Contribution of Lysosomes to the Subcellular Distribution of Basic Drugs in the Rat Liver

Junko Ishizaki,[1] Koichi Yokogawa,[1] Masako Hirano,[1] Emi Nakashima,[1] Yoshimichi Sai,[2] Shoji Ohkuma,[2] Tohru Ohshima,[3] and Fujio Ichimura[1,4]

Received December 25, 1995; accepted March 12, 1996

Purpose. We examined the subcellular distribution of the basic drugs, chlorpromazine (CPZ), imipramine (IMP) and biperiden (BP), in rat liver, and evaluated the contribution of lysosome (Lys) to their intracellular distribution in comparison with that of mitochondria (Mit).

Methods. In an in vivo distribution, the concentrations of CPZ, IMP and BP in the liver subcellular fractions were determined. In an in vitro study, uptake of [^{3}H]IMP into Lys and Mit fractions was determined in the presence or absence of several agents.

Results. The distribution of these drugs 10 min after administration was quite similar. However, the relative specific contents (the drug concentration per protein of each fraction divided by that of the total homogenate) in Lys were 7.3, 9.6 and 4.2, respectively for CPZ, IMP and BP, whereas those in the other organella were only 0.4 ~ 1.7. In an in vitro uptake study, the dose response of IMP uptake into Lys was biphasic, while that into Mit fractions was monophasic. The binding of IMP to the high affinity sites of Lys was pH dependent and disappeared in 50 mM NH_4Cl or 50 μM CPZ, both of which increased the intralysosomal pH. the low affinity sites were not affected by these drugs.

Conclusions. The results indicated that Lys has the highest affinity for the basic drugs in the liver and that its contribution to their subcellular distribution depends on the intralysosomal pH, which is also affected by these drugs. The importance of these effects may become significant in combination therapy using various basic drugs.

INTRODUCTION

Several investigations have studied the factors that contribute to the distribution of basic drugs. We showed that the distribution volume of several basic drugs in the body depended on their lipophilicity and that it was correlated with the volume of fat tissue (1,2). Nishiura et al. have suggested that acidic phospholipids participated in the tissue distribution of basic drugs (3). On the other hand, Yoshida et al. have reported that lung mitochondrial monoamine oxidase has specific binding sites for basic drugs and might function as a drug reservoir (4). However, lysosomes (Lys) may also contribute to the distribution of basic drugs, because these drugs become protonated and accumulate in Lys in membrane impermeable forms, thereby increasing the intralysosomal pH through proton consumption (5). Thus, the basic tricyclic antidepressants, the basic beta adenoreceptor antagonist and the basic anti-neoplastic agent all accumulate in Lys of cultured cells lines (6,7,8). Amiodarone, chloroquine (CQ), imipramine (IMP), and chlorpromazine (CPZ), which are basic drugs with cationic amphiphilic structures, also accumulate in Lys and impair phospholipid metabolism (9).

Studies on the distribution of the antimalarial drug, mefloquine, in vivo, have shown their preferential accumulation within Lys (10). However, the intracellular distribution of these and other basic drugs has not been extensively studied. We examined the subcellular distribution in the rat liver, of selected lipophilic basic drugs that are widely used clinically, namely CPZ, IMP and biperiden (BP) and evaluated the contribution of Lys to their intracellular distribution in comparison with that of mitochondria (Mit).

MATERIALS AND METHODS

Materials

Fluorescein isothiocyanate-dextran (FD, Av. Mol. Wt.: 70,000) and Percoll were purchased from Sigma (St. Louis, MO) and Pharmacia (Uppsala, Sweden), respectively. CPZ and IMP were obtained from Sigma (St. Louis, MO) and [^{3}H]IMP (24 Ci/mmol) was from Amersham International Ltd. (Bucks, UK). BP was supplied from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals were of reagent grade and used without further purification.

Animals

Male Wistar rats (280 ± 30 g; mean ± SD) were obtained from Sankyo Laboratory Animal Co., Toyama, Japan.

