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SHORT COMMUNICATIONS

Effect of chronic pentobarbital treatment on high affinity uptake of L-glutamate by cultured glial cells

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It has been shown that hamster astroblast glial cells (line NN) grown in culture can become tolerant to barbiturates [1]. When cells were grown in the presence of pentobarbital for periods of several weeks the rate of oxygen consumption was found to be significantly increased and the sensitivity to a challenging dose of pentobarbital was found to be reduced in these barbiturate cultivated cells compared to those of cells grown in a drug-free medium. The development of this cellular adaptation was accompanied by morphological changes of the cells as well as alterations in some of their metabolic functions, particularly a progressive increase in glutamate dehydrogenase activity [2]. NN cells have also been shown to accumulate L-glutamate by a "high affinity" uptake system [3]. In addition to a low K_m this uptake is characterized by a relatively high V_{max} and is likely to be mediated by an active transport system. The main objective of the present study was to examine the possibility of an alteration in L-glutamate uptake in parallel with the observed increase of glutamate dehydrogenase (GDH, EC 1.4.1.2) activity as a function of time in the presence of pentobarbital.

The preparation and properties of cells cultivated in the presence of pentobarbital were reported previously [1, 2]. The cells were grown in Falcon flasks (75 cm^2) in the presence of 5-ethyl-5-(1-methyl-butyl)-barbiturate sodium salt (pentobarbital, Abbot, U.S.A.) for periods of 2, 4 and 8 weeks. The method used to study glutamate uptake was described elsewhere [3]. Briefly, the cells were transferred from the Falcon flasks into Petri dishes (35 mm in diameter, Nunclon, Denmark) 48 hr prior to the uptake experiment. Since cells have a lower rate of growth in the presence of the barbiturate [1] the number of cells seeded per Petri dish was adjusted for treated cells in order to obtain a final cellular density approximately equal to that of controls. For the same reason sensitivity of the uptake of glutamate to morphine was established, since morphine has been also shown to reduce growth rate without biochemical changes comparable to those of pentobarbital [2]. Immediately before the experiment the growth medium was substituted by 3 ml of phosphate-buffered Krebs–Ringer solution. The cells were preincubated for 5 min at 25°C, after which labelled substrate ([^{3}H] L-glutamate, 24.1 Ci/mmol, New England Nuclear GmbH, Dreieichenhein, Germany) was added to 0.5 μCi per dish and the incubation was continued for 10 min. The uptake was terminated by rapid removal of the medium, washing (2 ×) with substrate-free medium and freezing of the cells. Subsequently, the cells were extracted with 2 ml of distilled water, homogenized, and a 1 ml aliquot was taken for determination of radioactivity. Protein was estimated in each sample according to the method of Lowry *et al.* [4]. The uptake was studied at five low concentrations: 2.67, 3.57, 5.0, 8.33 and 25.0 μM and four high concentrations: 500, 1000, 1500 and 2000 μM. The coefficient of diffusion (k_{diff}) (an increase in the diffusion rate per unit increase in the external concentration of substrate) was determined at high concentrations of substrate where the uptake was linear with concentration [3]. The calculated diffusion component was then subtracted from the uptake velocities obtained at lower concentrations and thus corrected values were used to compute the kinetic constants [6].

It was established that the time course of uptake in control and barbiturate cultivated cells was linear over a period of a least 10 min at both the lowest and the highest concentrations studied (Fig. 1). In some experiments the number of cells seeded per Petri dish was varied and the relation between uptake of L-glutamate and amount of protein per dish was studied. At substrate concentrations of 1 μM and 2000 μM the uptake was directly proportional to protein content (between 200 and 700 μg of protein per dish) for control as well as barbiturate pretreated cell preparations.

