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INHIBITION OF HYPOXANTHINE TRANSPORT BY CHLORPROMAZINE AND SKF 525-A IN CELL CULTURE

ERIK DYBING

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

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Abstract—Chlorpromazine (CPZ, 0·15 mM) inhibited the uptake and incorporation of hypoxanthine into TCA-soluble and TCA-insoluble fractions in the MH_1C_1 rat hepatoma cell line to 38 and 49 per cent of control values, respectively. Diethylaminoethyl diphenylvalerate (SKF 525-A, 0·20 mM) also reduced hypoxanthine uptake and incorporation in the TCA-soluble and TCA-insoluble fractions to 38 and 41 per cent, respectively. When nucleic acid synthesis was blocked with actinomycin D and cytosine arabinoside, hypoxanthine uptake was also diminished; CPZ (0·15 mM) further reduced hypoxanthine uptake in the TCA-soluble fraction.

The cells showed a time dependent uptake and incorporation of thymidine and uridine; CPZ also inhibited the uptake and incorporation of these nucleosides, but to a much smaller extent than hypoxanthine. CPZ (0·15 mM) reduced the thymidine uptake into the TCA-soluble fraction to 71 per cent of controls and those of uridine to 83 per cent of controls. The inhibitory effects of CPZ and SKF 525-A on hypoxanthine and nucleoside uptake and incorporation were equally evident in a fibroblast-derived cell line. It is concluded that CPZ and SKF 525-A inhibit the transport of hypoxanthine and to a lesser degree the transport of thymidine and uridine in the cell cultures.

CHLORPROMAZINE (CPZ) and diethylaminoethyl diphenylvalerate (SKF 525-A) both inhibit the incorporation of ^{14}C-alanine into protein and decrease the accumulation of ^{14}C-alanine in rat hepatoma cells grown in culture.[1] Both drugs were also found to inhibit the uptake as well as the efflux from the cells of the nonmetabolizable α-aminoisobutyric acid (AIB), indicating an inhibition of the transport of these amino acids. Similar effects were found using promazine, thioridazine, and imipramine;[2] it was suggested that the term membrane stabilization could be extended to this system of cells in culture. CPZ[3] and SKF 525-A[4] probably also inhibit the uptake of *p*-aminophenol (PAP) and *p*-nitrophenol (PNP) into the cultured cells.

Nucleosides are taken up from the external incubation medium by mammalian cells grown in culture,[5] although they are not required for the cellular synthesis of nucleic acids or growth. The precise mechanisms of transport of nucleosides into cells are not exactly known. In Novikoff hepatoma cells they are thought to be taken up by specific transport mechanisms distinct from the phosphorylating reactions.[6] In several hamster cell lines, however, phosphorylation may act as a regulating factor for pyrimidine nucleoside uptake.[7]

The fact that CPZ and SKF 525-A interact with such different uptake mechanisms as those for amino acids and lipophilic foreign compounds, suggests that the inhibition may be rather unspecific in nature. Further information about these inhibitory actions might be obtained by investigating whether these drugs also influence the transport of nucleic acid precursors in cell culture.

MATERIALS AND METHODS

Methods of cell culture. The clonal strain MH_1C_1 of rat hepatoma cells was grown in Dulbecco's modified Eagles's medium supplemented with 15% horse serum, 2·5% foetal calf serum and antibiotics (approximately 300,000 cells/ml at subculture time). In some experiments the R5 fibroblast cell line derived from rat thyroid was used.