Subcellular Fractionation

The livers of the rats injected with basic drugs were fractionated according to Sai et al. (11). Briefly, the rats were first injected i.p. with FD at a dose of 100 mg/100 g of body weight and starved overnight. Each of the basic drugs was then administered i.v. (3.2 mg/kg) to the rats 10 min before sacrifice. The excised liver was perfused with ice-cold 0.25 M sucrose and homogenized with four volumes of ice-cold 0.25 M sucrose. All subsequent steps were performed at 4°C. To obtain the nuclear fraction (P_1), the homogenate was centrifuged at 340 × g for 5 min. The supernatant was centrifuged again under the same conditions. The combined precipitates represented P_1. The resulting post nuclear supernatant was then incubated at 37°C for 5 min in the presence of 1 mM $CaCl_2$ to swell the Mit. Thereafter, the post-nuclear fraction (P_2) was pelleted by centrifugation at 10,000 × g for 30 min. The microsomal fraction (P_3) was isolated from the resulting supernatant by centrifugation at 100,000 × g for 60 min. The final supernatant represented the cytosol fraction (Sup). The P_2 was resuspended in iso-osmotic Percoll (in 0.25 M sucrose pH 7.4) at a density of 1.075 g/ml, and centrifuged at 60,000 × g for 15 min. The gradients were separated into 10 fractions of similar volume. The protein content, various enzyme activities and drug concentrations were measured in each fraction.

[1] Hospital Pharmacy, Kanazawa University, 13-1 Takara-machi, Kanazawa, 920, Japan.
[2] Department of Biochemistry, Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa, 920, Japan.
[3] Department of Legal Medicine, School of Medicine, Kanazawa University, Takara-machi, 13-1, Kanazawa 920, Japan.
[4] To whom correspondence should be addressed.

0724-8741/96/0600-0902$09.50/0

Enzyme and Protein Assay

N-acetyl-β-D-glucosaminidase (NAGA), cytochrome *c* oxidase and protein were assayed according to Sai et al. (11).

Determination of Basic Drugs

The concentrations of CPZ, IMP and BP in the liver subcellular fractions were determined by GLC as described (12) with slight modifications.

Calculation of Drug Concentrations in Each Organelle

The contribution of intact cells to the drug uptake in P_1 fraction was determined using the relative ratio of NAGA in the P_1 fraction to total homogenate and subtracted from the results. The drug concentrations in the Lys (fractions 9 and 10 of the Percoll gradient) and Mit fractions (fractions 1 to 4 of the Percoll gradient) were calculated by multiplying the drug concentration in the P_2 fraction by the relative fraction of Lys and Mit in P_2, respectively.

Preparation of Lys and Mit Fractions for Drug Binding Studies

After Percoll density gradient centrifugation, the P_2 fraction of the mitochondrial layer (close to the top of the tube) and the lysosomal layer (close to the bottom of the tube) were pooled and centrifuged at 100,000 × g for 1 h. The broad turbid layers in the middle of the tubes were collected, diluted with ten volumes of 0.25 M sucrose, and centrifuged at 10,000 × g for 30 min. After washing once under the same conditions to remove the Percoll, the pellets were resuspended (120–170 μg protein/ml) in a chilled buffer consisting of 0.2 M sucrose, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM ATP, 20 mM HEPES-TMAH (pH 7.4) and 5 μg/ml each of chymostatin, leupeptin, antipain and pepstatin.

[^{3}H]IMP Uptake

Uptake of [^{3}H]IMP into Lys and Mit fractions was determined in 1 ml of each fraction containing 0.1–100 μM [^{3}H]IMP. After an incubation at 37°C for 10 min, particle-associated IMP was separated by rapid filtration through Whatman GF/B glass fiber filters (Whatman, Inc, Clifton, NJ). The filters were placed in vials containing 16 ml of scintillation cocktail (ACS-II, Amersham Corp., Arlington Heights, IL), warmed at 20°C for 12 hr and the levels of radioactivity were assayed by liquid scintillation spectrometry (Aloka LSC-3600, Aloka, Japan, liquid scintillation counter).

