Role of Endocytosis and Lysosomal pH in Uptake of N-(Phosphonacetyl)-L-aspartate and Its Inhibition of Pyrimidine Synthesis

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ABSTRACT

The mechanism of uptake and retention of N-(phosphonacetyl)-L-aspartate (PALA) was examined. Uptake of [3H]PALA by Ehrlich ascites tumor cells appeared to be biphasic. A small, variable quantity of PALA associated with cells within 5 min; the significance of this rapid uptake component is unclear. Between 15 min and 5 h, uptake was linear and consistent from experiment to experiment. The properties of the slow phase of PALA uptake are consistent with fluid-phase endocytosis. The intracellular PALA concentration approached the extracellular level very slowly, at a rate of approximately 1%/hr. The velocity of PALA uptake in these cells was proportional to the concentration in the media from 10^-6 to 10^-2 M. Uptake of PALA was identical to that of the extracellular marker inulin. Uptake of both PALA and inulin was inhibited by colchicine and stimulated by phorbol myristate acetate. The microtubule antagonist and the phorbol ester are known to, respectively, inhibit and stimulate endocytosis in other cell types. Phorbol myristate acetate enhanced the ability of PALA to inhibit incorporation of [14C]bicarbonate into pyrimidine nucleotides, presumably through an increase in PALA uptake. This inhibitory action of PALA was almost completely blocked by two agents known to neutralize lysosomal pH, NH4Cl and methylamine. These results suggest that intracellular PALA is initially compartmentalized in a pinosomal vesicle which may later fuse with cellular lysosomes. Neutralization of lysosomal pH prevents the protonation of some or all of the four negatively charged groups found in the structure of PALA which may be necessary for its diffusion across the lysosomal membrane and eventual inhibition of aspartate transcarbamylase. Since partitioning of the fully charged molecule into the lipid phase of the plasma membrane for diffusion out of the cell should be minimal, the effects of PALA on cellular metabolism are expected to be prolonged.

INTRODUCTION

PALA^3 is an analogue of the putative transition state intermediate for the reaction catalyzed by ATCase (8). Inhibition of this enzyme blocks pyrimidine synthesis and results in decreased pyrimidine nucleotide pools (34, 35). It is highly effective against some murine solid tumors that are refractory to most chemotherapeutic agents (22, 23) but ineffective against murine leukemias (22). In clinical trials to date, PALA has been disappointing as single-agent therapy. This may be related, at least in part, to the capacity of human tumors to obtain pyrimidines by salvage pathways. In the future, PALA may be useful as part of a combination protocol with agents that inhibit pyrimidine transport or salvage enzymes. PALA has been shown to enhance the metabolism and cytotoxicity of 5-azacytidine (17), 1-ß-D-arabinofuranosylcytosine (16), and 5-fluorouracil (1, 18, 28, 31, 32) in tissue culture by lowering competing pyrimidine nucleotide pools. Several clinical trials of PALA with 5-fluorouracil have been reported recently (7, 10, 33, 50). The low hematological toxicity of PALA increases the likelihood of its use in combination therapy.

Some factors which determine relative sensitivity of tumor and host cells to PALA have been explored in several laboratories (20, 21, 23, 27, 30). Natural and acquired resistance to PALA has most often been correlated with elevated ATCase levels (23, 25, 27). This correlation is not a simple one. Small increases in ATCase result in large increases in the PALA concentration required for growth inhibition (23). Among tumors that are sensitive to PALA, there is little correlation between ATCase levels and growth inhibition (21, 30). Clearly, other properties of the cell must also be important.

