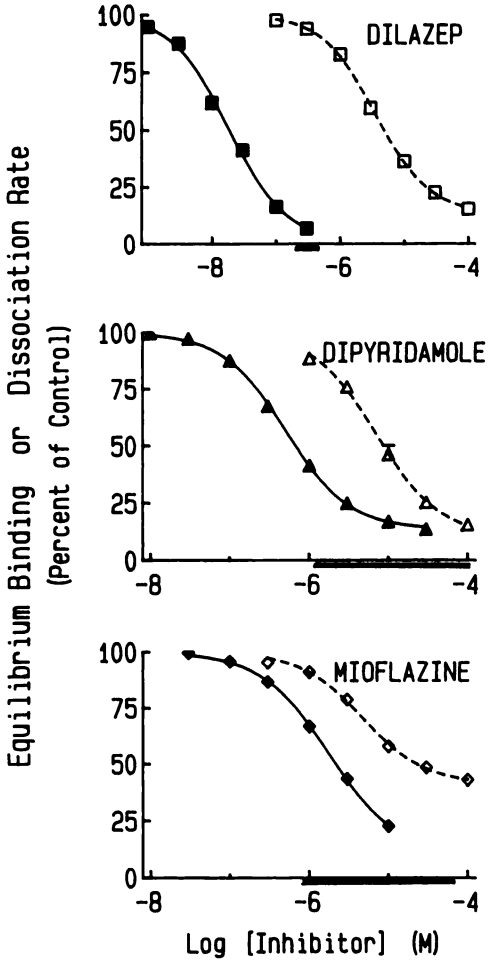

Fig. 5. Inhibition of [^{3}H]NBMPR binding and [^{3}H]NBMPR dissociation by dilazep, dipyridamole, and mioflazine. Equilibrium binding studies were conducted by incubation of membranes with [^{3}H]NBMPR in the presence of the indicated concentrations of inhibitors for 1 hr; data are expressed as the percentage of the binding observed in the absence of inhibitor (*solid symbols*). Rate constants for [^{3}H]NBMPR dissociation in the presence of 10 μM NBTGR plus the indicated concentrations of test inhibitor were obtained by linear regression analysis of data plotted as shown in Fig. 4. Data are expressed as a percentage of the dissociation rate induced by 10 μM NBTGR alone (*open symbols*). The concentrations of displacers that produced half-maximal effects (IC_{50}) on [^{3}H]NBMPR dissociation rate and equilibrium binding are shown in Table 3. Each *point* is the mean ± standard error from at least five experiments. *Double line below the abscissa*, the range of concentrations over which the compound inhibits both the rate of dissociation of [^{3}H]NBMPR and the equilibrium binding of [^{3}H]NBMPR.

N-(2,6-dichlorophenyl)-1-piperazineacetamide 2HCl], was also found to be ineffective in modifying the rate of dissociation of [^{3}H]NBMPR from hamster liver membranes (22). In general, the relative potencies of a series of compounds for inhibiting the rate of dissociation of [^{3}H]NBMPR from Ehrlich cell membranes was quite different from their relative potencies for inhibiting the equilibrium binding of [^{3}H]NBMPR (see Table 3). These results are indicative of interactions with distinct binding sites. A recent investigation on [^{3}H]dilazep binding to mouse lymphoma cells (28) also generated data supporting the existence of multiple forms of inhibitor binding sites on the nucleoside transport complex. Both high and low affinity binding sites for [^{3}H]dilazep were identified. Whereas the high affinity site appeared to coincide with the [^{3}H]NBMPR binding

TABLE 3

Comparison of the effects of nucleoside transport inhibitors on the rate of dissociation of [^{3}H]NBMPR with their effects on the equilibrium binding (1hr)of [^{3}H]NBMPR to Ehrlich cell membranes

The rate of dissociation (k_{-1}) of [^{3}H]NBMPR (0.65 nM) was determined in the presence of NBTGR (10 μM) and a range of concentrations of the indicated compounds. Equilibrium binding of [^{3}H]NBMPR (0.65 nM) was assessed as described in the text, using 1-hr incubation times at room temperature (22°); K_i values were derived from plots of inhibitor concentration versus percentage of control equilibrium binding. IC_{50} values for inhibition of [^{3}H]NBMPR dissociation were interpolated from sigmoid curves fitted to plots of inhibitor concentration versus percentage of control dissociation rate (see Fig. 5). Each value represents the mean ± standard error from the number of experiments indicated in parentheses. Ratio represents the IC_{50} value for inhibition of [^{3}H]NBMPR dissociation divided by the K_i value for inhibition of equilibrium binding.

