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CELL MEMBRANE MODIFICATIONS IN RABBIT ISOLATED HEPATOCYTES FOLLOWING A CHRONIC AMITRYPTILINE TREATMENT

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Abstract—The influence of a chronic amitryptiline pretreatment on the transport of the same drug through the cell membrane has been studied in rabbit isolated hepatocytes, comparing the amount of amitryptiline metabolized by intact hepatocytes with that by disrupted ones. The results show that the amitryptiline metabolism in intact hepatocytes from chronically pretreated rabbits is significantly lower than that in the disrupted ones at all incubation times. By contrast this difference is not present between intact and disrupted hepatocytes from untreated rabbits. These findings support evidence for the hypothesis that the amitryptiline pretreatment affects the permeability of the hepatocyte membrane, thus preventing the penetration of the drug itself into the cells and consequently decreasing its metabolism.

IN PREVIOUS experiments *in vivo* we found a delayed disappearance of amitryptiline and concomitant lower level of its metabolites in rabbit organs after chronic amitryptiline pretreatment. By contrast, *in vitro*, a marked increase in the metabolizing activity of rabbit organ homogenates from chronically pretreated animals, has been reported.[1]

The delayed rise of amitryptiline concentration and the lower levels of metabolites *in vivo*, which are hardly reconcilable with the increase of amitryptiline metabolism *in vitro*, have been tentatively explained through some permeability changes in the cell membrane induced by the chronic pretreatment.

The aim of the present experiment was to confirm the existence of these membrane phenomena using an *in vitro* system which allowed a direct contact of the drug with the cell membrane and, at the same time, possessed a good drug metabolizing activity. We have, therefore, employed a suspension of metabolically and structurally intact isolated hepatocytes, obtained from untreated and chronically pretreated rabbits and we have evaluated the metabolism of amitryptiline when added either to hepatocytes with intact cell membrane or to hepatocytes with disrupted cell membrane.

MATERIALS AND METHODS

Animals. Twelve male rabbits, Fulvo di Borgogna strain, 2·0–2·2 kg body wt, were used.

The animals were divided in two groups; the first group (7 rabbits) was treated for 40 days with a daily parenteral dose of 2·5 mg/kg of amitryptiline–HCl. The second group (5 rabbits) were used as an untreated control group.

The animals were housed in a thermally controlled room and tap water and food were given *ad lib.* during this period. All the animals were fasted the day before the experiment.

Twenty-four hr after the last chronic drug administration, two animals of the first group were sacrificed in order to evaluate the residual amounts of amitryptiline and its metabolites in the liver (chronic blanks), the other five animals were used for the preparation of the isolated hepatocytes.

The five rabbits of the second group were also used for the cell isolation procedure.

Reagents. All the chemicals were of analytical grade, collagenase (Type 1) and hyaluronidase (Type 1) were supplied by Sigma Chemical Co.

Liver cell isolation procedure. The suspension of rabbit hepatocytes was prepared according to the enzymatic perfusion technique of Berry, suitably modified for rabbit liver.[2]

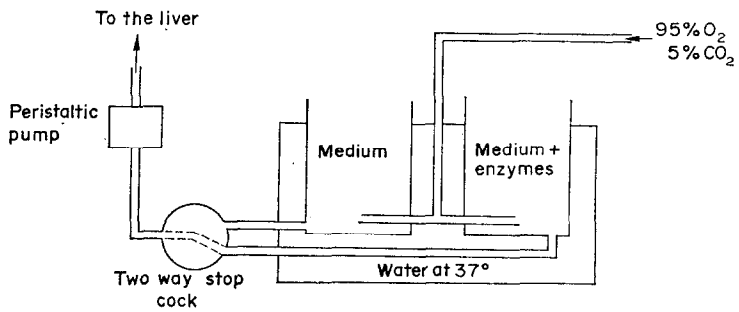

FIG. 1.

Two reservoirs containing respectively 1000 ml of a Ca^{2+} free Hanks's solution[3] and 600 ml of the same with added 0·05% collagenase and 0·10% hyaluronidase were held at 37°: the solutions, saturated with a mixture of 95% O_2 and 5% CO_2, were connected to a peristaltic pump according to the scheme reported in Fig. 1.

The heparinized (5000 i.u.) animals were killed by decapitation, the abdomen was rapidly opened and the portal vein canulated with a Teflon cannula (i.d. 2 mm); the inferior vena cava was ligated just above the level of the renal veins. The chest was opened and the thoracic portion of the inferior vena cava canulated with a Teflon cannula (i.d. 3 mm) just before the right atrium. All operations were completed in about 3 min.