Experimental procedure. All experiments were performed in ordinary serum-containing growth medium. Replicate flasks, 5–7 days after sub-culture containing 1–2 mg cell protein, were incubated with ^{14}C-hypoxanthine (0·1 μCi/ml, 2×10^{-6} M), ^{3}H-thymidine (0·2 μCi/ml, 10^{-6} M), or ^{3}H-uridine (0·2 μCi/ml, 10^{-6} M) alone or with the simultaneous addition of CPZ (or SKF 525-A) at 37° for up to 15 min. To ^{3}H-thymidine and ^{3}H-uridine were added appropriate amounts of unlabeled thymidine and uridine, respectively. Two flasks in each experiment served as correction for zero time uptake, i.e. radioactivity associated with the cells at the beginning of the incubations. Values from these were subtracted from activities measured at the different time intervals. After 5, 10 or 15 min the incubation media were decanted and the cells were immediately washed four times with 5 ml of ice-cold barbital–NaCl-buffer pH 7·4. The cells were then suspended in 3·0 ml ice-cold 0·02% EDTA-barbital–NaCl-buffer, pH 7·4, precipitated with 3·0 ml ice-cold 20% TCA and allowed to stand on ice for 1 hr. After centrifugation for 10 min at 10,000 *g* and 4°, duplicate aliquots of the supernatant, containing radioactivity associated with the TCA-soluble pool, were counted in 15 ml BBOT scintillation fluid in a Tri-Carb liquid scintillation spectrometer, Model 3003. In experiments with ^{14}C-hypoxanthine and ^{3}H-uridine the TCA-insoluble pellet was solubilized in 1·0 ml 1 N NaOH at 37° overnight, and aliquots were counted in 15 ml BBOT. In experiments with ^{3}H-thymidine the radioactivity assumedly associated with DNA was extracted with 0·5 ml 5% TCA at 90° for 20 min, and aliquots of this extract were counted; the remaining insoluble material for determination of protein content was solubilized in 1·0 ml 1 N NaOH at 37° overnight. In some experiments with ^{14}C-hypoxanthine, some of the flasks were preincubated for 1 hr with actinomycin D 1 μg/ml and cytosine arabinoside 10 μg/ml and then incubated with ^{14}C-hypoxanthine plus actinomycin D and cytosine arabinoside without or with CPZ. In all experiments activities were related to cellular protein content determined according to Lowry *et al.*[8] using bovine albumin as standard.

Radioactivity and chemicals. ^{14}C-8-hypoxanthine (sp. act. 59 mCi/mmole, Amersham), ^{3}H-6-thymidine (sp. act. 28 Ci/mmole, Amersham), and ^{3}H-5-uridine (sp. act. 30 Ci/mmole, Amersham) were purchased through Norsk Atominstitutt, Kjeller. Chlorpromazine HCl was obtained from Dolder AG, Switzerland, through Norsk Medisinaldepot. 2-Diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) was a gift from the Smith, Kline & French Laboratories. Actinomycin D (Cosmegen Lyovac) was obtained from Merck, Sharp & Dohme; cytosine arabinoside, unlabeled thymidine and uridine from Sigma.

RESULTS

Figure 1 shows a time course plot of the uptake and incorporation of hypoxanthine into TCA-soluble and insoluble fractions in the MH_1C_1 cultures. The purine base is taken up by the cells in a time dependent manner, approximately 10 per cent of the

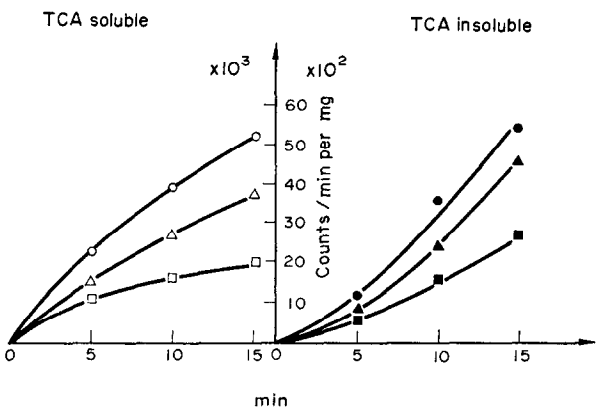

FIG. 1. Time course for hypoxanthine uptake and incorporation into TCA-soluble and TCA-insoluble fractions in MH_1C_1 cell cultures with/without CPZ 0·05 mM and 0·15 mM. Replicate sub-cultures were incubated with 0·1 μCi/ml ^{14}C-hypoxanthine (2×10^{-6} M) for 5, 10 and 15 min at 37°. Each point represents mean of values from duplicate flasks. Abscissa, time in minutes; ordinate, counts per minute and milligrams of cell protein. Open symbols TCA-soluble fraction, filled symbols TCA-insoluble fraction. (○) Controls; (△) CPZ 0·05 mM; (□) CPZ 0·15 mM.

radioactivity is further incorporated into TCA-insoluble material measured at 15 min. CPZ at concentrations of 0·05 and 0·15 mM reduces the amount of hypoxanthine transported into the cells. The reduction of radioactivity found in the TCA-soluble fraction in the presence of CPZ is followed by a reduction of radioactivity found in the TCA-insoluble fraction.

