Biochimica et Biophysica Acta, 373 (1974) 100–105

BBA 76782

CYCLOHEXIMIDE INHIBITION OF HYPOXANTHINE TRANSPORT IN CULTURED CELLS

ERIK DYBING

Institute of Pharmacology, University of Oslo, Blindern, Oslo 3 and The Department of Environmental Toxicology, National Institute of Public Health, Oslo 1 (Norway)

(Received May 14th, 1974)

SUMMARY

Cycloheximide preincubation inhibits hypoxanthine uptake into the acid-soluble fractions of cultured rat hepatoma cells (MH_1C_1) and human skin epithelial cells (NCTC 2544, HE cells) in a time- and dose-dependent manner. 50 % inhibition is seen after 4 h preincubation with 10^{-4} M cycloheximide of MH_1C_1 cells and after 2.5 h of HE cells. Adenine uptake is much less affected, after 10 h preincubation with 10^{-4} M cycloheximide it was reduced to 83 % and 67 % of controls in MH_1C_1 cells and HE cells respectively. Cycloheximide inhibits hypoxanthine uptake in a dose-dependent manner above 10^{-7} M, with 50 % inhibition in MH_1C_1 cells at $4 \cdot 10^{-7}$ M after 12 h preincubation and at 10^{-6} M in HE cells after 6 h preincubation. Puromycin mimics the action of cycloheximide. The inhibition of hypoxanthine uptake is not caused by reduction of the activity of hypoxanthine phosphoribosyltransferase in the two cell lines. 10^{-4} M cycloheximide preincubation for 10 h does not significantly reduce the uptake of the two non-metabolizable amino acids α-aminoisobutyric acid or 1-aminocyclopentane-1-carboxylic acid (cycloleucine). It is suggested that cycloheximide inhibits the synthesis of a rapidly turning over protein involved in hypoxanthine transport.

INTRODUCTION

Various mammalian cells are able to take up adenine and hypoxanthine from the extracellular incubation medium [1–3]. Evidence has been presented which suggest that adenine and hypoxanthine transport are separate events in rat hepatoma cells [4] and in human skin epithelial cells [5]. After cellular uptake it is assumed that adenine and hypoxanthine can serve as substrates for their respective phosphoribosyltransferases [6–8]; but it is believed that these reactions are distinct from those of cellular uptake [4, 5].

By the use of protein synthesis inhibitors it has been shown that amino acid transport is inhibited in embryonic chick bone [9], rat kidney cortex [10] and rat

Abbreviation: *P*-Rib-*P*-*P*, 5-phosphoribosyl-1-pyrophosphate.

diaphragm [11]. The underlying mechanisms for these effects were thought to be due to inhibition of the synthesis of a rapidly turning over protein involved in amino acid transport [9–11]. While investigating the effect of actinomycin D on purine transport [4, 12], it was noticed that cycloheximide preincubation inhibited the uptake of hypoxanthine in rat hepatoma cells in culture. This paper describes in some detail the effects of cycloheximide on purine uptake in cultured cells.

METHODS AND MATERIALS

Methods of cell culture. The clonal strain MH_1C_1 of rat hepatoma cells and the NCTC 2544 strain of human skin epithelial cells (HE cells) were grown as monolayer cultures as described [4, 12].

Cell culture experiments. All experiments were performed in growth medium without serum, after the cultures had been washed twice with warm serum-free medium (the cells do not divide in serum-free medium). Uptake into the acid-soluble fraction of the cells was measured after incubating with [^{14}C]hypoxanthine and [^{14}C]adenine for 15 min, with α-amino [^{14}C]isobutyric acid for 30 min and with [^{14}C]cycloleucine for 90 s as described [4, 12].

Assay of hypoxanthine phosphoribosyltransferase. Enzyme activity in vitro was assayed with a modification of the procedure described by Krenitsky et al. [8] with paper chromatography of the samples from the reaction mixtures in the system of Osnes et al. [13] as previously described [4]. In all experiments protein content was determined according to the method of Lowry et al. [14], using bovine albumin (Sigma) as standard.