The cell membrane has been shown to be a major determinant of the effectiveness of several other anticancer agents, most notably methotrexate. The significance of membrane transport as a determinant of PALA action is not yet clear. There have been few reports of direct measurements of PALA uptake, and these give conflicting results. Data on PALA uptake in human peripheral leukocytes are consistent with a carrier-mediated system (26), while PALA uptake in cultured Lewis lung carcinoma cells seems to be by passive diffusion (27). In these cell types and in other murine tumors as well (21), uptake was very slow. Jayaram et al. (21) reported that, after 2 hr of incubation, the concentration of PALA in Ehrlich cells was only 1% of that of extracellular level. Intracellular PALA appears to be retained in cells for prolonged periods following drug exposure. Numerous studies have shown sustained inhibition of ATCase and pyrimidine synthesis for several days after removal of the drug (20, 27, 34, 35, 49, 52). There has been no indication that PALA is converted to a less permeable metabolite. Kensler et al. (27) showed that resistance in Lewis lung cell lines correlated with an increased rate of loss of PALA from the tumor in vivo.

This report describes studies aimed at determining the mechanism of PALA uptake and retention. The slow and nonsaturable uptake of PALA was confirmed in the Ehrlich ascites tumor. Additional experiments strongly suggest that the mechanism of PALA uptake is not passive diffusion but fluid-phase endocytosis. The model that is most consistent with these results involves uptake of PALA into an endocytotic vesicle pinched off from the plasma membrane which eventually fuses with a lysosome. In an acid environment, PALA would be partially protonated and, with fewer negatively charged groups, PALA should partition into the lysosomal membrane at an increased rate. Only after escape from the lysosomal compartment does PALA reach its...
target enzyme in the cytoplasm. According to this hypothesis, loss of PALA from the cell and recovery of pyrimidine synthesis should be very slow since, at the neutral pH of the cytoplasm, PALA would be highly charged and unable to partition into the plasma membrane for efflux.

MATERIALS AND METHODS

Chemicals. Supplies of [14C]PALA and unlabeled PALA for preliminary experiments were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. PALA has also been obtained through the courtesy of Dr. Martin L. Black, Chemical Biological Information Department, Pharmaceutical Research Division, Warner-Lambert Co., Ann Arbor, MI. [3H]PALA was prepared from [3H]aspartate (8 or 34 Ci/mmol; Amersham Corp.) The [3H]aspartate was first purified by adsorption onto a 0.8- x 3-cm column of AG 50-X8 (Bio-Rad Laboratories, Richmond, CA) and elution with 2 M HCl. The acid was removed by lyophylization. The synthesis of [3H]PALA was carried out using a modification of the method described by Swyryd et al. (48). To eliminate the competition of another nucleophile with the amino group of aspartate for coupling with the acid chloride, the reaction was done in an organic solvent rather than in water. Phosphonocetamide chloride was prepared by reflux of 0.4 g of phosphonocetic acid (Sigma Chemical Co., St. Louis, MO) in 20 ml dry thionyl chloride (Aldrich Chemical Co., Milwaukee, WI) at 50° for 48 hr. The mixture was dried under a stream of argon and dissolved in 4 ml of freshly distilled dioxane. Purified [3H]aspartate was dissolved in 100 μl freshly distilled dimethylformamide containing 10 mg of dimethylaminopyridine (Aldrich). The deprotonation of aspartate by this reagent facilitates coupling. The reaction was started by adding 100 μl of the acid chloride solution to the [3H]aspartate mixture. After 2 hr at room temperature, the mixture was diluted with H2O and passed through a short column of AG 50 resin to remove unreacted [3H]aspartate. The column eluate was purified by chromatography on AG 1-X8 as described previously (48). Based on the amount of [3H]aspartate used in the reaction, the yield of [3H]PALA was about 80%. A slight brown color in the concentrated product suggested the presence of an impurity; however, the preparation was radiochemically pure by high-performance liquid chromatography (29).

NaH14CO3 was prepared from Ba14CO3 (ICN Radiochemicals, Irvine, CA). The solid Ba14CO3 was transferred to a 16- x 100-mm culture tube which was sealed with a rubber septum fitted with a disposable center well. The well contained 250 μl 1 N NaOH (approximately 3 times the equivalents of Ba14CO3) to capture the 14CO2 that was liberated upon injection of 1 N HCl into the bottom of the tube. After standing at room temperature for several hr, Na14CO3 was recovered from the center well and stored at -60°. [14C]Aspartate for assay of ATCase was obtained from ICN and purified as described above. [3H]inulin from ICN was purified on Bio-Gel P6 (Bio-Rad). The material eluting in the void volume was concentrated by lyophilization.