SKF 525-A also had an inhibitory effect on the transport and incorporation of hypoxanthine in MH_1C_1 cell cultures. Table 1 gives the values of the amount of radioactivity found in the TCA-soluble and insoluble fractions in the presence of SKF 525-A 0·05 and 0·20 mM compared to controls. SKF 525-A 0·20 mM reduces the activity in the TCA soluble fraction to 38 per cent and in the TCA-insoluble fraction to 41 per cent of controls, respectively.

The uptake of thymidine into the cold TCA-soluble pool and subsequent incorporation into the cold TCA-precipitable material of the MH_1C_1 cells is seen in Fig. 2. The greatest uptake velocity occurs during the first 5 min of incubation, but with a time dependent increase in intracellular radioactivity up to 15 min. The addition of CPZ 0·15 mM also reduces the amount of ^{3}H-thymidine found in the TCA-soluble fraction, but to a smaller extent than the effect noted on hypoxanthine transport (71 per cent of controls). Nine per cent of the activity of thymidine in the TCA-soluble fraction is associated with warm TCA-extractable material. CPZ inhibits the incorporation of thymidine into DNA to a similar degree as the inhibition of thymidine transport.

The effect of CPZ on uridine transport and incorporation is seen in Fig. 3. The inhibition of uridine uptake by CPZ was less than the inhibition of thymidine uptake.

CPZ and SKF 525-A inhibited hypoxanthine transport and incorporation in the fibroblast-derived cell line R5 (Table 2). As with the rat hepatoma cell line, the influence on thymidine and uridine transport was much less than on hypoxanthine transport.

TABLE 1. EFFECT OF SKF 525-A ON HYPOXANTHINE UPTAKE AND INCORPORATION INTO TCA-SOLUBLE AND INSOLUBLE FRACTIONS IN MH_1C_1 CELL CULTURES.

Control		With SKF 525-A 0·05 mM		With SKF 525-A 0·20 mM	
TCA soluble (counts/min $\times 10^3$)	TCA insoluble (counts/min $\times 10^3$)	TCA soluble (counts/min $\times 10^3$)	TCA insoluble (counts/min $\times 10^3$)	TCA soluble (counts/min $\times 10^3$)	TCA insoluble (counts/min $\times 10^3$)
46·51 ± 1·17	4·71 ± 0·55	32·07 ± 0·48	3·78 ± 0·27	17·69 ± 0·37	1·91 ± 0·06

Replicate sub-cultures were incubated with 0·1 μCi/ml ^{14}C-hypoxanthine (2×10^{-6} M) for 15 min at 37° with/without SKF 525-A 0·05 mM or 0·20 mM. Values are means ± S.D. from three flasks.

TABLE 2. EFFECT OF CPZ AND SKF 525-A ON HYPOXANTHINE, THYMIDINE AND URIDINE UPTAKE AND INCORPORATION INTO TCA-SOLUBLE AND INSOLUBLE FRACTIONS IN R5 CELL CULTURES.

	Control		With CPZ 0·15 mM		With SKF 525-A 0·20 mM	
Substrate added	TCA soluble (counts/min $\times 10^2$)	TCA insoluble (counts/min $\times 10$)	TCA soluble (counts/min $\times 10^2$)	TCA insoluble (counts/min $\times 10$)	TCA soluble (counts/min $\times 10^2$)	TCA insoluble (counts/min $\times 10$)
Hypoxanthine	85·6 ± 7·0	68·0 ± 4·2	39·3 ± 4·4	29·8 ± 2·3	31·2 ± 3·4	27·0 ± 5·5
Thymidine	20·1 ± 2·3	19·1 ± 2·0	18·9 ± 0·9	13·4 ± 1·6	15·9 ± 2·5	12·5 ± 3·3
Uridine	124·0 ± 1·9	343·8 ± 12·5	116·0 ± 9·4	339·5 ± 9·6	106·5 ± 6·6	318·3 ± 22·1

Parallel sub-cultures were incubated with 0·025 μCi/ml ^{14}C-hypoxyanthine (4×10^{-7} M), or 0·2 μCi/ml ^{3}H-thymadine (10^{-6} M), or 0·2 μCi/ml ^{3}H-uridine (10^{-6} M) without or in the presence of CPZ 0·15 mM cr SKF 525-A 0·20 mM for 15 min at 37°. Values are means ± S.D. from four flasks