Inhibitor	Rate of dissociation, IC_{50}	Equilibrium binding, K_i	Ratio
	μM	nM	
Dilazep	4.86 ± 0.14 (6)	2.3 ± 0.3 (4)	2,113
R75231	692 ± 12 (6)	65 ± 10 (5)	10,646
Dipyridamole	8.75 ± 0.27 (6)	96 ± 11 (7)	91
Mioflazine	23 ± 1 (6)	390 ± 20 (5)	59
Soluflazine	159 ± 3 (7)	790 ± 19 (5)	201

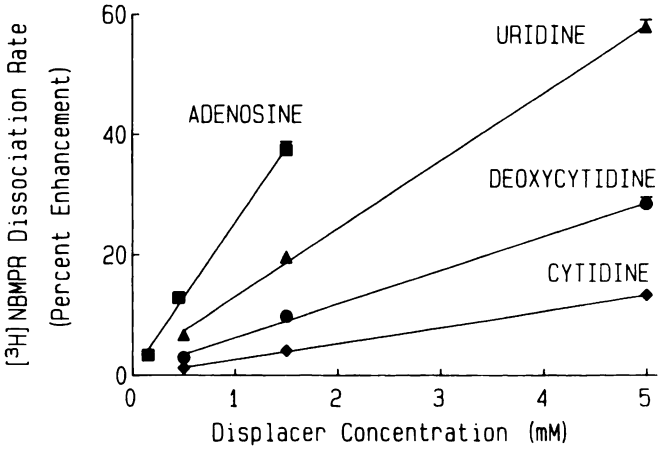

Fig. 6. Enhancement of the rate of [^{3}H]NBMPR dissociation from Ehrlich cell membranes by nucleosides. Rate constants for [^{3}H]NBMPR dissociation in the presence of 10 μM NBTGR plus the indicated concentrations of nucleosides were obtained by linear regression analysis of data plotted as shown in Fig. 4. Data are expressed as the percentage increase in the rate constant (k_{-1}) in the presence of nucleoside, compared with the k_{-1} obtained using NBTGR alone. Each *point* represents the mean ± standard error from five experiments. EC_{20} values (concentrations that induce a 20% enhancement in dissociation rate) derived via linear regression analysis of similar data obtained from experiments using a wide variety of nucleosides and benzodiazepines are shown in Table 4.

site, the low affinity binding site for [^{3}H]dilazep may be related to the "allosteric" inhibitor binding site characterized in the present study.

In contrast to the transport inhibitors discussed above, nucleoside substrates enhanced the rate of [^{3}H]NBMPR dissociation, with an order of effectiveness that paralleled their affinities for the transporter. It is possible that the overall binding affinity of [^{3}H]NBMPR reflects the average of two conformations in dynamic equilibrium. Nucleosides would force the equilibrium toward the fast dissociating conformation via interactions with the permeant site, whereas nonnucleoside ligands such as dipyridamole, which are not substrates for the transporter, would bind to the system and lock the conformation in the relatively high affinity state. The fact that benzodiazepines are also very effective enhancers of [^{3}H]NBMPR dissociation suggests that these agents inhibit nucleoside trans-

TABLE 4

Comparison of the capacities of a series of nucleoside analogues, transporter substrates, and benzodiazepines to enhance the rate of dissociation of [^{3}H]NBMPR with their inhibitory effects on the equilibrium binding of [^{3}H]NBMPR to Ehrlich cell membranes and the cellular accumulation of [^{3}H]uridine

The rate of dissociation of [^{3}H]NBMPR (k_{-1}) was determined in the presence of NBTGR (10 μM) and a range of concentrations of the indicated compounds. The concentrations that enhanced the dissociation of [^{3}H]NBMPR by 20% (EC_{20} values) were interpolated from linear plots of percentage of enhancement versus concentration of test agent (see Fig. 6). K_i values for the inhibition of the equilibrium binding of [^{3}H]NBMPR and the cellular uptake of [^{3}H]uridine (100 μM) were obtained as described in the text. Each value represents the mean ± standard error from five independent experiments.

