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ANION EXCHANGE MECHANISM FOR TRANSPORT OF METHOTREXATE IN L1210 CELLS[1]

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SUMMARY: Structurally diverse anions (folate, 5-formyltetrahydrofolate, AMP, ADP, thiamine pyrophosphate, phosphate, sulfate, and chloride) that are competitive inhibitors of methotrexate influx in L1210 cells also enhance the efflux of methotrexate from these cells. The increase in efflux reaches a maximum of 2- to 4-fold depending upon the anion employed, and the anion concentrations required for half-maximal stimulation of efflux are similar to their K_i values for inhibition of methotrexate influx. A competitive inhibitor of methotrexate uptake (fluorescein-diaminopentane-methotrexate) that is not transported by this system, does not increase methotrexate efflux. These results suggest that the efflux of intracellular methotrexate is coupled to the concomitant uptake of an extracellular anion.

Methotrexate enters L1210 cells via a high-affinity transport system whose primary substrate is 5-methyltetrahydrofolate (reviewed in ref. 1). Energy is coupled to this process, since free methotrexate can be concentrated within the cells to levels 50-fold higher than in the external medium (2). Various organic and inorganic anions competitively inhibit methotrexate influx and reduce steady-state levels of the drug (2-5), suggesting that the energy source for this system is an anion gradient (2,4). Uptake might then occur via an anion exchange mechanism, with the extrusion of intracellular anions into the medium and down a concentration gradient acting as the driving force. The present communication provides support for this exchange mechanism by demonstrating that the

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[2]Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; F-MTX, fluorescein-diaminopentane-methotrexate.

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transport of methotrexate out of the cell requires the concomitant uptake of an extracellular anion. Various organic and inorganic anions (in addition to folate compounds) can fulfill this ion requirement.

MATERIALS AND METHODS

Materials. [3',5'-9(n)-^{3}H]Methotrexate (250 mC$_i$/mmol), obtained from Amersham/Searle, was purified prior to use by thin-layer chromatography on cellulose sheets (Eastman 6064) using 0.1 M K-Hepes^2, pH 7, as the solvent. F-MTX was kindly provided by Dr. J. M. Whiteley. The buffers employed in transport determinations were either Hepes (160 mM Hepes and 2 mM $MgCl_2$ adjusted to pH 7.4 with KOH) or Hepes-sucrose (20 mM Hepes, 2 mM $MgCl_2$, and 225 mM sucrose adjusted to pH 7.4 with KOH); Mg^{++} was included in all buffers to maintain cellular integrity (2).

Methods. L1210 mouse leukemia cells were grown as described previously (6), washed with the desired buffer, and resuspended to a density of 2 x 10^7/ml. Methotrexate influx was determined by a previously described procedure (2) using assay mixtures (1.0 ml, final volume) that contained 2.0 μM [^{3}H]methotrexate (150,000 dpm/nmol) and the indicated additions. Transport was measured over a 5-min interval at 37°. Methotrexate efflux was measured (2) in cells that had been preincubated for 20 min at 37° in Hepes buffer containing 0.5 mM glucose and 5.0 μM [^{3}H]methotrexate, washed, and resuspended in the desired buffer.

Protein concentrations were measured by the biuret reaction (7) using bovine serum albumin as the standard.

RESULTS

Previous studies by Goldman (8) have shown that in L1210 cells [^{3}H]methotrexate influx can be increased by preloading the cells with structural analogs such as 5-formyltetrahydrofolate, 5-methyltetrahydrofolate, or folate. Extending these studies, we have now shown that the efflux of [^{3}H]methotrexate can also be increased by the addition of these same folate compounds to the external medium. The time dependence of this effect is illustrated in Fig. 1 for dl-5-formyltetrahydrofolate. Stimulation of methotrexate efflux is not confined, however, to folate compounds, since the same results can be achieved with a diverse group of anions (AMP, ADP, thiamine pyrophosphate, phosphate, sulfate, and chloride). For comparison, representative data for AMP have also been included in Fig. 1.

