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EFFECTS OF CHLORPROMAZINE AND ACTINOMYCIN D ON UPTAKE AND INCORPORATION OF CERTAIN AMINO ACIDS, HYPOXANTHINE AND THYMIDINE IN CULTURES OF HUMAN SKIN EPITHELIAL CELLS

ERIK DYBING

The Institute of Pharmacology, University of Oslo, Blindern, Oslo 3, Norway

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Abstract –Chlorpromazine (CPZ) $1{\cdot}5 \times 10^{-4}$ M inhibited the uptake and incorporation of alanine (25 and 3 per cent of controls respectively), the uptake of α-aminoisobutyric acid (AIB, 39 per cent of controls) and the uptake and incorporation of hypoxanthine (36 and 44 per cent of controls) into acid-soluble and insoluble fractions of human skin epithelial cells (HE cells, NCTC 2544) grown in culture. The uptake of phenylalanine and 1-aminocyclopentane-1-carboxylic acid (cycloleucine) was not inhibited by CPZ in the same dose range, but CPZ above 10^{-5} M inhibited the incorporation of phenylalanine into acid-insoluble material with 50 per cent inhibition at $6{\cdot}5 \times 10^{-5}$ M. Actinomycin D stimulated the uptake of thymidine into the acid-soluble fraction of the HE-cells, 5·0 μg/ml increased the uptake to 160 per cent of the controls. The uptake of hypoxanthine was inhibited by actinomycin D, 5·0 μg/ml reduced the uptake to 67 per cent of controls. Actinomycin D did not alter the uptake of AIB or cycloleucine.

CHLORPROMAZINE (CPZ) a drug that has membrane effects in a great number of biological systems (for review see Seeman),[1] has been shown to inhibit the uptake and incorporation of alanine and the uptake of α-aminoisobutyric acid (AIB)[2] as well as the uptake and incorporation of hypoxanthine, thymidine and uridine[3] in cultured MH_1C_1 rat hepatoma cells. There were differences in the degree of inhibition caused by CPZ, however, indicating that the mechanism of action of CPZ is not completely unspecific in nature.

Actinomycin D stimulates thymidine uptake but inhibits hypoxanthine uptake into acid-soluble fractions of the MH_1C_1 cell cultures,[4] effects that appear to be independent of the antibiotic properties of actinomycin D.

The present experiments were undertaken to see if the actions of CPZ and actinomycin D on uptake and incorporation of amino acids and nucleic acid precursors found in the MH_1C_1 rat hepatoma cells were demonstrable in cells derived from non-neoplastic tissue taken from another organ and species; cultures of cells from human skin epithelium were used in this study. Experiments were designed to test if CPZ had a different effect on the uptake and incorporation for different neutral amino acids.

METHODS AND MATERIALS

Methods of cell culture. HE cells (NCTC 2544) derived from human skin epithelium were grown as monolayers in Carrell flasks containing 10 ml of Dulbecco's

modified Eagles's medium supplemented with 15% horse serum (Gibco), 2·5% foetal calf serum (Gibco), penicillin 100 U/ml, streptomycin 0·1 g/ml and nystatin 60 U/ml. Sixteen subcultures were made from one full-grown Roux bottle, cells were used for experiments 2–3 days after subculture, each flask containing approx 2·5–3·5 mg cell protein.

Cell culture experiments. All experiments were performed in ordinary growth medium with serum. In experiments measuring uptake and incorporation of radioactive isotopes (^{14}C-alanine, ^{14}C-phenylalanine, ^{14}C-α-aminoisobutyric acid (AIB), ^{14}C-1-aminocyclopentane-1-carboxylic acid (cycloleucine), ^{14}C-hypoxanthine or ^{3}H-thymidine), replicate subcultures were incubated with isotopes alone or together with drugs to be tested for various lengths of time at 37°. CPZ was added directly together with the isotopes; in the actinomycin D experiments cells were preincubated for 2 hr with the drug and then incubated with isotopes and continuous presence of actinomycin D. Radioactivities were measured in cold PCA-soluble and PCA-insoluble material as described previously.[4,2] Cell protein was measured by the method of Lowry *et al.*[5] using bovine albumin (Sigma) as standard.