The liver was initially perfused with the enzyme-free Hanks's solution for 3 min, at a rate of 150–190 ml/min. This condition was usually satisfactory for a complete blanching of the tissue. Once the saline perfusion finished, turning the two-way stop-cock of the apparatus, the liver was again perfused for 3 min with the Hanks's plus enzyme solution at the rate of 150–190 ml/min; the enzyme perfusate was discarded. The liver was carefully removed, weighed and cut into several small pieces and placed in a large beaker containing 100 ml of enzyme medium at 37°.

The suspension obtained was rapidly transferred in a 500 ml glass flask, and shaken for 5–8 min at 37° in a Dubnoff shaker under air atmosphere (100 oscillations/min) to break up the cell clumps and to digest isolated nuclei and damaged cells.

The suspension was filtered through three layers of nylon gauze to remove large

fragments of hepatic tissue and cell clumps and the filtrate diluted to 200 ml with fresh medium (previously gassed with 95% O_2 and 5% CO_2, and without added enzymes) at 25°.

The cells of the diluted dispersion (D) were separated from debris by centrifuging at 50 *g* for 2 min. The supernatant was removed and the cells gently dispersed in 200 ml of gassed fresh medium. The washing procedure was repeated twice and the washed cells (C), separated from the final supernatant (S_3), were resuspended in the Hanks's Ca^{2+} free solution to a final volume of 200 ml. The yield of isolated hepatocytes in the final suspension varied from 3 to 4 $\times$ 10^6 cells/ml and corresponded to 7·5 $\pm$ 0·6 mg of protein/ml.

The recovery of isolated cells on the original liver wet weight basis varied from 10 to 15%; this low recovery seems mainly to depend on the short time (8 min) of treatment of the hepatic tissue with collagenase and hyaluronidase.

However, as reported in the literature,[4,5] a more prolonged exposure to these enzymes could be responsible for severe cell damage.

Structural and metabolic integrity of isolated hepatocytes. The structural integrity of the cell membrane of isolated hepatocytes was examined microscopically by means of tripan blue (0·25%) exclusion and by retention of soluble cytoplasmic enzymes, LDH and GPT within the cells. The assay of lactate dehydrogenase and glutamic pyruvic transaminase activities was performed according to the methods of Wroblewski and Ladue[6] and Henley and Pollard.[7]

The metabolic integrity of the isolated hepatocytes was tested by the ability of the cell to synthesize glucose from pyruvate and lactate. Glucose formation was determined according to Schmidt.[8]

Protein determinations were carried out by the method of Lowry *et al.*,[9] using bovine serum albumin as standard.

The dry weight of isolated cells was determined by mixing a measured portion of the cells suspension with an equal volume of 10% trichloracetic acid. The TCA insoluble material was centrifuged and weighed after drying at 100°.[10]

Amitryptiline metabolism in isolated hepatocytes. The metabolism of amitryptiline was studied in hepatocytes isolated from ten rabbits, five untreated and five chronically pretreated.*

In each experiment the cell suspension was divided in two equal fractions. The first one, which we called "disrupted cell fraction", was homogenized at 800 rev/min for 20 sec using a Potter–Elvehjem homogenizer with a Teflon pestle, with clearance of 0·15 mm; the second one was used unaltered and called "intact cell fraction".

Samples of 2 ml each (approx. 100 mg of protein per sample) from disrupted or intact cell fractions, were added to 1 ml of 6 $\times$ 10^{-2} M Tris–HCl buffer pH 7·4 containing 2 $\times$ 10^{-5} M G6P, 3 $\times$ 10^{-6} M $NADP^+$, 6 $\times$ 10^{-4} M $MgCl_2$ and placed in a thermostat controlled Dubnoff water bath at 37°. The reaction was started by the addition of 1 ml of Tris–HCl buffer containing 1 mg of amitryptiline–HCl. Duplicate samples of each fraction were incubated under air atmosphere for 20, 40 or 60 min. At the end of the incubation periods, the reaction was stopped by the addition of 0·1 ml of glacial acetic acid and the samples were subjected to enzymatic hydrolysis with β-glucoronidase and arylsulphatase at pH 4·9 for 24 hr.

* In these experiments the washed cells (C) were resuspended in Hanks's Ca^{2+} free solution to a volume of 30 ml (50 mg of protein/ml).

The hydrolysed samples were adjusted at pH 11 with 10 N NaOH and extracted twice with 10 vol. of *n*-heptane. The heptanic extracts of the duplicate samples of each experimental group combined, were evaporated to dryness *in vacuo* and the residue taken up in 1 ml of CH_3OH. The methylic extract, frozen at $-10°$ in order to allow the precipitation of interfering fatty materials, was centrifuged and subjected to chromatographic separation. After elution of the spots, the spectrophotometric determination of amitryptiline and all its basic metabolites (10 hydroxyamitryptiline, 10-11 hydroxyamitryptiline, nortryptiline, 10 hydroxynortryptiline, 10-11 hydroxynortryptiline) was performed according to the methods previously described.[11,12]

RESULTS AND DISCUSSION

The method of isolation described above gave a routinely high percentage (85–90 per cent) of viable hepatocytes as judged by tripan blue exclusion.

