TRANSPORT OF ADEFOVIR (PMEA) IN HUMAN T-LYMPHOBLASTOID CELLS

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The uptake of [^{3}H]PMEA by human T-lymphoblastoid cells (CCRF-CEM) apparently proceeds by fluid-phase endocytosis. The transport kinetics was shown to be nonconcentrative and nonsaturable. The uptake takes place even at a low temperature (4 °C), is strictly dependent on the intracellular level of ATP, is not substantially affected by cell suspension density and is not competitively inhibited by other PME derivatives.

Key words: Adefovir; Transport; Human cells.

9-(2-Phosphonomethoxyethyl)adenine (PMEA; Adefovir) represents the simplest structural type of acyclic nucleoside phosphonates, an important group of novel antiviral agents. The compound inhibits human immunodeficiency virus type 1 and 2 (HIV-1, HIV-2) (refs[1–4]), feline and simian immunodeficiency viruses (FIV and SIV) (refs[5–7]), Visna LP-BM5 complex[8], murine sarcoma virus (MSV) (refs[4,9]), RMLV (ref.[10]), human hepatitis B virus (HBV) (ref.[11]) and herpesviruses[12,13].

The principle of the antiviral effect of PMEA consists in the interference of its diphosphoryl derivative PMEApp with the replication of viral DNA, and/or with reverse transcription[14]. Similarly, the cytostatic effect of PMEA (refs[15–17]) can be interpreted by its interaction with the enzyme apparatus involved in DNA synthesis[18] because PMEApp is the substrate/inhibitor of replicative DNA polymerases α, δ and ε.

Adefovir (PMEA) and its oral form (bis-POM-PMEA; Adefovir Dipivoxil) are presently in an advanced stage of clinical trials for treatment of AIDS and of hepatitis B. Therefore, research is oriented not only on the antiviral effect of the drug but also on other processes that are not directly associated with its mode of action, but that influence its biological activity. Among them membrane transport plays a crucial role. Our recent study[19] showed that transport of PMEA into HeLa S3 cells *in vitro* is medi-

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ated by membrane receptor (50 kDa). It was the aim of our present work to establish whether such a transport mechanism occurs also in other cell types. In view of the relevance to the anti-HIV activity of PMEA the experimental model used here *in vitro* were T-lymphoblastoid cells.

EXPERIMENTAL

Materials

Acyclic nucleoside phosphonates and their phosphoryl and diphosphoryl derivatives were synthesized as described before[20–22].

[^{3}H]PMEA (630 GBq/mmol) was synthesized in the Institute for Research, Production and Uses of Radioisotopes in Prague (Czech Republic). [^{14}C]Carboxyinulin (308 MBq/mmol) was purchased from Amersham International (England).

Cytochalasin B and D, colchicin, oligomycin, and antimycin were products of Sigma (U.S.A.). The silicone oil, cultivation medium RPMI-1640 in lyophilized form, PBS, $NaHCO_3$, HEPES, glutamine and a mixture of penicillin/streptomycin were also from Sigma, mineral oil was from Fluka (Switzerland). The biological detergent Triton X-100 was obtained from New England Corp. (U.S.A.) and the liquid scintillator Aquasafe-500 was purchased from Zinsser Analytic (Austria).

Heat-inactivated fetal calf serum (FCS) was purchased from PAA Laborgesellschaft (Austria).

Methods

Cultivation of the CCRF-CEM cell line. Human lymphoblastoid cells CCRF-CEM (ATCC CCL 119) were grown in a synthetic medium RPMI-1640, containing 10% FCS, 2 mM glutamine, 15 mM HEPES and a mixture of penicillin/streptomycin in the usual concentration of 0.01%. The pH was adjusted with NaOH to 7.4. Cultivation was done in an incubator at 37 °C in humid atmosphere with 5% CO_2. The cells were passaged upon reaching a density of 10^6 cells/ml by dilution to 1.0–1.5 . 10^5 cells/ml. The doubling time of this cell line was 23–25 h. After staining with Trypan blue the cells were counted visually in a Bürker chamber.