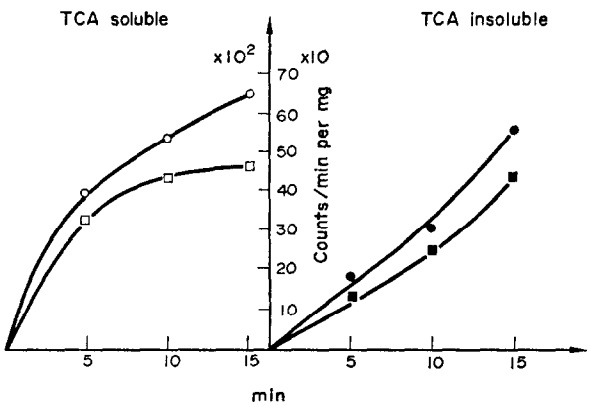

FIG. 2. Time course for thymidine uptake and incorporation into cold TCA-soluble and cold TCA-insoluble fractions in MH_1C_1 cell cultures with/without CPZ 0·15 mM. Replicate sub-cultures were incubated with 0·2 μCi/ml ^{3}H-thymidine (10^{-6} M) for 5, 10 and 15 min at 37°C. Each point is mean of results from duplicate flasks. (○) Controls; (□) CPZ 0·15 mM.

To gain more support for the assumption that CPZ was acting at the level of hypoxanthine transport, experiments using known inhibitors of nucleic acid synthesis were performed. Preincubation for 1 hr with actinomycin D, which inhibits DNA-dependent RNA synthesis, at a concentration of 1 μg/ml, reduced the amount of hypoxanthine incorporated in TCA-insoluble material to 5 per cent of controls. With preincubation and continuous presence of actinomycin D and the inhibitor of DNA synthesis cytosine arabinoside 10 μg/ml, hypoxanthine incorporation was inhibited to 1 per cent of controls. Figure 4 shows the effect of actinomycin D plus cytosine arabinoside without or with CPZ on the hypoxanthine transport and incorporation

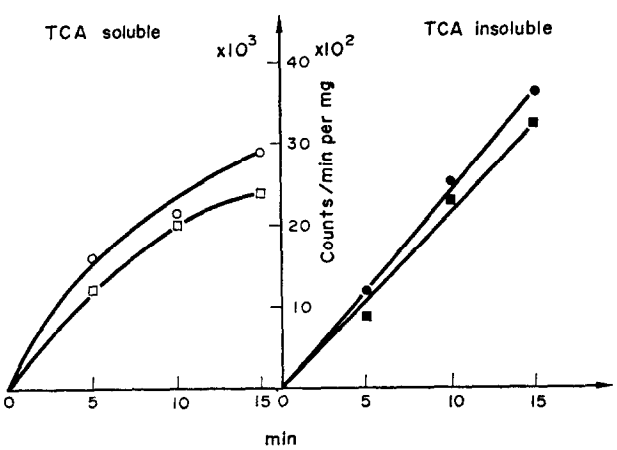

FIG. 3. Time course for uridine uptake and incorporation into TCA-soluble and insoluble fractions in MH_1C_1 cell cultures with/without CPZ 0·15 mM. Replicate sub-cultures were incubated with 0·2 μCi/ml ^{3}H-uridine (10^{-6} M) for 5, 10 and 15 min at 37°. Each point is mean of results from duplicate flasks. (○) Controls; (□) CPZ 0·15 mM.