The concentration dependence for the increase in methotrexate efflux by AMP and inorganic phosphate is shown in Figs. 2A and 2B. In each instance,

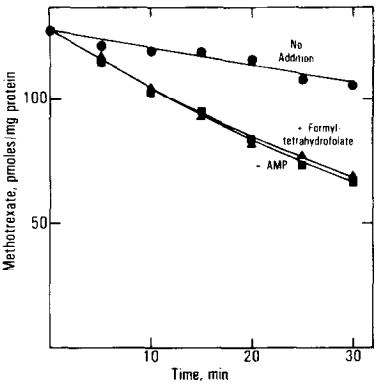

Fig. 1. Time-dependence for the stimulation of methotrexate efflux by AMP and 5-formyltetrahydrofolate. Cells preloaded with [^{3}H]methotrexate (see Materials and Methods) were suspended in Hepes-sucrose buffer containing the indicated additions and analyzed for [^{3}H]methotrexate efflux as a function of time (at 37^0).

efflux reached a plateau at high levels of these anions. Maximum stimulation was 3.7- and 3.2-fold for AMP and phosphate, respectively, as determined from a double-reciprocal plot (not shown) of the increase in efflux rate vs. anion concentration. Half-maximal stimulation of efflux was calculated to occur at 125 μM AMP and 1600 μM phosphate. The effects of these anions were not additive since the stimulation of efflux produced by high levels (1 mM) of AMP was not increased further by the simultaneous addition of excess (10 mM) phosphate (not shown). A similar analysis

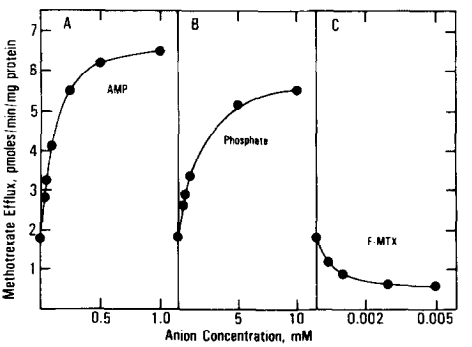

Fig. 2. Concentration dependence for the effect of AMP, phosphate, and F-MTX on methotrexate efflux. Cells preloaded with [^{3}H]methotrexate (see Materials and Methods) were suspended in Hepes-sucrose buffer and analyzed (at 37^0) for [^{3}H]methotrexate efflux as a function of anion concentration.

TABLE I

Kinetic Constants Derived from the Effects of Various Anions on Methotrexate Influx and Efflux.[a]

Anion	K_i for Methotrexate Influx	K_{stim} for Methotrexate Efflux	Maximum Stimulation of Efflux
	μM	μM	-fold
Methotrexate	0.6	0.8	2.7
Folate	20	30	2.0
5-Formyltetrahydrofolate	1.2	2.5	3.2
AMP	40	125	3.7
ADP	34	100	2.0
ATP	20	--[b]	--[b]
Thiamine pyrophosphate	5	8	4.0
Phosphate	600	1600	3.2
Sulfate	550	2000	2.8
Chloride	32000	40000	3.6
Bicarbonate	13000	--[c]	--[c]

[a] K_i values for inhibition of methotrexate influx were determined as described previously (5). Efflux parameters were calculated from a double-reciprocal plot of the stimulation in efflux rate vs. anion concentration. K_{stim} is defined as the concentration of anion required for a 50% stimulation of methotrexate efflux.

[b] Not determined; ATP (above 100 μM) appears to cause changes in the permeability of L1210 cells, as reported previously for other tumor cell lines (9).

[c] Not determined; the pH of the medium could not be maintained at 7.4 in the presence of high levels (above 20 mM) of bicarbonate, even with a 95% O_2/5% CO_2 atmosphere.

of [^{3}H]methotrexate efflux as a function of anion concentration showed that unlabeled methotrexate, folate, 5-formyltetrahydrofolate, ADP, thiamine pyrophosphate, sulfate, and chloride also stimulated efflux by a maximum of 2- to 4-fold (Table I). A comparison of values listed in Table I showed further that a close correlation existed between anion concentrations required for 50% stimulation of efflux (K_{stim}) and their corresponding K_i values for inhibition of methotrexate influx.