Determination of intracellular volume of CCRF-CEM. The cells were washed by transport medium RPMI-1640 (without FCS and the antibiotics), pelleted by centrifugation, resuspended in the same medium and divided to aliquots of different densities in 1 ml: 5 . 10^6; 1 . 10^7; 1.5 . 10^7; 2 . 10^7. The samples were preincubated 30 min in a rotary stirrer at 37 °C, centrifuged and suspended to PBS which contained (in 0.5 ml): 0.5 μl [^{3}H]H_2O (163 kBq/μl) and 7.5 μl [^{14}C]carboxyinulin (3 kBq/μl) (ref.[23]). Pulse-labelled cells (1 min at 37 °C) were exhaustively washed by PBS and radioactivity of ^{3}H and ^{14}C was determined in the scintillation spectrometer Beckman LS-6000. The intracellular volume of T-lymphoblastoids CCRF-CEM for calculation of the actual cytoplasmic concentration of PMEA was 3.38 μl/10^7 cells.

Cellular uptake of [^{3}H] PMEA. The cells were washed at the centrifuge (370 g, 5 min) in 10 ml transport medium RPMI-1640 at room temperature. After removing the supernatant the cell sediment was resuspended in the same medium. The suspension was distributed into microtubes in 450-μl aliquots and 50 μl [^{3}H]PMEA was added to the desired concentration. Incubation was done in a controlled CO_2-incubator using a rotary stirrer (RM-30, Developmental Workshop, Academy of Sciences, Prague) at 37 °C. At suitable time intervals the uptake process was terminated by centrifugation at 5 300 g for 1 min through an oil layer[24] (a mixture of silicone and mineral oil at final specific density of 1.05 g/ml of 150 μl volume). All tests were run in duplicate. The cell sediment (after passing through the oil layer) was washed by centrifugation (5 300 g, 1 min) in 1 ml PBS

at 4 °C. The cells were then disrupted with 2% Triton X-100 (300 μl per sample) and radioactivity was counted in an aqeous scintillator (4 ml per sample).

Intracellular ATP level. Cells were washed in 1 ml PBS at 4 °C and pelleted by centrifuging at 5 300 g for 1 min. The sediment was resuspended in 200 μl water at 4 °C and 200 μl 10% trichloroacetic acid (TCA) was added. After 10 min of vigorous stirring the precipitate was sedimented at 11 000 g (5 min). TCA was extracted from the supernatant with 400 μl of a mixture of 1,1,2-trichloro-1,2,2-trifluoroethane–trioctylamine (4 : 1, v/v). The two phases were separated by centrifugation at 11 000 g for 5 min and the upper aqueous layer was analyzed by HPLC. The cell pool (100 μl of an aqueous phase) was applied to a column (250 × 46 mm) of Silasorb C18 (5 μm). The elution buffer A contained 50 mM KH_2PO_4 and 3 mM tetrabutylammonium hydrogensulfate at pH 6.8 and buffer B [acetonitrile (20%, v/v) in buffer A]. The elution by a linear gradient of 5–100% buffer B for 70 min revealed peak of ATP. Its concentration was determined from a calibration curve after integration.

Intracellular metabolism of [^{3}H]PMEA. Cells washed with the transport medium RPMI-1640 were incubated with 10 μM [^{3}H]PMEA in CO_2-incubator for 6 h at 37 °C. The samples were processed in the same way as before. After removal of TCA the aqueous extract was analyzed by HPLC for PMEA metabolites as follows: the cell extract was applied to Silasorb C18 column (250 × 46 mm, 5 μm). Elution buffer C contained 50 mM KH_2PO_4 and 3 mM tetrabutylammonium hydrogensulfate at pH 3.1. Buffer D was identical with C except for the addition of 30% (v/v) acetonitrile. The column was eluted with a linear gradient from 15 to 60% of buffer D for 25 min at the flow rate of 1 ml/min. 0.5-ml fractions were collected and radioactivity was counted in an aqueous scintillator (4 ml per sample). PMEA, PMEAp and PMEApp were identified with the aid of standards.