Tumor Cells and Uptake Procedure. Ehrlich ascites tumor cells were grown in male CF-1 mice (Charles River Breeding Laboratories, North Wilmington, MA) and passed weekly by i.p. inoculation of 0.2 ml of undiluted ascites fluid. Cells were harvested 7 to 10 days after injection and washed 3 times with room temperature saline. For uptake studies, washed cells were suspended in Earle's salts medium (Flow Laboratories) that was supplemented with Dnase II (100 units/ml; Sigma) to reduce clumping. PALA was prevented from having any pharmacological effect during uptake experiments by inclusion of 0.1 μm uridine. Stopped Erlenmeyer flasks containing cells and medium were incubated in a shaking water bath. Uptake was started by the addition of [3H]PALA (1 μCi/ml) plus 1 μM unlabeled PALA, except as indicated. The method for quantitating PALA uptake was similar to that described by Goldman et al. (15) for methotrexate uptake. A 1-ml aliquot of the cell suspension was injected into 10 ml of 0° saline, and cells were sedimented by centrifugation. The cells were resuspended and washed 3 times to remove all extracellular 3H. The pellet was aspirated into the tip of a Pasteur pipet, transferred to a 1.5-ml polyethylene tare, and dried overnight at 50-60°. The dried samples were weighed on a Cahn Model 25 electrobalance (Cahn Instruments, Paramount, CA), transferred to 7-ml scintillation vials, and digested in 0.25 ml 1 N NaOH for 1 hr at 60°. The digest was neutralized with 0.25 ml 1 N HCl and counted in 4 ml Amersham aqueous counting scintillation fluid. Results of these experiments, obtained initially as nmol/g, dry weight, could be expressed as nmol/ml of cell H2O by applying the conversion factor of 3.32 ml/g, dry weight (13, 44). In some cases, the data were expressed as μl of media cleared per ml cell H2O. This conversion is based on the assumption that if cells take up 1 nmol and the media concentration is 1 nmol/μl, then it would appear that the cells had "cleared" 1 μl.

NaH14CO3 incorporation into Pyrimidine Nucleotides of RNA and DNA. A modification of the method described by Huisman et al. (9) was used. Reduction of the unlabeled NaH14CO3 concentration from 15 to 3 mw increased the NaH14CO3 specific activity. The medium was supplemented with 12 μM morpholinopropanesulfonic acid, pH 7.4, to replace the lost buffering capacity. To achieve maximum rates of uptake, cell density was kept below 5 x 10⁶/ml, and the cells were not chilled during preparation. In the original method, cells were treated with acid to drive off unreacted NaH14CO3 and heated to convert purine nucleotides to pyrimidine nucleoside monophosphates. The purine bases were selectively adsorbed on a cation-exchange resin, and the [14C]-labeled pyrimidine nucleotides in the eluate were counted. We observed unacceptably high blanks of acid-stable radioactivity with this method despite the use of freshly prepared NaH14CO3. Washing the cells in 0° saline prior to extraction reduced but did not sufficiently eliminate this blank. To avoid this difficulty, incorporation of NaH14CO3 into pyrimidines of nucleic acids was measured. A 1.0-ml aliquot of the cell suspension was dilute with 10 ml 0° saline to stop the incorporation. After centrifugation, the pellet was extracted twice with cold 0.5 M HClO4. After suspension in 1 ml 0.5 M HCl, the sample was heated at 100° for 30 min, diluted with 2 ml 0.1 M HCl, and applied to a 0.8- x 3-cm column of Dowex 50-X8 (Bio-Rad) that was prequillibrated with 0.1 M HCl. The column was washed twice with 2 ml 0.1 M HCl, and the radioactivity in the combined eluates was counted as a gel with 10 ml aqueous counting scintillation fluid.