Inhibition of IMP Uptake

To test the effect of basic drugs and ionophores on the uptake of IMP, the fractions were incubated in the presence or absence of these agents at 37°C for 5 min, after which [^{3}H]IMP (1 nM) was added. After a 10 min incubation at 37°C, the uptake of IMP was assessed as described above.

Data Analysis

The parameters of the Scatchard plots were estimated by the non-linear least-squares method using a NONLIN program (12) by a digital computer, FACOM-M360AP, at the Information Processing Center, Kanazawa University. The data were analyzed using Fisher's *t* test for comparison of unpaired means of two sets of data. The number of determinations (N) is noted in the figure. A p value of 0.1 or less was used to indicate a significant difference between sets of data.

RESULTS

Subcellular Distribution of Basic Drugs in Rat Liver

The distribution of selected maker enzymes and protein in the fractions obtained after differential centrifugation is shown in Fig. 1-(A). The P_2 fraction contained the highest relative specific activities for both NAGA and cytochrome *c* oxidase, showing the preferential accumulation of Lys and Mit in this fraction. About 50% of the total content of NAGA and cytochrome *c* oxidase accumulated in P_1, but with relative specific activities close to unity. Hence, P_1 is considered to be contained in some intact cells. The disintegration of Lys during the fractionation procedures used in this study was slight judging from the very low NAGA activity in the supernatant.

The distribution of the two marker enzymes and protein on the Percoll gradient is shown in Fig. 1-(B). About 90% of NAGA in P_2 was at the bottom (fractions 9~10) of the gradient with relative specific activities of 3.5–14 (relative to P_2) or 13–52 (relative to homogenate), indicating the presence of highly purified Lys in these fractions which we refer to as Lys. About 86% of cytochrome *c* oxidase activities accumulated in the top (fractions 1~4) well separated from Lys, which we refer to as the Mit fraction. The Lys (fractions 9~10) and Mit (fractions 1~4) fractions contained about 9.5 and 77%, respectively, of the protein content of P_2.

Table I summarizes the subcellular distribution of CPZ, IMP and BP (nmol/g tissue and relative distributions (%)) in the rat liver 10 min after administration of each drug at 3.2 mg (100~114 μmol)/kg. The total amounts of the drugs accumulated at 10 min in the liver were: 8.2% (CPZ), 2.4% (IMP) and 1.9% (BP), respectively, of the administration with their plasma concentrations being 1.51 μM (CPZ), 1.34 μM (IMP) and 1.10 μM (BP) respectively. However, these basic drugs were similarly distributed: about 30~40% in the nuclei, 30% in Mit, 10~20% in the cytosol and only 10% each in Lys and microsomes.

In Fig. 2, the relative specific contents of these drugs are plotted against the relative protein contents of the fractions expressed in a cumulative manner. The relative specific contents of CPZ, IMP and BP in Lys were 7.3, 9.6 and 4.2, respectively, while those in the other subcellular organella were only 0.4~1.7, indicating the more preferential accumulation of these drugs in Lys than in any other organella fractions including Mit.

Characteristics of Uptake of the Basic Drug to the Lys and Mit Fractions

In the following studies, the ability to take up drug was compared between Lys and Mit fractions in vitro. Figure 3 shows the time courses of the uptake of IMP (1 μM) into Lys and Mit fractions at 37°C. IMP was rapidly taken up into both these fractions reaching maximal values within 1 min. The maximal amount of IMP taken up in the Lys and Mit fractions