Compound	Rate of dissociation, EC_{20}	Equilibrium binding K_i	[^{3}H]Uridine uptake, K_i
	μM	μM	μM
Diazepam	41 ± 1	13 ± 1	15 ± 2
Chlordiazepoxide	95 ± 4	—	—
Triazolam	100 ± 3	—	—
Cyclopentyladenosine	115 ± 3	0.34 ± 0.08	1.1 ± 0.2
CV-1808	123 ± 7	0.38 ± 0.07	0.33 ± 0.06
2′-Deoxyadenosine	511 ± 25	44 ± 2	41 ± 4
2-Chloroadenosine	644 ± 60	29 ± 2	32 ± 3
Adenosine	786 ± 47	69 ± 5	52 ± 2
Thymidine	809 ± 26	466 ± 28	173 ± 15
Uridine	1620 ± 50	594 ± 31	156 ± 47
2′-Deoxycytidine	3460 ± 180	858 ± 48	254 ± 15
Cytidine	7510 ± 140	5260 ± 772	569 ± 63

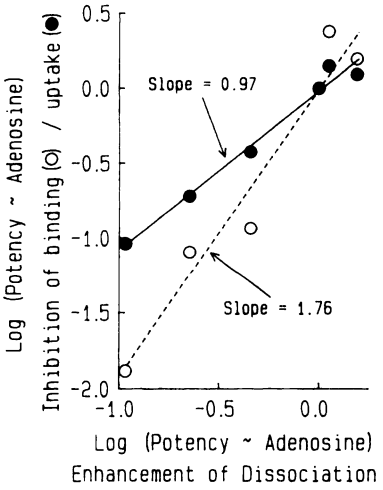

Fig. 7. Correlation of the relative potencies of a variety of nucleosides for enhancing the rate of dissociation of [^{3}H]NBMPR from Ehrlich cell membranes with (a) their relative potencies for inhibiting [^{3}H]uridine uptake by Ehrlich cells (●) and (b) their relative potencies for inhibiting the equilibrium binding of [^{3}H]NBMPR to Ehrlich cell membranes (○). Assays were conducted at 22°, as described in the text, using the following nucleosides (in increasing order of potency; plotted from *left* to *right*): cytidine, 2′-deoxycytidine, adenosine 2-chloroadenosine and 2′-deoxyadenosine. Data for each measured parameter are expressed as the logarithm of the potency of each nucleoside relative to the potency of adenosine; the intersection point for the two correlations corresponds to adenosine (log relative potency = 0). The definitive potencies of these nucleosides for enhancing [^{3}H]NBMPR dissociation, inhibiting [^{3}H]NBMPR equilibrium binding, and inhibiting [^{3}H]uridine uptake are shown in Table 4.

port and [^{3}H]NBMPR binding by competing for the permeant site of the transporter. These results also imply that benzodiazepines can induce a nucleoside-like conformation change in the transporter.

The effects of compounds on the rate of association of [^{3}H]NBMPR with Ehrlich cell membranes are also compatible with a model invoking allosteric modulation of [^{3}H]NBMPR binding site affinity. All competitive inhibitors of [^{3}H]NBMPR binding would be expected to inhibit the rate of [^{3}H]NBMPR association, but this effect should be linearly related to their ability to inhibit the equilibrium binding of [^{3}H]NBMPR. This expected relationship was not observed in the present study. Agents that enhanced [^{3}H]NBMPR dissociation, such as diazepam and adenosine, had less than the expected effect on the rate of [^{3}H]NBMPR association, whereas agents that inhibited dissociation, such as dipyridamole and mioflazine, decreased the rate of association of [^{3}H]NBMPR dramatically (see Figs. 2 and 3). A similar effect of dipyridamole on the rate of association of [^{3}H]NBMPR has been reported using rat and guinea pig lung membranes (19), indicating that this phenomenon is not peculiar to Ehrlich cells.

The dramatic effects of agents such as dipyridamole and mioflazine on the rate of [^{3}H]NBMPR association highlight an important methodological consideration. Traditionally, [^{3}H]NBMPR binding assays utilize incubation times of 1 hr or less (for example, see Ref. 16). However, it is clear from this study that in the presence of high concentrations of dipyridamole or mioflazine incubation times well in excess of 1 hr are required to attain [^{3}H]NBMPR binding equilibrium. The effects of these inhibitors, and possibly others, on [^{3}H]NBMPR association and dissociation kinetics, if not considered in experimental design or analysis, may lead to artifactual inhibitor K_i values and Hill coefficients, as well as apparent discrepancies between the relative potencies of agents as inhibitors of [^{3}H]NBMPR binding and nucleoside transport. This is particularly important for studies involving the relatively dipyridamole/mioflazine/dilazep-insensitive transporters found in rat and mouse tissues (12, 16, 22).

In conclusion, the binding of [^{3}H]NBMPR is subject to allosteric modulation via ligand interactions with distinct sites on the nucleoside transporter. This modulation may reflect, in part, ligand-induced changes in the dynamic equilibrium between two (or more) conformation states involved in the process of nucleoside translocation. The pharmacological relevance of these allosteric interactions is evident from the results of this study; however, their role in the functioning of the nucleoside transporter under physiological conditions is unknown. The answer to this question awaits detailed molecular characterization of the purified and reconstituted transporter components.

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