RESULTS AND DISCUSSION

The mechanism of transport of acyclic nucleoside phosphonates into eukaryotic cell raises some questions. Character of the P–C bond in the molecule of these compounds makes dephosphorylation highly improbable so that cleavage by 5′-ectonucleotidases and subsequent transport of nucleosides apparently does not take place[25]. It was shown recently that in various cells PMEA (Adefovir) is taken up by one or another type of endocytosis. The first results obtained with T-lymphoid cells (CCRF-CEM) showed that unlike with HeLa S3 cells[19], receptor-mediated endocytosis was not involved.

In the starting experiments we have analyzed the time course of PMEA accumulation in T-lymphoblastoids (Fig. 1). The uptake is rather slow, the rate decreases with time. After placing the cells in a solution of non-radioactive PMEA the intracellular level of labelled PMEA decreased: after additional 60 min the intracellular level amounted to 58% of the original value.

The uptake of PMEA depended on its extracellular concentration which ranged from 0.1 to 1 000 μM (Fig. 2). In an independent experiment the distribution of [^{3}H]PMEA metabolites was examined using 10 μM [^{3}H]PMEA at 37 °C, for 6 h. The intracellular pool contained 86% PMEA, 5% PMEAp and 9% PMEApp, reflecting an unusually slow process of intracellular phosphorylation, hardly affected by the membrane transport[19].

The time dependence of [^{3}H]PMEA uptake is, in contrast with HeLa cells, distinctly nonlinear (Fig. 1). The linear dependence of [^{3}H]PMEA uptake on its extracellular

concentration shows that the process is not concentrative and not saturable over the whole range of concentrations used (Fig. 2), even at concentrations that clearly exceed the IC_{50} value of 25 μM. The uptake of [^{3}H]PMEA by T-lymphoblastoid cells is temperature-dependent at various concentrations of PMEA. Figure 3 relates to samples taken after 180 min of incubation; the transport was linear at all the temperatures tested and did not cease even at 4 °C when the rate amounted to 31% of that found at 37 °C with 3 μM PMEA after 30 min and to 10% after 180 min. Since receptor-mediated endocytosis is a strictly temperature dependent process, which is fully inhibited at 10 °C (ref.[19]) it may be concluded that this transport involves a fluid-type pinocytosis.

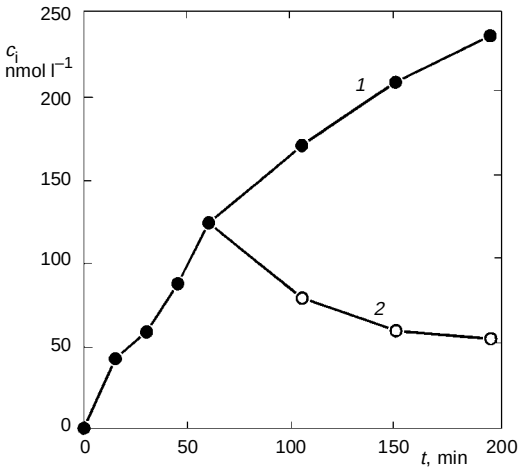

FIG. 1
Time course of PMEA uptake by T-lymphoblastoid cells incubated with 3 μM [^{3}H]PMEA at 37 °C (*1*); after 60 min a part of the culture was centrifuged and the medium replaced with nonlabelled PMEA at the same concentration (*2*). c_i Intracellular concentration of PMEA; t incubation time

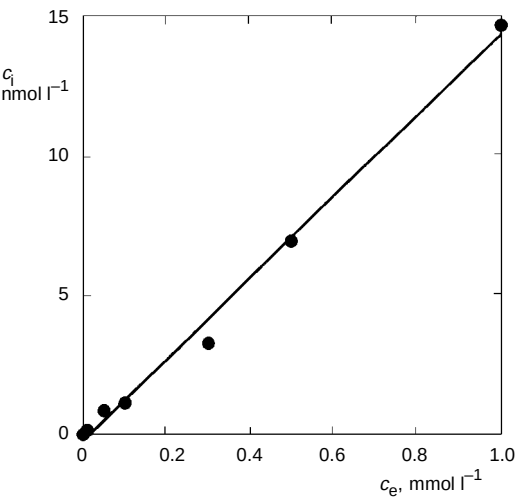

FIG. 2
Concentration dependence of PMEA uptake. CCRF-CEM cells were incubated at different concentrations of [^{3}H]PMEA for 30 min at 37 °C. c_i Intracellular concentration of PMEA; c_e extracellular concentration of PMEA