Radioisotopes and chemicals. [8-^{14}C]Hypoxanthine (62 Ci/mole), [8-^{14}C]adenine (59 Ci/mole) and 1-aminocyclopentane-1-[^{14}C]carboxylic acid (cycloleucine, 53 Ci/mole) were purchased from the Radiochemical Centre, Amersham; α-amino[3-^{14}C]isobutyric acid (10.2 Ci/mole) from New England Nuclear. Cycloheximide, puromycin, IMP (sodium salt), AMP (sodium salt), unlabelled hypoxanthine, adenine, α-aminoisobutyric acid and 1-aminocyclopentane-1-carboxylic acid (cycloleucine) were all obtained from the Sigma Company. 5-Phosphoribosyl-1-pyrophosphate (*P*-Rib-*P*-*P*, tetrasodium salt) was delivered from Calbiochem, Cosmegen Lyovac (Merck, Sharp and Dohme) was used as actinomycin D source.

RESULTS

Preincubation of cultures of MH_1C_1 cells and HE cells with 10^{-4} M cycloheximide for up to 10 h leads to decreased uptake of hypoxanthine and adenine into the acid-soluble fraction (Fig. 1). There are significant differences in the degree of inhibition of the same substrate in the two cell lines and between the two substrates in the same cell line, however. Hypoxanthine uptake is much more reduced than that of adenine, 50 % inhibition of hypoxanthine uptake was noted after approx. 2.5 h preincubation in HE cells and after about 4 h in the MH_1C_1 cells. After 10 h preincubation with cycloheximide, adenine uptake was reduced to 67 % of controls in HE cells and to 83 % of controls in MH_1C_1 cells; extrapolation to 50 % inhibition would give 15 h and 28 h, respectively.

The possible mechanism for the effect of cycloheximide preincubation on

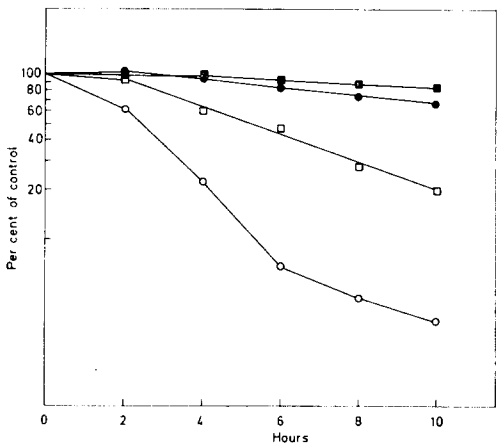

Fig. 1. Semi-logarithmic plot of the effect of cycloheximide preincubation on hypoxanthine and adenine uptake in MH_1C_1 and HE cell cultures. Replicate subcultures were preincubated with 10^{-4} M cycloheximide for 0, 2, 4, 6, 8 and 10 h and then incubated with $4 \cdot 10^{-7}$ M [^{14}C]hypoxanthine or [^{14}C]adenine (0.025 μCi/ml) for 15 min at 37 °C. Each point represents the mean of duplicate flasks in per cent of duplicate controls (ordinate); abscissa, time in h. ○—○, hypoxanthine in HE cells; □—□, hypoxanthine in MH_1C_1 cells; ●—●, adenine in HE cells; ■—■, adenine in MH_1C_1 cells.

hypoxanthine uptake in the cells was investigated using another inhibitor of protein synthesis, puromycin. After 4 h preincubation in the MH_1C_1 cells, the uptake was reduced to 68 % of controls after 10^{-4} M cycloheximide, to 63 % of controls after 10^{-4} M puromycin and to 59 % of controls when the two drugs were added together. After 2 h preincubation in HE cells, the same additions caused inhibition of hypoxanthine uptake to 62 %, 58 % and 60 % of controls respectively. Thus, it seems that the

