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Short Communication

Cellular uptake of phosphonylmethoxyalkylpurine derivatives

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Summary

The cellular uptake of the phosphonylmethoxyalkylpurine derivatives HPMPA and PMEA has been studied in H9 cells. The two compounds exhibited an identical pattern of permeation in this cell line. Uptake did not occur via the nucleoside transport system, but through a different mechanism which, for its slow kinetics and temperature-dependence, is compatible with an endocytosis-like process. The amount of cell-associated drug increased up to one hour post-incubation.

HPMPA; PMEA; Cellular uptake; Endocytosis; Nucleoside transport

Introduction

The phosphonylmethoxyalkylpurine derivatives (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) exhibit a potent and selective activity against a great variety of viruses, mainly DNA viruses and retroviruses (De Clercq et al., 1986, 1987; Pauwels et al., 1988). The target for the antiviral action of these compounds is viral DNA synthesis (Votruba et al., 1987). Several aspects of the drugs' pharmacological action, however, including the process by which the compounds get inside the cells, have not been clarified. Like nucleotides, these are negatively charged molecules, and may not easily be taken up by the cells

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because of the electrostatic repulsion between the drug and the exterior of the cell membrane. Nevertheless, phosphonylmethoxyalkylpurines such as HPMPA and PMEA must access the intracellular compartment to achieve their antiviral activity.

Cell permeation studies were performed with HPMPA and PMEA to explain the apparent paradox of theoretically unpermeant molecules which are endowed with biological activity. H9 cells were chosen since lymphocytes are known to be the host cells for a number of different viruses that are sensitive to the compounds. Moreover, cells that are in suspension, in contrast with adherent cells, can be quickly separated from hydrophilic metabolites through phase partition. This would avoid washing procedures that would otherwise cause redistribution effects between the intracellular and the extracellular compartments. Thus, drug permeation experiments could be performed at initial-rate kinetics.

Materials and Methods

S-(paranitrobenzyl)-6-thioinosine (NBTI) and dipyridamole were obtained from Sigma (St. Louis, MO, U.S.A.). Tritiated thymidine and adenosine (specific activity 16 Ci/mmol) were from Amersham (Bucks., U.K.). Tritiated HPMPA and PMEA were obtained from Moravek Biochemical Company (specific activity 27 Ci/mmol). They were shown to be pure by reverse-phase HPLC, using the unlabelled compounds and adenosine as references. H9 cells (a kind gift from Prof. P.M. Cereda, Pavia) were routinely cultured in RPMI-1640 medium containing 20% FCS (Gibco). Permeation studies were performed essentially as reported previously (Palú et al., 1990). Briefly, 10^7 H9 cells/ml in RPMI 1640 medium were simultaneously mixed by means of a dual syringe device with 30 pmol of tritiated compounds and, where required, also with 0.5 μM NBTI/dipyridamole.

For short kinetics experiments, intended to test the occurrence of facilitated diffusion and carried out at 21°C over a time period of 2–120 s, final suspensions (200 μl) were directly dispensed into microhemocytometer tubes that contained 50 μl of a dense silicon oil/paraffin mixture (86/14, d = 1.13g/ml) overlayed onto 25 μl of 25% TCA. For longer assay periods, cells were incubated with drugs at either 37 or 4°C in separate vials before being dispensed into microhemocytometer tubes for phase separation. This was achieved within 2 s by centrifuging the samples in an Eppendorf 5414 microfuge at 12 000 × g for 30 s (Young and Jarvis, 1983). The bottom of the tubes containing the cell pellets in TCA was eventually sliced off to count cell-associated radioactivity by scintillation spectrometry. Cell volumes (intracellular and extracellular spaces) were calculated as reported elsewhere (Belt, 1983).

Results and Discussion

The results of the rapid kinetics of permeation of HPMPA and PMEA are shown in Fig. 1. For comparison, results are also shown for adenosine and thymidine (Fig. 1, inset). The natural nucleosides enter the cells quickly: cellular uptake slows down between 10 and 30 s, at a time at which equilibrium between intracellular (i.c.) and extracellular (e.c.) compartments is reached. Beyond this point, further drug influx occurs concomitantly with drug metabolism and efflux which start once the drug is inside the cell (Paterson et al., 1981). From the data presented in Fig. 1 (inset) it is also clear that the nucleoside uptake process in H9 cells, as also seen with other cell populations, is sensitive to inhibitors of the nucleoside transport system (Lauzon and Paterson, 1977; Plagemann et al., 1980; Paterson et al., 1981; Belt, 1983).