The data reported in Table 1 show that the GPT and LDH specific activity measured in various tissue fractions was as high in the final cell suspension as in the initial dispersion. According to Berry, if most of the soluble enzymes had been lost from the cells into the suspending medium during the isolation procedure and the subsequent incubation period of 30 min, the specific activity of the residual LDH and GPT of the cells would have been substantially lower than that present in the initial dispersion.[2] Therefore the ability of isolated hepatocytes to retain cytoplasmic enzymes gives evidence of their structural and functional integrity.

Moreover, the results reported in Table 1, demonstrate that the isolated cells were able to convert lactate and pyruvate to glucose. The maintenance of this function, as suggested by Hems, is a critical test to assess the metabolic integrity of liver cells, since such an anabolic process involves the mitochondrial and cytoplasmic cell fractions.[13]

Thus both the satisfactory retention of highly diffusible enzymes and the unimpaired metabolic activity of the cells give convincing evidence that the cells obtained, in spite of the limitations of cell isolation procedures, had survived and were biochemically intact.

The results of amitryptiline metabolism in isolated hepatocytes from control and chronically pretreated rabbits reported in Tables 2 and 3, suggest that:

(1) in "chronic" hepatocytes the amitryptiline metabolism is markedly higher than in "untreated" ones at all the observation times. Such an enhancement of the amitryptiline metabolism which is comparable to that previously observed in rabbit organs homogenates from chronically pretreated animals,[1] probably depends on an increased activity of the drug metabolizing enzymes induced by the chronic amitryptiline treatment.

(2) (a) in untreated animals there are no significant differences in the amount of amitryptiline metabolized either by intact or by disrupted hepatocytes; (b) in chronically pretreated rabbits, the intact hepatocytes, when compared to the disrupted ones, exhibit a significant decrease of the amount of metabolized amitryptiline.

On the basis of these findings we can conceivably argue that, under our experimental conditions, the drug transport into the cells is not significantly modified by the presence of the intact hepatocytes membrane, this is because the amitryptiline metabolism in intact hepatocytes is not significantly different from that of the same hepatocytes with disrupted cell membrane.

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TABLE 1. LACTATE DEHYDROGENASE AND GLUTAMIC PYRUVIC TRANSAMINASE ACTIVITIES* IN RABBIT LIVER FRACTION

Tissue fraction	Protein (mg)	LDH Activity (U/ml)	LDH Sp. act. (U/mg)	GPT Activity (U/ml)	GPT Sp. act. (U/mg)
(D) Dispersion	2900 ± 141·8	82505 ± 5603·7	5690 ± 187·2	4654 ± 174·2	321 ± 11·4
(S_3) Supernatant	38 ± 3·8	1235 ± 141·5	5880 ± 189·6	74 ± 9·8	350 ± 16·6
(C) Cells	1500 ± 100·2	43265 ± 3472·2	5782 ± 174·6	2475 ± 173·1	330 ± 11·2
(C′) Cells after 30 min†	1479 ± 149·9	43039 ± 3654·5	5754 ± 199·9	2423 ± 180·2	324 ± 10·7

Synthesis of glucose by isolated liver cells

Incubation time (min)	Protein (mg)	Addition	Glucose formed‡ (μmoles)	Rate of glucose formation (μmoles/g dry wt/min)
60	50 ± 1·78	None	0·22 ± 0·08	0·1
		Pyruvate	7·89 ± 1·01	2·6
		Lactate	7·04 ± 0·92	2·3

Cells were incubated at 37° in bicarbonate and glucose-free Hanks's medium containing 10 mM phosphate buffer, pH 7·4. Substrate was added at a final concentration of 10 mM. Gas phase air. Total volume 4 ml.

* One unit is the amount of enzyme required to bring about the conversion of 1 mμmole of substrate per min at 25°. The specific activity is defined as the number of the enzyme units per milligram of protein. Mean ± S. E. in hepatocytes isolated from ten different rabbits, five untreated and five chronically pretreated.

† The specific activity LDH and GPT was assayed after centrifugation and resuspension in fresh medium of the cells incubated at 37° for 30 min.

‡ Mean ± S. E. in hepatocytes isolated from ten different rabbits, five untreated and five chronically pretreated.