Preparation of Cell Extracts and Assay for ATCase. Washed cells were suspended at a density of 100 x 10⁶/ml in 50 ml 250 mM N2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, plus 0.1% Triton X-100 and then frozen at -20°. The suspension was thawed, vortexed, and centrifuged at 2000 x g for 10 min before assay was done on ATCase in the supernatant. Addition of Triton to extracts prepared without detergent had no effect on enzyme activity; however, inclusion of Triton in the freeze-thaw buffer increased extraction efficiency and uniformity. ATCase activity was assayed as described by Friedman et al. (12). The method for assay of [3H]PALA binding to ATCase was based on that of Penesky (38) as modified by Fry et al. (14). Enzyme extract and high-specific-activity [3H]PALA were mixed, brought to a volume of 150 μl with N2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, and incubated for 30 min at 37°. A minicolumn of Sephadex G-50-fine was prepared in the barrel of a tuberculin syringe plugged at the bottom with fibrous polyethylene. The column was centrifuged at 1000 x g for 3 min to remove excess fluid. A sample of the PALA-ATCase mixture (100 μl) was applied, and the column was centrifuged at 50 x g for 5 min, then at 1000 x g for 3 min. The protein-bound [3H] PALA was collected directly into a 4-ml scintillation vial for counting. Unbound [3H]PALA was quantitatively retained. To ensure that the enzyme was saturated, the concentration of [3H]PALA was increased until the level of binding was constant. The intracellular ATCase concentration was calculated by assuming 1.07 ml cell H2O/10⁶ cells (44).

RESULTS

Measurement of PALA Uptake. In previous reports of PALA uptake (21, 26, 27), methods were used for separation of cells...
from the media that were developed originally for rapidly transported substrates (51). With this procedure, uptake is terminated by rapid centrifugation of cells through a layer of oil, and intracellular PALA is calculated from the total counts in the pellet less the counts in the extracellular space. The extracellular space is determined in parallel experiments with an extracellular marker such as [3H]inulin. Application of this method to substrates that are taken up slowly is difficult. The extracellular space is 10 to 15% of the total aqueous space in the pellet (42). Jayaram et al. (21) reported that for Ehrlich cells intracellular PALA reached only 0.5 to 1% of the extracellular concentration after 1 hr. Thus, after 1 hr, intracellular radiolabel would represent less than 7% of total counts, and each step in sample processing requires great precision. In our hands, long incubations were necessary before [3H]PALA in cell pellets prepared by this procedure exceeded the level expected based on measurement of the [3H]-inulin space. For the studies reported here, cells were washed free of extracellular label in 0° saline. For this approach to be valid, loss of radiolabel during the wash steps with cold saline must be negligible. This was confirmed by comparison studies with the 2 methods which gave similar results for long incubations. Further, comparison of cellular [3H]PALA in samples suspended in 0° saline for 90 min and in samples that were washed as rapidly as possible revealed no difference.

Kinetics of PALA Uptake. Chart 1 shows a time course for uptake and efflux of [3H]PALA. Uptake was relatively rapid initially but later slowed to a rate that remained constant for at least 5 hr. The net rate of uptake of 1mm [3H]PALA after 30 min was fairly consistent from experiment to experiment [0.192 ± 0.036 (S.D.) μmol/min/liter cell H2O]. Although 1 μCi of [3H]PALA was used per sample, initial rates were difficult to study due to the low levels of radioactivity (typically, 200 to 300 dpm). Most often, the more rapid initial rate was observed as the y-intercept extrapolated from longer time courses. This y-intercept was highly variable [12.69 ± 7.18 (S.D.) μmol/liter cell H2O] and did not correlate with the 30 to 240 min rate (r = 0.013). The experiment illustrated in Chart 1 reveals a higher than average initial uptake. More typical initial uptakes are shown in Charts 3 and 4. A part of this rapid uptake may be due to the presence of a small but variable fraction of dead or dying cells with damaged membrane integrity. The mean initial uptake would correspond to 1.3% of the cells rapidly accumulating PALA to an intracellular concentration equal to the extracellular level. Exposure of cells to 0.5 mm PALA for 5 min had no effect on NaH14CO3 incorporation into pyrimidine nucleotides. The radiolabeled PALA accumulated over 2.5 hr was not lost when cells were centrifuged and resuspended in fresh media. Intracellular PALA was not all bound to ATCase, since nonexchangeable drug far exceeded the [3H]PALA-binding capacity of cell extracts [0.129 ± 0.07 (S.E.) μmol/liter cell H2O]. Chart 2 summarizes results of uptake time courses performed at PALA concentrations between 10^-6 and 10^-2 m. Over this range, the net rate of [3H]PALA uptake between 30 and 240 min and the magnitude of the y-intercept were proportional to the PALA concentration.