Initially, the direct effects of pentobarbital and morphine (Merck, Germany) on the uptake of L-glutamate and L-aspartate (at 1 μM substrate concentration) were measured

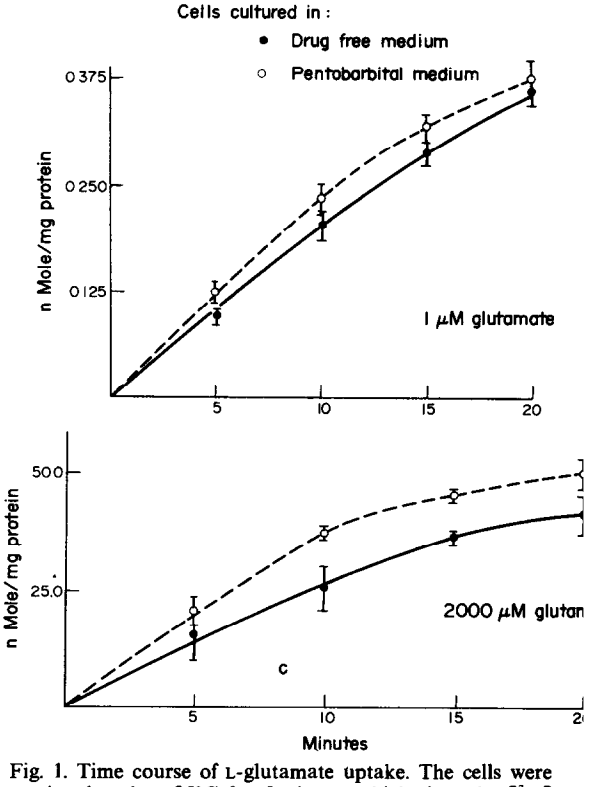

Fig. 1. Time course of L-glutamate uptake. The cells were pre-incubated at 25°C for 5 min, at which time the [^{3}H] L-glutamate was added (0.5 μCi per dish in case of 1 μM L-glutamate and 1 μCi per dish in case of 2000 μM L-glutamate) and incubation continued for periods of time indicated. The accumulated radioactivity is plotted as nmole per mg of protein. Each point is a mean of four separate incubations and vertical bars indicate extent of standard deviations.

in control as well as in barbiturate cultivated cells. For these uptake studies pentobarbital or morphine were added to the incubation medium at the beginning of the preincubation time and were present throughout the experiment. Uptake in four separate preparations for each concentration was compared to four control preparations (drug absent from the incubation medium). As can be seen in Fig. 2 pentobarbital inhibited glutamate uptake in control and barbiturate-treated cells. The amount of inhibition progressively increased with increasing concentrations of the drug and 50 per cent inhibition (IC_{50}) was reached at 1.9 mM and 3.7 mM pentobarbital for control and barbiturate cultivated (5 weeks, 5×10^{-4} M Pentobarbital) cells, respectively. These results are interesting since it has been shown that the presence of pentobarbital in the incubation medium does not affect uptake or release of L-glutamate by brain slices [5]. Morphine at 1 mM concentration had no statistically significant effect on L-glutamate uptake ($P < 0.02$, by Student's t-test).

The kinetic properties of L-glutamate uptake were studied in cells grown in the presence of 0.5 mM pentobarbital for 2, 4 and 8 weeks, and compared to those obtained in control cells which had been grown for the same periods of time and the same number of subcultures in drug-free medium (Table 1).

In barbiturate cultivated cells there is a consistent and progressive increase in the value of V_{max} without any significant change in K_m over the period of 8 weeks. This could be interpreted as a progressive increase in the number of sites of the active transport. The possible mechanism of pentobarbital in inducing this effect remains unclear, however, it may reflect an increased demand for substrate caused by the increased activity of glutamate dehydrogenase. The increase in V_{max} is too large to be interpreted solely as a compensation for the direct inhibition by pentobarbital. This is further substantiated by the fact that the uptake of L-glutamate by cells cultivated for 5 weeks in the presence of pentobarbital was less sensitive to the acute inhibitory effect of the drug (Fig. 1).