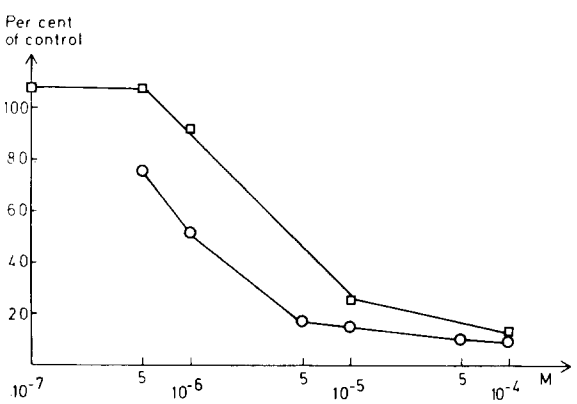

Fig. 2. Log dose–response plot of the cycloheximide effect on hypoxanthine uptake in MH_1C_1 and HE cell cultures. Replicate subcultures were preincubated without or with various concentrations of cycloheximide for 12 h in MH_1C_1 cells and 6 h in HE cells; and then incubated with $4 \cdot 10^{-7}$ M [^{14}C]hypoxanthine (0.025 μCi/ml) for 15 min at 37 °C. Each point represents the mean of duplicate flasks in per cent of duplicate controls (ordinate); abscissa, logarithm of cycloheximide concentration in moles/l. ○—○, HE cells; □—□, MH_1C_1 cells.

cycloheximide effect can be mimicked by puromycin, and that the two drugs are not acting in an additive manner.

The preceding experiments were done with very high doses of cycloheximide, accordingly effects via mechanisms different from protein synthesis inhibition could come into account. The next figure (Fig. 2) shows log dose–response curves of varying concentrations of cycloheximide on the uptake of hypoxanthine. The preincubation time in MH_1C_1 was here 12 h and in HE cells 6 h. chosen so as to get a high degree of uptake inhibition and at the same time to be at a presumably linear part of the preincubation time curve (Fig. 1). Cycloheximide clearly inhibits hypoxanthine uptake in a dose-dependent manner in both cell lines, but with differences in the degree of inhibition. 50 % inhibition was noted at $4 \cdot 10^{-6}$ M in the MH_1C_1 and at 10^{-6} M in HE cells. In all experiments uptake rates are related to total cellular protein in control cultures not treated with cycloheximide. Protein content of cells treated with the drug was reduced with an average of 3.4 % per h in MH_1C_1 cells and 3.6 % per h in HE cells. When taking this into account, the log dose–response plots (Fig. 2) were moved to the left, but the differences in the degree of action between the two cells lines were still evident.

The possibility that cycloheximide could affect the uptake of hypoxanthine through inhibition of its intracellular conversion to IMP was tested. After 18 h preincubation of MH_1C_1 cells with 10^{-4} M cycloheximide, hypoxanthine phosphoribosyltransferase activity was in fact slightly increased, to 110.9 ± 3.2 % of controls. A similar effect was seen also after preincubation of cells with $8 \cdot 10^{-7}$ M actinomycin D. Hypoxanthine phosphoribosyltransferase activity in fully grown, stationary cultures of HE cells was assayed after preincubation of the cells for 2, 4, 6, 8 and 10 h with 10^{-4} M cycloheximide, in this experiment no alteration in the rate of phosphoribosyltransferase was seen. In other experiments using growing cultures of HE cells, an increase in hypoxanthine phosphoribosyltransferase activity in cycloheximide-pretreated cells was also seen.

The possible effects of cyclohexamide preincubation on other cellular uptake processes, namely those of neutral amino acids, were also investigated. Table I shows the effect of 10^{-4} M cycloheximide preincubation for 10 h on the uptake of the two non-metabolizable amino acids α-aminoisobutyric acid and 1-aminocyclopentane-1-carboxylic acid (cycloleucine) into the acid-soluble fraction of HE cells. Cyclo-

TABLE I

EFFECT OF CYCLOHEXIMIDE ON THE UPTAKE OF α-AMINOISOBUTYRIC ACID AND CYCLOLEUCINE IN HE CELL CULTURES

Replicate subcultures were preincubated without or with 10^{-4} M cycloheximide for 10 h and then incubated with $2 \cdot 10^{-5}$ M butyric[^{14}C]-α-aminoisobutyric acid (0.025 μCi/ml) for 30 min or $2 \cdot 10^{-4}$ M [^{14}C]cycloleucine (0.05 μCi/ml) for 90 s at 37 °C. Values given in cpm/mg cell protein are means ±S.D. from three flasks.