Kamen et al. (24) have shown that the presence of a very small amount of radiolabeled contaminant can greatly distort the results and interpretation of transport experiments if the contaminant is preferentially accumulated. Ehrlich cells were exposed to [3H]PALA for 4 hr and extracted with 100° H2O. High-performance liquid chromatography (29) revealed a single peak which cochromatographed with unlabeled PALA. This result is in agreement with reports that have shown that PALA is not metabolized (48, 52).

Similarities of PALA Uptake and Fluid-Phase Endocytosis. In some preliminary oil centrifugation experiments using [3H]-inulin for measurement of the extracellular H2O in cell pellets, the "extracellular space" seemed to increase if the exposure to [3H]-inulin was prolonged. To examine this further, the rates of uptake of [3H]PALA and [3H]inulin were compared using the 0° saline wash procedure (Chart 3). To normalize the data for the 2 substrates, the results are expressed as μl/min/ml of cell H2O. A paired t test on data from 7 experiments indicated that there was no difference between the PALA rate [0.214 ± 0.048 (S.D.) μl/min/ml] and the inulin rate [0.241 ± 0.054 (S.D.) μl/min/ml] or in the magnitude of the y-intercepts. Although not statistically significant, slower
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Chart 3. Comparison of the rates of uptake of \(^{3}H\)PALA and \(^{3}H\)inulin. Cells were incubated with 1 mM \(^{3}H\)PALA or \(^{3}H\)inulin (10 ng/ml) in the absence (O, •) or presence (△, ○) of 10 \(\mu\)M colchicine. To permit comparison, the data were normalized as the volume of extracellular media apparently taken up or “cleared” of radiolabel.

PALA uptake might have been due to electrical repulsion of PALA from negatively charged cell surfaces.

A mechanism that would allow for identical rates of uptake for otherwise impermeant substrates is fluid-phase endocytosis. Nonsaturable uptake kinetics would be expected with this mechanism. Steinman et al. (46) reported that L-cell mouse fibroblasts interiorize 25% of their volume in 3 to 6 hr. This could be expressed as 0.7 to 1.4 \(\mu\)l/min/ml cell H2O, a value which is similar in magnitude to the rates of PALA and inulin uptake. Besterman et al. (5) have demonstrated biphasic uptake of \(^{14}C\)sucrose by endocytosis in macrophages and fibroblasts which is similar to the uptake pattern for \(^{3}H\)PALA in Chart 1. Additional experiments were performed to characterize further the PALA uptake process and test this hypothesis.

Endocytosis is known to be temperature and energy dependent (47). Chart 4A illustrates that reducing the temperature to 27° slowed \(^{3}H\)PALA uptake by about one-half. At 0°, net uptake was almost totally stopped, although there was a significant \(y\)-intercept. Combinations of inhibitors of glycolysis and oxidative phosphorylation produced unexpected effects (Chart 4B). KCN plus NaF initially inhibited uptake but later caused a large increase in the uptake rate. A similar effect was seen using \(^{3}H\)inulin as the substrate (not shown). One interpretation of these results may be that after exposure to energy inhibitors for 2 hr or more the cell membrane becomes somewhat leaky, allowing PALA to rapidly accumulate to the level of the extracellular media.