Radioisotopes and chemicals. The following radioactive isotopes were purchased through Norsk Atominstitutt: [U-^{14}C] alanine (sp. act. 10 mCi/m-mole, Amersham), [3-^{14}C] α-aminoisobutyric acid (AIB, sp. act. 10·2 mCi/m-mole, New England Nuclear), [carboxylic acid-^{14}C] 1-aminocyclopentane-1-carboxylic acid (cycloleucine, sp. act. 53 mCi/m-mole, Amersham), [8-^{14}C] hypoxanthine (sp. act. 59 mCi/m-mole, Amersham), [U-^{14}C] phenylalanine (sp. act. 513 mCi/m-mole, Amersham) and [6-^{3}H] thymidine (sp. act. 28 Ci/m-mole, Amersham).

Chlorpromazine hydrochloride was obtained from Dolder AC, Switzerland. Unlabelled alanine, AIB, cycloleucine, thymidine and hypoxanthine were purchased from the Sigma Company; actinomycin D (Cosmegen Lyovac^{R}) from Merck, Sharp & Dohme.

RESULTS

Table 1 shows the effect of CPZ $1·5 \times 10^{-4}$ M on the uptake and incorporation of the amino acid alanine, the uptake of the nonmetabolizable amino acid AIB and the uptake and incorporation of the purine base hypoxanthine into HE cells. The uptake of alanine and AIB into the acid-soluble fraction was reduced to 26 and 39 per cent of controls, respectively, after incubation with CPZ $1·5 \times 10^{-4}$ M for 2 hr,

TABLE 1. EFFECT OF CPZ ON THE UPTAKE AND INCORPORATION OF ALANINE, AIB AND HYPOXANTHINE INTO ACID-SOLUBLE AND ACID-INSOLUBLE FRACTIONS OF HE CELLS*

Radioisotope added	Control		With CPZ 0·15 mM	
	PCA-soluble (cpm/mg × 10^2)	PCA-insoluble (cpm/mg × 10^2)	PCA-soluble (cpm/mg × 10^2)	PCA-insoluble (cpm/mg × 10^2)
^{14}C-alanine	30·47 ± 1·32	27·39 ± 0·75	7·83 ± 0·14	0·75 0·03
^{14}C-AIB	54·41 ± 1·11	—	21·20 ± 0·90	
^{14}C-hypoxanthine	169·80 ± 7·00	23·72 ± 0·93	61·70 ± 9·90	10·35 ± 1·76

* Replicate subcultures were incubated with ^{14}C-alanine 0·08 μCi/ml, $3·2 \times 10^{-4}$ M for 2 hr; ^{14}C-AIB, 0·08 μCi/ml, $1·5 \times 10^{-5}$ M for 2 hr; or ^{14}C-hypoxanthine 0·025 μCi/ml, 4×10^{-7} M for 15 min at 37° without, or in the presence of, CPZ $1·5 \times 10^{-4}$ M. Values are means ± S.D. from three flasks.

whereas the incorporation of alanine into acid-insoluble material was inhibited to 3 per cent of controls. After 15 min CPZ $1{\cdot}5 \times 10^{-4}$ M reduced hypoxanthine uptake and incorporation to 36 and 44 per cent of controls, respectively.

A time course study of the effect of CPZ on the uptake and incorporation of alanine and phenylalanine is shown in Fig. 1. Most of the apparent uptake of phenylalanine occurs during the first 15 min of incubation. CPZ 10^{-4} M was found not

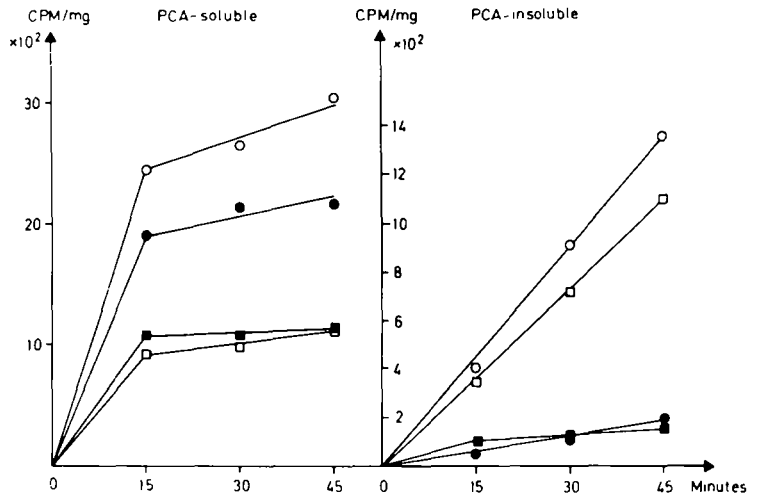

FIG. 1. Time course of the effect of CPZ on the uptake and incorporation of alanine and phenylalanine into acid-soluble and acid-soluble fractions of HE cells. Replicate subcultures were incubated with ^{14}C-alanine 0·0625 μCi/ml, $2{\cdot}5 \times 10^{-4}$ M or 14C-phenylalanine 0·0625 μCi/ml, $4{\cdot}1 \times 10^{-4}$ M for 15, 30 and 45 min at 37° without, or in the presence of, CPZ 10^{-4} M. Each point represents the mean of values from duplicate flasks. Ordinate, counts/min and mg cell protein; abscissa, time in min. (○) alanine; (□) phenylalanine; (●) alanine + CPZ; (■) phenylalanine + CPZ.