Cell suspension density (in the range of 1–20 . 10^6 cells/ml) did not have any dramatic effect on the transport although, after a small initial maximum, the intracellular PMEA concentration showed a decreasing tendency (Fig. 4). This finding, too, indicates that receptor-mediated endocytosis[19] is not involved in this process.

To examine whether an active carrier is involved in the transport process we tested its dependence on ATP generation. From the group of selected inhibitors[26,27] (2,4-dinitrophenol, KCN + iodoacetic acid, oligomycin and antimycin) we found antimycin and oligomycin were most effective, depressing the intracellular ATP level by 80%. The rate of uptake of [^{3}H]PMEA by T-lymphoblastoid cells preincubated with 0.1 μM antimycin and 10 μM oligomycin was decreased, by approximately 80 and 70%, respec-

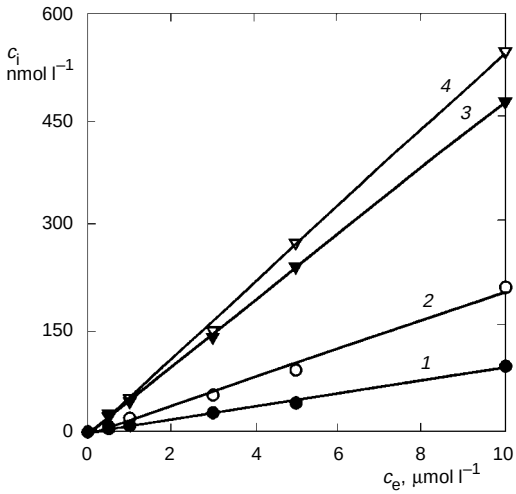

FIG. 3
Dependence of PMEA uptake rate on temperature. T-Lymphoblasts were incubated with 3 μM [^{3}H]PMEA at 4 (*1*), 20 (*2*), 30 (*3*) and 37 (*4*) °C for 3 h. Coordinates as in Fig. 2

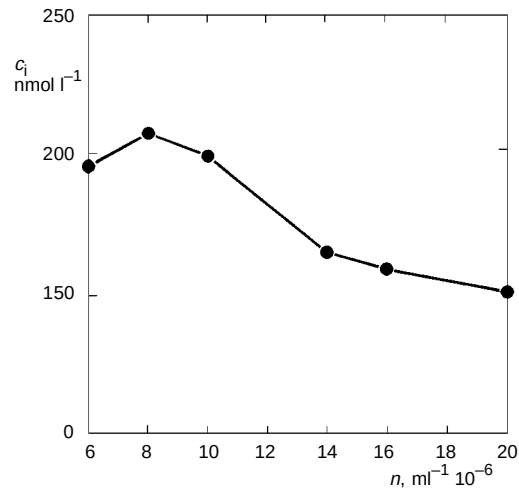

FIG. 4
Dependence of PMEA transport on cell suspension density. T-Lymphoblasts were incubated with 3 μM [^{3}H]PMEA for 30 min at 37 °C. c_i Intracellular concentration of PMEA; *n* cell count

tively (Table I). In spite of the slight difference between the effect of these two antibiotics which is apparently due to their different mode of action it is evident that the uptake of [^{3}H]PMEA by T-lymphoblastoid cells is strictly dependent on the intracellular level of ATP. This fact excludes nonspecific permeation (simple diffusion), which is energy-independent but does not exclude the ATP-driven carrier transport (see below). The remaining alternative which involves ATP in the uptake process could be some sort of endocytosis or potocytosis. However, since potocytosis associated with the so-called caveolae[28] has not been observed with plasma membranes of lymphoid cells, this type of transport appears to be highly improbable[29].