Methotrexate efflux was not stimulated (Fig. 2C) by a fluorescein derivative of methotrexate (F-MTX) that is a potent competitive inhibitor of methotrexate influx, but is not transported via this system (10). F-MTX, in fact, reduced the efflux rate by 3-fold. The concentration of F-MTX required for half-maximal inhibition of efflux by 50% (0.5 μM) was similar to its K_i value for inhibition of influx (0.3 μM), indicating that the effects of F-MTX were due to an interaction with the methotrexate transport system.

It is important to note that the above efflux experiments were performed in cells preloaded with [^{3}H]methotrexate and then suspended in isotonic sucrose containing 20 mM Hepes. These conditions were necessary to eliminate interfering anions. The latter include components (e.g., chloride, phosphate, and bicarbonate) of commonly used saline buffers. Hepes also has a weak ability (K_i = 120 mM) to interfere with methotrexate transport. Efflux of methotrexate in cells suspended in Hepes-sucrose buffer ($t_{1/2}$ = 70 min) was much slower than in either 160 mM Hepes ($t_{1/2}$ = 33 min) or phosphate-buffered saline ($t_{1/2}$ = 11 min) (2), but was somewhat faster than in sucrose solutions containing only 5 mM Hepes ($t_{1/2}$ = 90 min).

DISCUSSION

Methotrexate transport in L1210 cells is highly dependent upon the ionic composition of the assay medium. K_t values for half-maximal rates of influx are reduced nearly 10-fold when various buffered saline solutions are replaced with either Hepes (2) or Hepes-sucrose. The basis for this effect is that the former buffers contain various anions, such as phosphate, chloride, and/or bicarbonate, that interact with the methotrexate binding site (2) (see also Table I). Other organic and inorganic anions also competitively inhibit methotrexate influx (2-5). With the inorganic anions, K_i values are in the millimolar range, while much lower values are observed for adenine nucleotides and thiamine pyrophosphate (Table I).

Rates of methotrexate efflux also fluctuate with the composition of the external medium. Removal of various anions decreases efflux (2), while an increase is observed upon re-addition of various anions to the medium (cf. Figs. 1 and 2; Table I). These anion effects result from a direct interaction with the methotrexate transport system since stimulation of efflux by 50% occurs at anion concentrations similar to their K_i values for inhibition of methotrexate influx (Table I). It also appears likely that these anions enter the cell in exchange for methotrexate and do not simply enhance methotrexate release at the external cell surface, since F-MTX, a competitive transport inhibitor that is not taken up via the methotrexate carrier system (10), does not increase methotrexate efflux. The latter compound actually inhibits efflux (Fig. 2C), apparently by trapping the binding site on the carrier protein at the outer membrane surface.

The present results support the hypothesis that the 5-methyltetrahydrofolate/methotrexate transport system of L1210 cells facilitates the exchange of anions across the cell membrane. While a variety of organic and inorganic anions appear to exchange for intracellular methotrexate (cf. Table I), anions within the cell that might exchange for extracellular folate compounds have not been identified. In the latter regard, organophosphate compounds (4) and inorganic phosphate (2,3) have been suggested as candidates for these intracellular exchange anions in vivo. The exchange process also appears to be obligatory, since the presence of extracellular anions is required for methotrexate efflux. Finally, the present results suggest that both influx and efflux of methotrexate occur via the same carrier protein. If separate routes are operative (11-12), they would have to occur via a similar mechanism and also exhibit comparable sensitivities to a variety of anions (cf. Table I). The fact that p-chloromercuriphenylsulfonate inhibits in parallel both methotrexate influx and efflux (13) further supports the single-carrier hypothesis.

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