The phosphonyl nucleoside derivatives behaved quite differently from the natural nucleosides, for they seem to gain a rather poor access to the i.c. compartment even after a 2-min incubation period. Furthermore, the cellular uptake of HPMPA and PMEA is apparently not affected by inhibitors of the nucleoside transport system (Fig. 1). When the cells are incubated at 37°C in the presence of the phosphonates for longer time-periods (Fig. 2), cellular

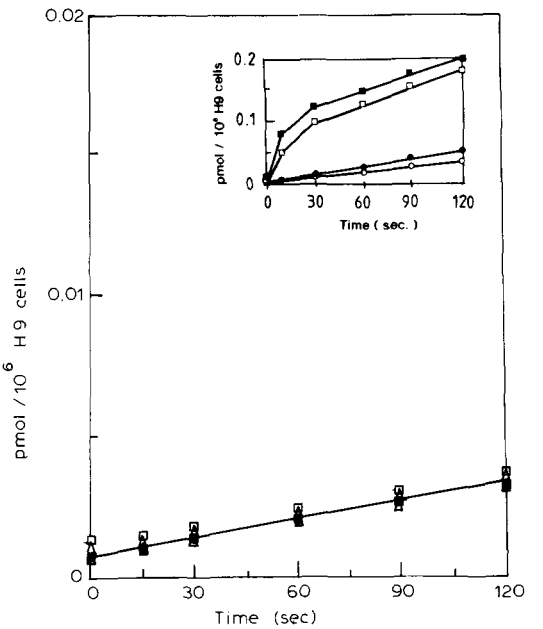

Fig. 1. Cellular uptake of [^{3}H]HPMPA and [^{3}H]PMEA (~ 30 pmol assay) by H9 cells at 21°C in the absence of inhibitors of the nucleoside transporter (▲, HPMPA; △, PMEA) and the presence of a 0.5 μM NBTI/dipyridamole concentration (□, HPMPA; ×, PMEA). Inset: Cellular uptake of [^{3}H]adenosine and [^{3}H]thymidine (~ 30 pmol assay) by H9 cells at 21°C, in the absence of inhibitors of the nucleoside transporter (■, adenosine; □, dThd) and in the presence of a 0.5 μM NBTI/dipyridamole concentration (●, adenosine; ○, dThd).

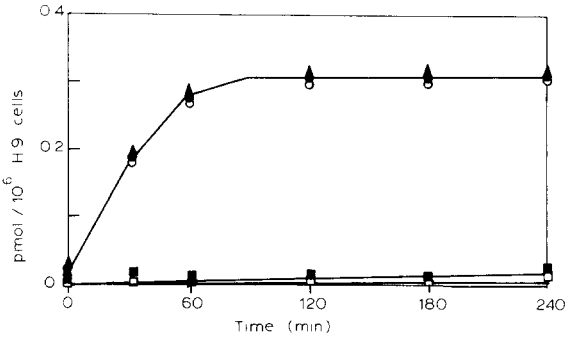

Fig. 2. Cellular uptake of [^{3}H]HPMPA and [^{3}H]PMEA (~ 30 pmol assay) by H9 cells at 37°C (▲, HPMPA; △, PMEA) and 4°C (■, HPMPA; □, PMEA). Experiments at 37°C were also performed in the presence of a 0.5 μM NBTI/dipyridamole concentration (●, HPMPA; ○, PMEA).

uptake increased linearly for about 60 min to reach a plateau phase thereafter. Once again, this cellular uptake was not affected by NBTI/dipyridamole. On the other hand, uptake of both compounds was virtually abolished if the incubation with the cells was carried out at 4°C for 1 h.

Our data thus provide evidence that the phosphonylmethoxyalkylpurine derivatives HPMPA and PMEA are not taken up by H9 cells via the nucleosides transport system (i.e. facilitated diffusion). A functional alteration of this system in H9 cells is unlikely, for these cells are normally permeable by natural nucleosides and sensitive to inhibitors of their transport system. The hydrophilic nature of HPMPA and PMEA and their very poor cell permeation ability in short-time kinetics do not seem to favour the possibility that the phosphonates enter the cells via passive diffusion. If this phenomenon were to occur, it would probably have faster kinetics, which, like facilitated diffusion, would rapidly lead to an equilibrium between i.c. and e.c. drug concentrations. Also, one should not expect dramatically different uptake kinètics at 4°C versus 37°C if passive diffusion should occur. Instead, our data point to a different kind of uptake process that is rather slow and temperature-dependent, unlike passive or facilitated diffusion. Slow kinetics and temperature dependence are two features that are characteristic of endocytosis (Loke et al., 1989; Yakubov et al., 1989). The presence of a plateau phase also suggests an equilibrium with exocytosis. Thus, the equilibrium between influx and efflux, which is reached after 1 h incubation, would correspond to the time it takes for the endo-exocytic vesicles to complete their traffic from and to the cell membrane. In view of their slow kinetics of cellular uptake and of the need to be activated inside the cell, HPMPA and PMEA thus incur some delay before they can exert their antiviral action. Our interpretation of the above-presented data would favor an endocytic mode of cellular uptake for phosphono compounds. This mode notwithstanding, the drugs reach quite a high intracellular concentration. In fact, the i.c. HPMPA concentration reached after 1 h incubation is higher

(0.6 μM) than the e.c. (0.15 μM) concentration in molar terms, considering an internal volume of 0.5 μl for 10^6 H9 cells. Whether this apparent concentration process is due to an active transport different from endocytosis, to distribution within hydrophilic compartments of H9 cells, or to some metabolic conversion, is not clear as yet. Further studies on the uptake of these drugs by different cell types, including virus-infected cells, as well as a more thorough dissection of the process by which they are taken up by the cells, may increase our understanding of the pharmacological behavior of the phosphonates at the cellular level.

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