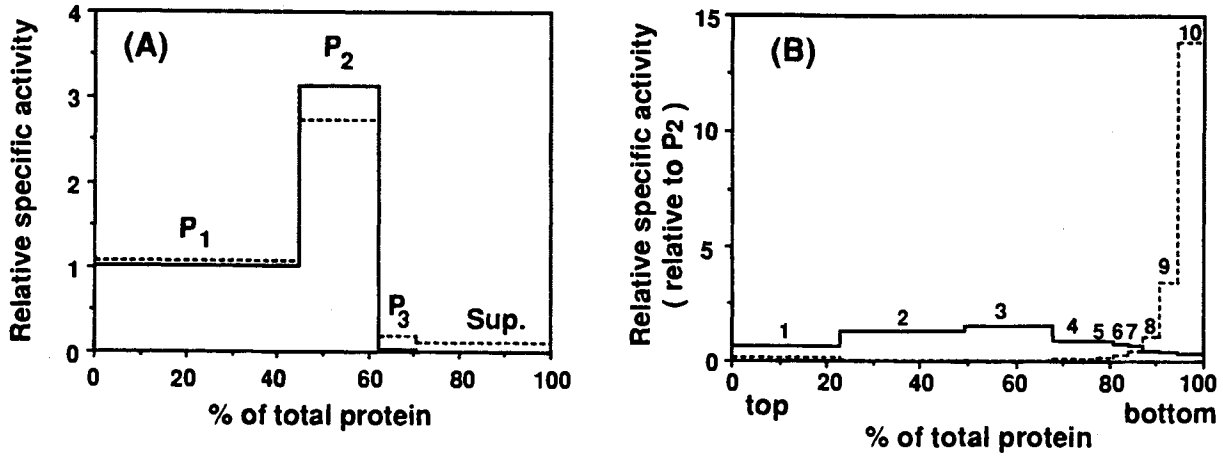

Fig. 1. Distribution of marker enzymes and protein in rat liver. Rat livers were separated first by differential centrifugation and the P_2 fractions were further separated by Percoll density gradient centrifugation as described under Materials and Methods. The relative specific activities of NAGA and cytochrome *c* oxidase are plotted against the relative protein content of each fraction expressed in a cumulative manner. (A) Differential centrifugation: Nuclei (P_1), 10,000 × g precipitate fractions (P_2), Microsomes (P_3) and Cytosol (Sup.). (B) Percoll density gradient centrifugation of P_2. Values represent the mean of three experiments. ——, cytochrome *c* oxidase; -----, NAGA.

was 7.1 and 1.5 nmol/mg protein, respectively, showing about 5 times more preferential uptake in the Lys than in the Mit fractions.

Figure 4 shows Scatchard plots of IMP uptake into the Lys and Mit fractions at 37°C. At least two types of IMP binding sites are evident in Lys, namely high affinity/low capacity sites and low affinity/high capacity sites. On the other hand, in Mit fractions, there are only low affinity/high capacity sites. In the presence of 50 mM NH_4Cl, however, the uptake of IMP into Lys changed, indicating the presence of only one type of binding site. The dissociation constants (K_d, μM) and maximum number of binding sites (B_{max}, nmol/mg protein) calculated using the NONLIN program for IMP uptake into Lys and Mit fractions in the presence and absence of 50 mM NH_4Cl or 50 μM CPZ. The high affinity site is obvious only in Lys (K_d 0.897 ± 0.241, B_{max} 4.90 ± 0.97), which disappeared in the presence of NH_4Cl or CPZ. On the other hand, the low affinity sites in both Lys (K_d 258 ± 39, B_{max} 804 ± 97) and Mit fractions (K_d 421 ± 129, B_{max} 832 ± 241) did not disappear with these bases (NH_4Cl or CPZ). But the value of K_d for low affinity sites of Lys was significantly smaller than that of Mit fractions ($p < 0.001$). The values shown as mean ± SD.

Effect of Basic Drugs and Ionophore on the Uptake of IMP into Lys and Mit Fractions

Figure 5 summarizes the effect of CQ, BP, CPZ, NH_4Cl and nigericin (NIG) on the uptake of IMP in Lys and Mit fractions at 37°C. In Lys, both BP and CPZ were inhibitory at concentrations lower than those of NH_4Cl and CQ, the representative Lys inhibitors. NIG, a K^+/H^+-exchanging ionophore, was also inhibitory. In Mit, on the other hand, the inhibitory effects of these agents were at most 20%, except for CPZ which was about 50% inhibitory.