Significance of Acidic Lysosomal pH for PALA Inhibition of Pyrimidine Synthesis. The hypothesis that \(^{3}H\)PALA is taken into cells via endocytosis implies that it does not immediately enter the compartment containing ATCase. In fluid-phase endocytosis, a portion of the plasma membrane surrounds a small volume of the media and pinches off to form a vesicle called a pinosome (or endosome). This pinosome migrates in the cell and eventually fuses with a lysosome, where the pH is approximately 4.5 (37). In this environment, some of the 4 negatively charged groups on the molecule would be protonated. With a decreased net charge, PALA may partition more readily into the lipid phase of the membrane and escape into the cytoplasmic compartment containing the target enzyme. This reasoning suggests that neutralization of lysosomal pH should block the inhibitory action of PALA on de novo pyrimidine synthesis.

A number of lysosomotropic amines have been shown to neutralize lysosomal pH, the simplest of these being NH4+. Ehrlich cells were treated briefly with NH4Cl prior to exposure to PALA. After 2 hr with or without PALA, the rate of incorporation of NaH\(^{14}\)CO3 into pyrimidine nucleotides was measured as described in "Materials and Methods" (Chart 6). NH4Cl had no effect on NaH\(^{14}\)CO3 incorporation in contrast to published...
The addition of NH₄Cl dramatically decreased the inhibitory effect of pyrimidine synthesis by NH₄Cl in these cell types may be the dose-response curve for PALA inhibition of ATCase in cell culture (27). It was concluded that the mechanism of PALA uptake in carcinoma cells was nonsaturable and independent of temperature (28). Several direct measurements of PALA uptake have been made (26, 29), which showed (a) a rapid uptake component, (b) a much slower phase of uptake which sustained a constant rate for at least 5 hr, and (c) an equilibrium would be achieved for a tetravalent anion such as PALA pK₄ values for aspartate are 3.86 and 2.09. Ethyl phosphonate has pK₄ values of 7.85 and 2.43 (11). Assuming PALA pK₄ values are similar, it can be calculated that at pH 7.4 only about one molecule of PALA in 10⁶ is completely uncharged. However, the acid environment of the lysosome seems critical for PALA action. Lysosomotropic amines, such as NH₄⁺ and methylamine, which neutralize lysosomal pH (37), blocked the inhibitory action of PALA on pyrimidine synthesis. NH₄Cl and methylamine have also been shown to protect cells from the effects of diphtheria toxin by preventing its release from lysosomes after endocytosis (9).

Since release of PALA from vesicular compartments within the cell may be slow, conclusions about the concentration of inhibitor acting on ATCase cannot be based on assays of total cellular PALA. Experiments designed to measure the inhibitory effect of PALA or pyrimidine synthesis indicate that high concentrations of total intracellular drug are required. The data from Chart 6 show that a 90-min exposure to 200 μM PALA inhibited NaH¹⁴CO₃ incorporation into pyrimidine nucleotides by only 70%. From knowledge of the rate of net uptake, not including the rapid uptake component, total cellular PALA should be about 2.7 × 10⁻⁶ mol/liter cell H₂O. This is approximately 20 times the ATCase concentration and 2000 times the ATCase Kᵣ of 1.3 × 10⁻⁹ M (2). The incomplete inhibition may be due in part to the need to inhibit a large fraction of total enzyme to make ATCase rate limiting for pyrimidine synthesis. Also, there may be competition for PALA binding sites by carbamyl phosphate if it accumulates to high levels behind the block. A third factor may be that the drug concentration in the compartment containing the target enzyme was considerably lower than the total cellular level.

Endocytosis of PALA is consistent with reports that intracellular drug levels are achieved in vivo that equal or exceed the extracellular level (52). Uptake via passive or facilitated diffusion is not. Cells maintain an electrical potential across the cell membrane that tends to exclude negatively charged molecules. For the Ehrlich cell with a membrane potential of −15.6 mV (13), calculations using the Nernst equation indicate that electrochemical equilibrium would be achieved for a tetravalent anion such as PALA when the intracellular concentration is less than 10% of the extracellular level. A higher level would usually be considered evidence for intracellular binding or active transport against lipid bilayer membranes may be too impermeable to highly charged hydrophilic molecules to allow passive diffusion of PALA at even this slow rate. An alternative hypothesis consistent with slow nonsaturable uptake is fluid-phase endocytosis (pinocytosis). This hypothesis is supported by the observations that the rate of uptake was (a) temperature dependent, (b) identical to that of the extracellular marker, inulin, (c) quantitatively similar to endocytic rates in other cell types (46, 47), (d) inhibited by the microtubular antagonist, colchicine, and (e) stimulated by phorbol myristate acetate. Colchicine is known to partially inhibit while phorbol myristate acetate stimulates endocytosis (39).