Table 2. Amounts* of metabolized amitryptiline from intact (I) and disrupted (D) hepatocytes of untreated rabbits at various incubation times

Time (min)		Amitryptiline	10 OH Amitryp. + 10–11 OH Amitryp.	Nortriptyline	10 OH Nortryp. + 10–11 OH Nortryp.	Total metabolites	Recoveries (% ± S.E.)
20	D	70·1 ± 0·56	8·6 ± 0·26	9·9 ± 0·37	1·6 ± 0·04	20·1 ± 0·53	90·1 ± 0·12
	I	69·8 ± 0·43	9·2 ± 0·24	9·5 ± 0·17	1·6 ± 0·05	20·3 ± 0·93†	90·1 ± 0·17
40	D	59·1 ± 0·89	13·6 ± 0·37	15·0 ± 0·55	2·2 ± 0·07	30·9 ± 0·87	90·0 ± 0·17
	I	58·6 ± 0·75	14·0 ± 0·32	15·0 ± 0·54	2·2 ± 0·08	31·2 ± 0·76†	89·8 ± 0·31
60	D	50·8 ± 0·68	17·7 ± 0·40	18·6 ± 0·37	2·8 ± 0·13	39·0 ± 0·78	89·8 ± 0·21
	I	50·1 ± 0·47	18·2 ± 0·19	18·9 ± 0·35	2·8 ± 0·10	39·9 ± 0·88†	90·0 ± 0·11

* Data are expressed as micrograms of unchanged amitryptiline and metabolites/10 mg of protein; mean ± S. E. in hepatocytes isolated from five different rabbits.

The experiment was performed adding 1 mg of amitryptiline/100 mg of protein of the incubation mixture (see Methods).

The statistical evaluation was performed with the analysis of the variance, between the values from intact and disrupted hepatocytes.

† F not significant ($P > 0·05$).

TABLE 3. AMOUNTS* OF METABOLIZED AMITRYPTILINE FROM INTACT (I) AND DISRUPTED (D) HEPATOCYTES OF CHRONICALLY PRETREATED† RABBITS AT VARIOUS INCUBATION TIMES

Time (min)		Amitryptiline	10 OH Amitryp. + 10–11 OH Amitryp.	Nortryptiline	10 OH Nortryp. + 10–11 OH Nortryp.	Total metabolites	Recoveries (% ± S. E.)
20	D	64·3 ± 0·45	12·4 ± 0·16	11·0 ± 0·36	2·3 ± 0·04	25·7 ± 0·40	90·0 ± 0·12
	I	66·1 ± 0·39	11·5 ± 0·15	10·3 ± 0·37	2·1 ± 0·05	24·4 ± 0·45‡	90·9 ± 0·07
40	D	45·8 ± 0·35	20·6 ± 0·16	18·3 ± 0·15	4·9 ± 0·08	43·8 ± 0·47	89·7 ± 0·15
	I	48·8 ± 0·40	19·6 ± 0·28	17·5 ± 0·16	4·3 ± 0·11	41·4 ± 0·38§	89·4 ± 0·17
60	D	36·9 ± 0·54	24·3 ± 0·41	22·0 ± 0·19	6·7 ± 0·13	53·0 ± 0·56	89·9 ± 0·06
	I	39·7 ± 0·65	22·6 ± 0·16	21·1 ± 0·41	6·5 ± 0·07	50·2 ± 0·45§	90·0 ± 0·24

* Data are expressed as micrograms of unchanged amitryptiline and metabolites/10 mg of protein; mean ± S. E. in hepatocytes isolated from five different rabbits.
The experiments were performed adding 1 mg of amitryptiline/100 mg of protein of the incubation mixture (see Methods).

† The animals were pretreated for 40 days with a daily i.m. dose of 2·5 mg/kg of amitryptiline. Twenty-four hr after last chronic drug administration, the residual amounts of amitryptiline and metabolites/gram of tissue were practically negligible.
The statistical evaluation was performed with the analysis of the variance, between the values from intact and disrupted hepatocytes.

‡ F significant at 5% level.

§ F significant at 1% level.

Consequently the decreased amount of the amitryptiline metabolized by the intact hepatocytes from chronically treated rabbits in comparison to that observed in the disrupted cells from the same animals, can only be due to the effect of the chronic treatment on the membrane of the hepatocyte. Very likely the chronic amitryptiline pretreatment, by inducing changes in the membrane permeability, inhibits the penetration of the same amitryptiline into the hepatocytes and consequently decreases the quantity of the metabolized drug.

In conclusion the results of the present experiment confirm the data reported in our previous publication[1] and substantiate the existence of permeability modifications in the cell membrane induced by the chronic amitryptiline treatment.

In addition when considering the inhibitory effect of amitryptiline on its transport similar to that on catecholamines reuptake,[14–18] we can suggest that this rather aspecific effect of the drug on the cell membrane, could interfere also with the active transport of various substances into the cells.

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