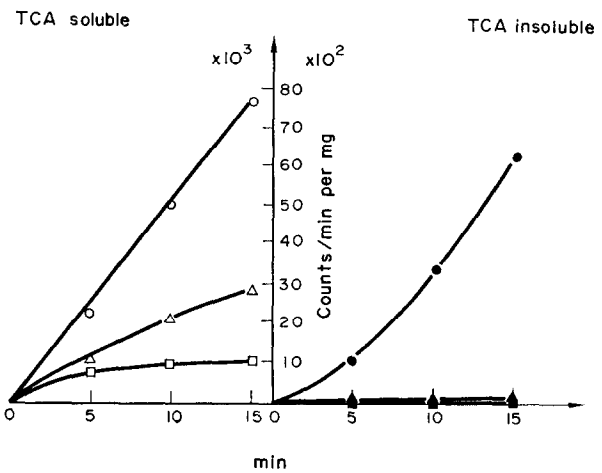

FIG. 4. Effect of CPZ, cytosine arabinoside and actinomycin D on hypoxanthine uptake and incorporation into TCA-soluble and insoluble fractions in MH_1C_1 cell cultures. Replicate sub-cultures were preincubated for 1 hr at 37° with/without actinomycin D 1 μg/ml and cytosine arabinoside 10 μg/ml, then incubated with 0·1 μCi/ml ^{14}C-hypoxanthine (2×10^{-6} M) with/without actinomycin D and cytosine arabinoside, or with actinomycin D and cytosine arabinoside plus CPZ 0·15 mM for 5, 10 and 15 min at 37°. Each point is mean of duplicate flasks. (○) Controls; (△) actinomycin D + cytosine arabinoside; (□) actinomycin D + cytosine arabinoside + CPZ 0·15 mM.

as compared to controls without any drugs. Actinomycin D plus cytosine arabinoside under these conditions also diminished the transport of hypoxanthine into the cells, 36 per cent of control values were found at 15 min in the TCA-soluble fraction. CPZ gave an additional reduction of the amount of hypoxanthine found in this fraction with only 12 per cent of the activity seen in the controls without any drugs at 15 min.

DISCUSSION

Drugs that reduce the uptake of nucleosides, such as persantine[9] or cholchicine,[5] give a parallel degree of inhibition of uptake and incorporation into trichloroacetic acid soluble and insoluble material. The effects of CPZ and SKF 525-A on the transport and incorporation of the purine base hypoxanthine in the MH_1C_1 as well as in the R5 cell line show a similar pattern.

Mizel and Wilson[5] noted little or no effect of cytosine arabinoside on thymidine uptake and actinomycin D on uridine uptake respectively into the TCA-soluble pools of HeLa cells; apparently they did not preincubate the cells with the inhibitors of DNA or RNA synthesis. Actinomycin D plus cytosine arabinoside, however, after 1 hr preincubation reduced the amount of radioactivity found in the TCA-soluble fraction by as much as 64 per cent. Elucidation of the underlying mechanism for this effect has not been attempted at the present time. It could mean, however, that the synthesis of a rapidly turning-over transport protein was inhibited. Inhibition by puromycin of amino acid transport via such a mechanism has been seen in experiments with embryonic chick bone,[10] rat kidney cortex[11] and rat diaphragm.[12] This effect became first apparent after 1–2 hr of preincubation with puromycin. Actinomycin D, although inhibiting *l*-lysine incorporation in the rat diaphragm,[12] had no effect on the uptake of AIB. However, the point to be stressed here is that CPZ further reduces

the accumulation of hypoxanthine into TCA-soluble material even when the incorporation into TCA-insoluble material virtually was abolished.

The effects of CPZ and SKF 525-A seen on base and nucleoside transport could be a result of interaction with specific carriers. Plagemann and Roth[6] claim that the transport of uridine as well as that of choline in Novikoff rat hepatoma cells are reactions distinct from phosphorylation, and that permeation is the rate-limiting step in the incorporation of these precursors by whole cells into the intracellular pools of phosphorylated intermediates. It is not known if this is true also for the purine base hypoxanthine in the MH_1C_1 cells; at any rate hypoxanthine must achieve both a ribosyl and a phosphoric acid residue before subsequent incorporation of the corresponding nucleotide (inosine phosphate) into nucleic acids. The possibility that CPZ and SKF 525-A inhibit nucleotide incorporation into TCA-insoluble material and thereby through feedback mechanisms reduce the pool of soluble intermediates should also be taken into consideration.

In addition to the transport inhibition by CPZ and SKF 525-A reported here, both drugs have been found to inhibit the glucuronidation of PAP and PNP presumably at the uptake level[3,4] as well as to inhibit amino acid transport.[1] As these substances presumably are taken up by cells via different mechanisms, it seems conceivable that CPZ and SKF 525-A exert their effects in a rather unspecific manner. This does not readily explain, however, why the transport of thymidine and uridine is much less affected by CPZ and SKF 525-A than that of hypoxanthine. Seeman *et al.*[13] found that CPZ inhibited the passive Na^+-transport in frog sartorius muscle as well as in human erythrocytes, and they related this to the membrane expansion known to be caused by CPZ.

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