to reduce this process. The incorporation of phenylalanine into acid-insoluble material was strongly inhibited, however, with 14 per cent of control values incorporated after 45 min. In contrast to phenylalanine the uptake of alanine was reduced by addition of CPZ 10^{-4} M; after 45 min the acid-soluble fraction contained 71 per cent of the activity of the controls. Here again incorporation into acid-insoluble materials was considerably inhibited by CPZ to 14 per cent of control values.

A log-dose response curve of the effect of CPZ on the incorporation of phenylalanine into acid-insoluble material of HE cells is shown in Fig. 2. At doses above 10^{-5} M CPZ there is a dose dependent inhibition of phenylalanine incorporation, with 50 per cent inhibition at $6{\cdot}5 \times 10^{-5}$ M.

The uptake of AIB into HE cell cultures seems to follow Michaelis–Menten kinetics (Fig. 3) with an apparent K_m of $2{\cdot}6 \times 10^{-3}$ M and a V_{max} of 0·42 μmoles/mg cell protein and hr. Addition of CPZ gives a noncompetitive pattern in the inhibition of the AIB uptake without altering the apparent K_m.

The nonmetabolizable amino acid cycloleucine shows a time dependent increase in uptake into HE cells during the first 90 sec of incubation (Fig. 4), whereas no further increase in intracellular radioactivity can be found after 5 min. Activity associated with the acid-soluble fraction at 90 sec is 74 per cent of the activity measured at 5 min. CPZ did not influence the uptake of cycloleucine.

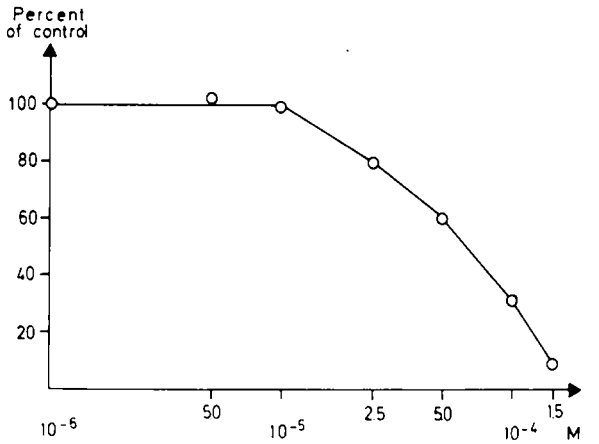

FIG. 2. Semi-logarithmic plot of the CPZ effect on the incorporation of phenylalanine into the acid-soluble fraction of HE cells. Replicate subcultures were incubated with ^{14}C-phenylalanine 0·05 μCi/ml, 3·6 × 10^{-4} M for 2 hr at 37° without, or in the presence of, varying concentrations of CPZ. Each point represents the mean of values from duplicate flasks. Ordinate, values as a percentage of incorporated activity/mg cell protein of duplicate controls; abscissa, substrate concentrations in moles/l.