The specificity of the transport process was further characterized by testing the interference of structurally related phosphonate analogues. It had been shown for the HeLa S3 cells that the transport of PMEA by receptor-mediated endocytosis[19] is highly competitive with its structural analogues; in the present study we investigated the influence of both related pyrimidine and purine compounds, *viz.* PME derivatives of cytosine (PMEC), uracil (PMEU), thymine (PMET), 2-aminopurine (PMEMAP), 2,6-diaminopurine (PMEDAP), hypoxanthine (PMEHx) and guanine (PMEG). In these experiments, [^{3}H]PMEA concentration was set at 3 μM while the analogues were used at 1 : 1, 1 : 3 and 1 : 6 ratios, respectively. Table II demonstrates that these compounds exert no inhibition on PMEA transport. This finding also excludes the carrier mechanism which would ostensibly show high degree of inhibition by structurally related PMEA analogues.

To check the possible role of cytoskeleton in the PMEA transport T-lymphoblastoids were preincubated with compounds known to depolymerize microtubules and microfilaments (colchicine, cytochalasin B and D). Colchicin stimulated the transport of [^{3}H]PMEA into the culture cells by as much as 300% while the two cytochalasins were without effect (not shown). The maximum intracellular concentration of PMEA was found with 5 mM colchicin (Fig. 5). Since cytochalasin D and colchicin are known to

TABLE I
Uptake (30-min) of 3 μM [^{3}H]PMEA after a 15-min preincubation with inhibitors of ATP synthesis

Inhibitor	Intracellular level of ATP in percent of control	Intracellular concentration of [^{3}H]PMEA μM
None	100	0.0583
100 μM antimycin	20.1	0.0175
10 μM oligomycin	21.7	0.0291

inhibit the uptake of only those substances that are taken up in non-clathrin-coated vesicles[30] other vesicles are apparently in play here.

Finally, we have compared the time course of [^{3}H]PMEA uptake with that of [^{14}C]carboxyinulin which serves as a marker for fluid-phase pinocytosis. The identical course of these uptake curves (not shown) again corroborates that contrary to HeLa cells[19], [^{3}H]PMEA (Adefovir) is taken up by the T-lymphoblastoid cells by fluid-phase endocytosis.

TABLE II
Effect of base-modified PME-derivatives (at concentrations of 3, 6, and 18 μM) on the uptake of 3 μM [^{3}H]PMEA after a 30-min incubation at 37 °C

PME derivative of	Percentual uptake of [^{3}H]PMEA		
	3 μM	6 μM	18 μM
Hypoxanthine	86	95	98
Guanine	104	98	92
2-Aminopurine	98	99	108
2,6-Diaminopurine	97	93	91
Cytosine	86	89	99
Thymine	106	101	102
Uracil	90	96	103

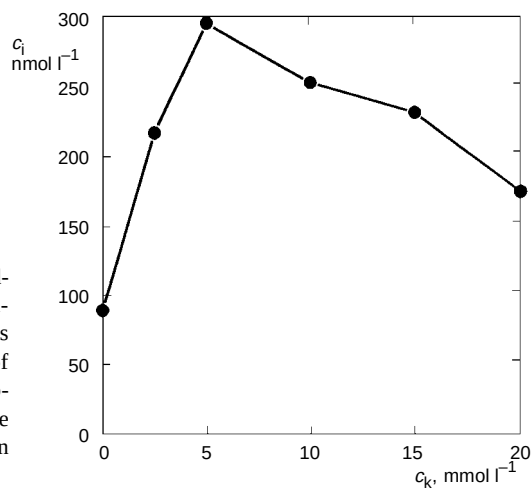

FIG. 5
Effect of colchicin (a cytoskeleton-degrading substance) on PMEA transport. The intracellular concentration of [^{3}H]PMEA was determined after a 15-min preincubation of T-lymphoblasts with colchicin and subsequent 30-min incubation in the presence of [^{3}H]PMEA. c_i Intracellular concentration of PMEA; c_k concentration of colchicin

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