If PALA enters cells by endocytosis it would be localized initially in a vesicle, usually described as a pinosome or endosome, which may eventually fuse with a lysosome. At the acid pH of the lysosome, PALA would be partially protonated. A decrease in its net charge should allow it to partition into membrane lipids and diffuse across the lysosomal membrane at an increased rate. The pK₄ values for aspartate are 3.86 and 2.09. Ethyl phosphonate has pK₄ values of 7.85 and 2.43 (11). Assuming PALA pK₄ values are similar, it can be calculated that at pH 7.4 only about one molecule of PALA in 10⁶ is completely uncharged. At a lysosomal pH of 4.5 (22), this ratio drops to about one in 10⁶. Although the fraction of uncharged molecules is still quite low, the acid environment of the lysosome seems critical for PALA action. Lysosomotropic amines, such as NH₄⁺ and methylamine, which neutralize lysosomal pH (37), blocked the inhibitory action of PALA on pyrimidine synthesis. NH₄Cl and methylamine have also been shown to protect cells from the effects of diphtheria toxin by preventing its release from lysosomes after endocytosis (9).

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an electrochemical gradient. Uptake via the mechanism proposed here also allows for eventual accumulation of PALA to high levels.

The hypothesis that PALA enters cells via endocytosis and release from lysosomes is consistent with numerous in vitro and in vivo observations that the effects of PALA as an inhibitor of ATCase and pyrimidine synthesis are long-term (20, 27, 36, 37, 49, 52). For example, Yoshida et al. (52) observed that after mice were given injections of a single dose of PALA, drug levels in liver and spleen remained essentially constant for at least 72 hr and eventually exceeded the plasma concentration by an order of magnitude. Tsuboi et al. (49) demonstrated that, after exposure of HT-29 cells (human colonic epithelial tumor) to PALA for 1 day, de novo pyrimidine synthesis was undetectable for at least 6 days after removal of PALA. The data presented here suggest that an acid environment is necessary to permit PALA to diffuse across membranes. The rate of diffusion from the cytoplasm to the outside is probably very slow indeed, despite a considerable electrochemical gradient. The mechanism for PALA escape and eventual reversal of its pharmacological effect may be exocytosis, a process that is poorly understood at this time (5, 6).

Appreciation of the mechanism of PALA uptake may help to explain the selective action of PALA against some slow-growing murine solid tumors. The prolonged maintenance of effective intracellular drug concentrations is probably important in the treatment of tumors with a low fraction of dividing cells. Fluid-phase endocytosis is known to be cell cycle dependent; uptake of horseradish peroxidase by hepatoma cells was greatest in G1, may be exocytosis, a process that is poorly understood at this time.

Other phosphate drugs may also depend on endocytosis for entry into cells. The rate of uptake of [14C]phosphonacetate by L1210 cells and PHA-stimulated human leukocytes (4) was similar to the rate of [3H]PALA uptake reported here. L-cell mouse fibroblasts, which may have a relatively high rate of endocytosis, took up [14C]phosphonacetate much more rapidly and were much more sensitive to inhibition of DNA synthesis by phosphonacetate. To achieve 50% inhibition of DNA synthesis with intact L1210 required a 6-hr exposure to 5 mM phosphonacetate, but less than 0.1 mM drug immediately produced 50% inhibition in permeabilized cells. It should be appreciated in preclinical development of very hydrophilic agents that although endocytosis is one potential mechanism for uptake of any compound from the media or from plasma, entry into the cytoplasm of the cell will require escape from an intracellular vesicular compartment, it will be slow, and it may be variable among different cell types.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of James P. Rathmell in the synthesis of [3H]PALA.

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