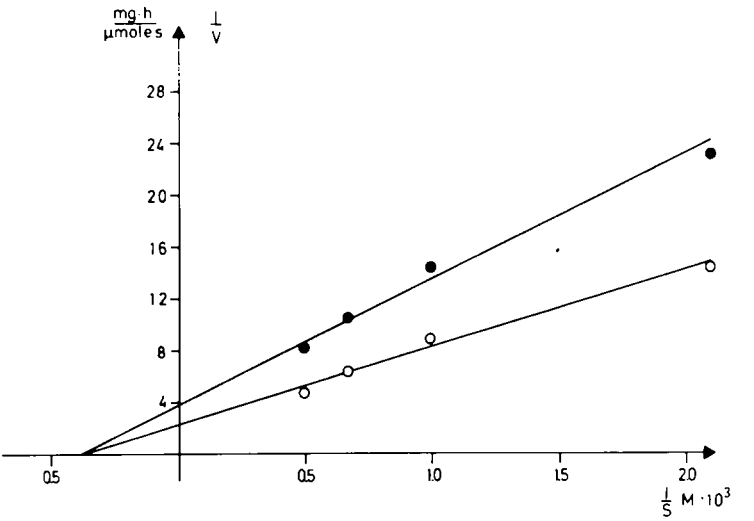

FIG. 3. Double reciprocal plot of the CPZ effect on the uptake of AIB into the acid-soluble fraction of HE cells. Replicate subcultures were incubated with ^{14}C-AIB 0·5, 1·0, 1·5 and 2·0 × 10^{-3} M, 68 CPM *n*-mole, for 30 min at 37° without, or in the presence of, CPZ 1·5 × 10^{-4} M. Each point represents the mean of values from three flasks. Ordinate, inverse of the velocity in mg and hr/n-moles × 10^{-2}, abscissa, inverse of the molar substrate concentration M. (○) Control; (●) with CPZ.

Table 2 gives the result of preincubation and continuous presence of actinomycin D at doses of 1·0, 2·0 and 5·0 μg/ml on the uptake of thymidine and hypoxanthine. Actinomycin D at 1·0, 2·0 and 5·0 μg/ml gave a dose dependent stimulation of thymidine (2·5 × 10^{-7} M) uptake to 119, 125 and 160 per cent of controls, respectively. In another experiment with four times higher thymidine concentration (10^{-6} M) the same doses of actinomycin D increased thymidine uptake to 110, 115 and 120 per cent of controls, respectively. The apparent K_m for thymidine uptake in HE cells is

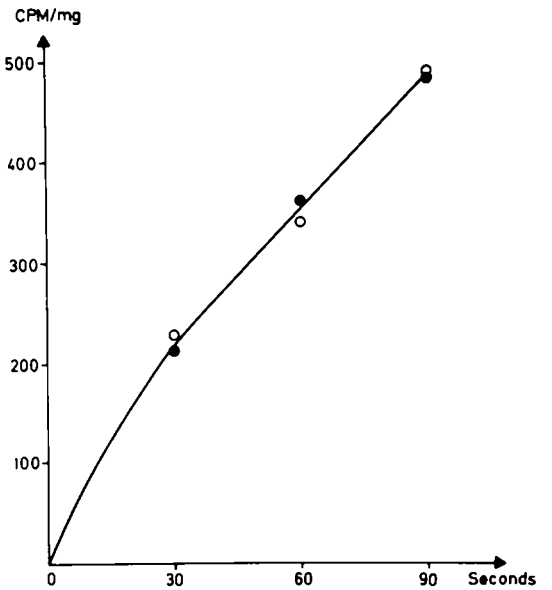

FIG. 4. Time course of the effect of CPZ on the uptake of cycloleucine into the acid-soluble fraction of HE cells. Replicate subcultures were incubated with ^{14}C-cycloleucine 0·05 μCi/ml. 10^{-6} M for 30, 60 and 90 sec at 37° without, or in the presence of, CPZ $1{\cdot}5 \times 10^{-4}$ M. Each point represents the mean of values from duplicate flasks. Ordinate, cpm and mg cell protein; abscissa, time in sec. (○) Control; (●) with CPZ.

TABLE 2. EFFECT OF ACTINOMYCIN D ON THE UPTAKE OF THYMIDINE AND HYPOXANTHINE INTO THE ACID-SOLUBLE FRACTION OF HE CELLS*

Precursor added	Control (cpm/mg × 10^2)	With actinomycin D		
		1·0 μg/ml (cpm/mg × 10^2)	2·0 μg/ml (cpm/mg × 10^2)	5·0 μg/ml (cpm/mg × 10^2)
Thymidine	64·52 ± 4·94	76·68 ± 3·22	80·59 ± 4·04	103·26 ± 1·90
Hypoxanthine	60·14 ± 3·92	44·25 ± 3·51	43·26 ± 2·56	40·51 ± 1·52

* Replicate subcultures were preincubated without or with actinomycin D 1·0 μg/ml, 2·0 μg/ml or 5·0 μg/ml for 2 hr and then incubated with ^{3}H-thymidine 0·05 μCi/ml, $2{\cdot}5 \times 10^{-7}$ M or ^{14}C-hypoxanthine 0·0125 μCi/ml, 2×10^{-7} M for 15 min at 37° without, or in the presence of, actinomycin D. Values are means ± S.D. from three flasks.