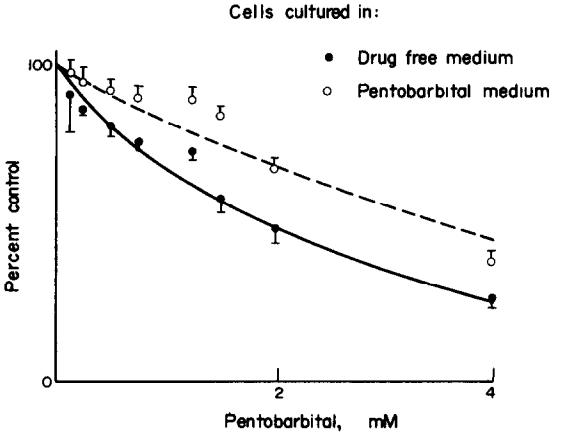

Fig. 2. Direct inhibition of L-glutamate (1 μM) uptake by pentobarbital as a function of drug concentration. The uptake is expressed as percentage of control. The solid and doubled lines represent uptake by control and barbiturate treated cells, respectively. Each point is a mean of four separate incubations and vertical bars indicate extent of standard errors. The values of controls were 0.16 ± 0.01 mmol/mg and 0.21 ± 0.02 mmol/mg for control and barbiturate-treated cells, respectively.

Table 1. Kinetics of L-glutamate uptake by glial cells (clone NN)

Time of treatment	Control	Treated
2 weeks		
K_m	19.2 ± 2.0	14.3 ± 1.3
V_{max}	0.203 ± 0.012	0.263 ± 0.009*
k_{diff}	1.4	1.8
4 weeks		
K_m	12.5 ± 1.9	16.3 ± 2.2
V_{max}	0.237 ± 0.012	0.399 ± 0.018*
k_{diff}	1.3	1.7
8 weeks		
K_m	16.7 ± 2.7	16.0 ± 5.1
V_{max}	0.253 ± 0.016	0.543 ± 0.061*
k_{diff}	1.4	1.0

Cells were grown in presence of 5×10^{-4} M pentobarbital for the time periods indicated. For experimental details see [1]. Kinetic constants and standard errors were calculated using Fortran programs of Cleland [6] and PDP 8/11 computer. K_m is expressed in μM, V_{max} in nmol/min/mg protein and k_{diff} in pmol/min/mg protein/μM. Four separate incubations were carried out in parallel between control and treated cells at each concentration, i.e. each pair of K_m and V_{max} values and their corresponding standard errors are based on 20 experimental values (obtained at lower concentrations): k_{diff} is based on 16 experimental values (obtained at higher concentrations). For details see [3].

*$P < 0.001$. Statistical significance calculated as suggested by Cleland [6].

In conclusion, high affinity uptake of L-glutamate by normal glial cells grown in culture was found to be inhibited by relatively high concentrations of pentobarbital when the drug is added directly to the incubation medium. This direct inhibition was reduced in glial cells previously grown in the presence of the barbiturate for several weeks. More significantly, there was a substantial time dependent increase in V_{max} of L-glutamate uptake in barbiturate cultivated cells without any significant change in K_m. This increase in V_{max} after continuous exposure to pentobarbital is likely to be related to metabolic changes, an effect of the drug on membrane structure or a combination of both.

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REFERENCES

1. B. F. Roth-Schechter and P. Mandel, *Biochem. Pharmac.* **25**, 563 (1976).
2. B. F. Roth-Schechter, L. Laluet, G. Tholey and P. Mandel, *Biochem. Pharmac.* **26**, 1307 (1977).
3. V. J. Balcar, J. Borg and P. Mandel, *J. Neurochem.* **28**, 87 (1977).
4. D. H. Lowry, N. J. Rosebrough, A. L. Farr and A. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
5. R. W. P. Cutler and D. S. Dudzinski, *Brain Res.* **67**, 546 (1974).
6. W. W. Clelland, *Adv. Enzymol.* **29**, 1 (1967).