Amino acid	Control (cpm/mg × 10^2)	With cycloheximide (cpm/mg × 10^2)
α-Aminoisobutyric acid	14.40±0.89	16.17±0.07
Cycloleucine	3.90±0.12	3.41±0.49

heximide caused a slight increase in α-aminoisobutyric acid uptake to 112 % of controls, whereas that of cycloleucine was slightly decreased to 87 % of controls. Thus neither uptake was much affected by the treatment of cycloheximide which reduced hypoxanthine uptake to only 3 % of controls.

DISCUSSION

The present experiments strongly point to the involvement of protein synthesis in the cellular uptake of purines, directly or indirectly. An explanation near at hand is that cycloheximide inhibits the synthesis of a rapidly turning over transport or control protein for hypoxanthine uptake; in analogy with the explanation for the effects of protein synthesis inhibitors on amino acid uptake in embryonic chick bone, rat kidney cortex and rat diaphragm [9–11]. Here the transport of the non-metabolizable α-aminoisobutyric acid was thought to be mediated by a protein with short-half-life, inhibition of the synthesis of this protein was associated with significant reduction of amino acid uptake provided that sufficient time was allowed for its physiological catabolism [3]. These findings are in contrast with the small effect of cycloheximide on the uptake of α-aminoisobutyric acid and cycloleucine presented here. $8 \cdot 10^{-7}$ M actinomycin D for 2 h, has earlier been shown not to reduce the uptake of α-aminoisobutyric acid or cycloleucine in HE cells [15].

Cycloheximide, in the present report, is not thought to compete with hypoxanthine binding sites and thus impair uptake, judging from the time-dependent increase in the effect, and that omission of cycloheximide from the medium during uptake does not alter the uptake inhibition once it is developed. The great differences in the effect of cycloheximide on hypoxanthine vs adenine uptake in the two cell lines observed, fit into the earlier assumption that the uptake of hypoxanthine and adenine are processes different from each other [4, 5].

Cycloheximide is not found to reduce the activity of the enzyme which converts hypoxanthine to IMP, hypoxanthine phosphoribosyltransferase, so that the observed effects on hypoxanthine uptake are not due to altered rates of its subsequent phosphorylation. However, changes in the relative level of other nucleotides in the intracellular pool could possibly also have an influence on the uptake of hypoxanthine; this has at present not been investigated. Cycloheximide did not affect the pool size of purine deoxyribonucleoside phosphates in the myxomycete *Physarum* [16]. The apparent stability of hypoxanthine phosphoribosyltransferase is remarkable, the observed increase of its activity in some experiments after cycloheximide preincubation is thought to be due to inhibition of enzyme degradation or because other proteins decrease more rapidly than this enzyme.

The underlying mechanism for the cycloheximide effect is thought to be different from that noted using actinomycin D [4, 12], this drug inhibited hypoxanthine uptake through an effect at the level of the plasma membrane presumably independently of RNA synthesis.

As mentioned, cycloheximide reduced the total protein content of the cells compared to controls without cycloheximide. If the uptake rates after cycloheximide preincubation are corrected for this reduction and instead related to protein content at zero time, the degree of inhibition of hypoxanthine uptake becomes even more pronounced. The inhibitory effects of cycloheximide on hypoxanthine uptake is not

associated with gross impairment of cellular function, judging from the small effect on amino acid uptake.

ACKNOWLEDGEMENT

The present investigation was supported by the Norwegian Research Council for Science and the Humanities (NAVF) and the Norwegian Cancer Society.

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