$6{\cdot}7 \times 10^{-7}$ M and the V_{max} 2·5 n-moles/mg cell protein and hr. Table 2 also gives the effect of actinomycin D on the uptake of hypoxanthine in HE cells. There is a dose dependent inhibition of uptake to 74, 72 and 67 per cent of controls.

Preincubation with actinomycin D 1·0 μg/ml for 2 hr had no apparent effect on the uptake of the two nonmetabolizable amino acids AIB and cycloleucine (Table 3).

DISCUSSION

CPZ $1{\cdot}5 \times 10^{-4}$ M inhibits the uptake and incorporation of alanine, the uptake of AIB and the uptake and incorporation of hypoxanthine into the HE cell cultures to about the same extent as was seen using the MH_1C_1 rat hepatoma cell cultures.[2,3]

TABLE 3. EFFECT OF ACTINOMYCIN D ON THE UPTAKE OF AIB AND CYCLOLEUCINE INTO THE ACID-SOLUBLE FRACTION OF HE CELLS*

Amino acid added	Control (cpm/mg × 10^2)	With actinomycin D (cpm/mg × 10^2)
AIB	13·7 ± 0·5	13·6 ± 1·4
Cycloleucine	5·7 ± 0·4	6·3 ± 0·8

* Replicate subcultures were preincubated without or with actinomycin D 1·0 μg/ml for 2 hr and then incubated with ^{14}C-AIB 0·025 μCi/ml, 4×10^{-4} M for 30 min or ^{14}C-cycloleucine 0·05 μCi/ml, 10^{-6} M for 90 sec at 37° without, or in the presence of, actinomycin D. Values are means ± S.D. from duplicate flasks.

No inhibition of the uptake of phenylalanine or cycloleucine in HE cells by CPZ in the same dose range could be found, however, Oxender and Christensen[6] using Ehrlich ascites cell have described a system with two distinct mediating sites termed alanine-preferring (A-system) and leucine-preferring (L-system) for the transport of neutral amino acids. The neutral amino acids are apparently transported preferentially by one of the two systems, alanine and AIB being substrates mainly for the A-system and phenylalanine a substrate mainly for the L-system. If similar amino acid transport systems exist in HE cells, it is possible that CPZ only interacts with an A-system. Cycloleucine transport is mediated by both systems in the Ehrlich ascites cell[6], the affinity of cycloleucine for an A-system in HE cells might be low, however. The possibility that CPZ may change apparent alanine uptake indirectly through alteration in the size of the alanine pool must also be taken into consideration. As CPZ affects phenylalanine incorporation whereas it apparently does not reduce phenylalanine uptake, this suggests that for alanine, CPZ inhibits both net uptake and incorporation. Peterson *et al.*[7] have shown that CPZ inhibits the incorporation of leucine into isolated synaptosomal particles.

The uptake of thymidine was stimulated in a dose-dependent manner by actinomycin D in cultures of cells derived from non-neoplastic tissue of another organ and species than that in which this actinomycin D effect was first demonstrated.[4] This suggests that the ability of actinomycin D to alter the uptake of thymidine and hypoxanthine might be found in other eukaryotic cells. Thymidine uptake in HE cells is stimulated to a much smaller extent than in the rat hepatoma cells, however (actinomycin D 1·0 μg/ml preincubation for 2 hr gave 238 per cent of controls in MH_1C_1 cells vs 119 per cent in HE cells; 5·0 μg/ml gave 371 vs 160 per cent, respectively). The apparent K_m for thymidine uptake is considerably lower in HE cells compared to the MH_1C_1 cells (apparent K_m's $6{\cdot}7 \times 10^{-7}$ M vs $5{\cdot}9 \times 10^{-6}$ M). The inhibition of hypoxanthine uptake in HE cells caused by actinomycin D is also considerably less pronounced in HE cells than in the rat hepatoma cells (actinomycin D 1·0 μg/ml after 2 hr preincubation reduced hypoxanthine uptake to 74 vs 48 per cent of controls respectively).

The uptake of the two nonmetabolizable amino acids AIB and cycloleucine was not altered by actinomycin D 1·0 μg/ml pretreatment for 2 hr. Elsas *et al.*[8] accordingly

found no inhibition of AIB uptake in rat diaphragms after preincubation with actinomycin D 10 μg/ml for 3 hr, whereas Risser and Gelehrter[9] reported that actinomycin D 0·4 μg/ml after 2·5 hr preincubation in HTC rat hepatoma cells inhibited AIB uptake to 80 per cent of controls. The apparent K_m for AIB uptake in this cell line was found to be $2{\cdot}18 \times 10^{-3}$ M.

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