DISCUSSION

In studying the subcellular distribution of basic drugs, not only the distribution of organella themselves but also the redistribution of drugs during the cell fractionation procedure should be carefully controlled. At first, we examined the drug efflux in the process of tissue homogenation by changing the dilution fold. When the livers were homogenized with ten fold volumes (4~40 vol.) of ice-cold 0.25 M sucrose, the percentage of drug efflux into the supernatant after centrifugation was about 13% under all conditions. When the P_2 was resuspended

Table I. Subcellular Distribution of Chlorpromazine(CPZ), Imipramine(IMP), and Biperiden(BP) in the Rat Liver 10 min After Administration of 3.2 mg/kg of Each Basic Drug

	nmol/g tissue %					
	CPZ		IMP		BP	
Nuclei	8.55 ± 1.04	28.9	3.20 ± 0.52	31.8	2.98 ± 0.21	44.5
Mitochondria	10.0 ± 0.6	33.8	2.98 ± 0.56	29.7	1.84 ± 0.19	27.5
Lysosomes	3.17 ± 0.18	10.7	1.28 ± 0.31	12.8	0.464 ± 0.100	6.93
Microsomes	3.07 ± 0.26	10.4	1.34 ± 0.34	13.3	0.676 ± 0.172	10.1
Cytosol	4.81 ± 0.71	16.2	1.25 ± 0.64	12.5	0.738 ± 0.130	11.0
total	29.6		10.1		6.69	

Note: Values represent the means ± SEM of three experiments.

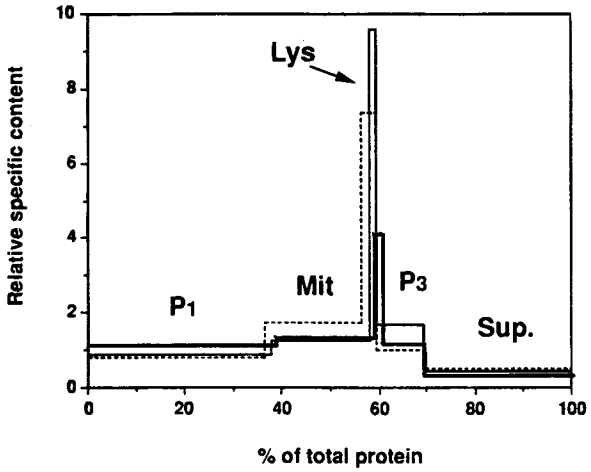

Fig. 2. Subcellular distribution of chlorpromazine(CPZ), imipramine(IMP) and biperiden(BP) in rat liver. Rats were injected i.v. with 3.2 mg/kg of each basic drug 10 min before sacrifice and their livers were fractionated as described under Materials and Methods. Their relative specific contents of each drug are plotted against the relative protein content of each fraction expressed in a cumulative manner. The contribution of intact cells in P_1 fraction was subtracted as described under Materials and Methods. ----- CPZ; ——, IMP; ▬, BP.

in 1 to 8 ml of 0.25 M sucrose and centrifuged, again the drug efflux rates in the supernatant were almost constant (about 20%) (unpublished observation). Therefore, we assumed that considerable redistribution of the drug did not occur during the fractionation procedure. However, to minimize the possible redistribution of drugs, no washing procedures were performed in this study. The volume of the solvent was also minimized.

The amounts of CPZ, IMP and BP taken up in the liver (per wet weight) were in the order, CPZ > IMP > BP (Table I), which was also the order of their lipophilicity: the octanol-water partition coefficients of the nonionized form of these drugs were 5.19, 4.77 and 4.25 in log values, respectively (2). However the subcellular distribution of each of these drugs was

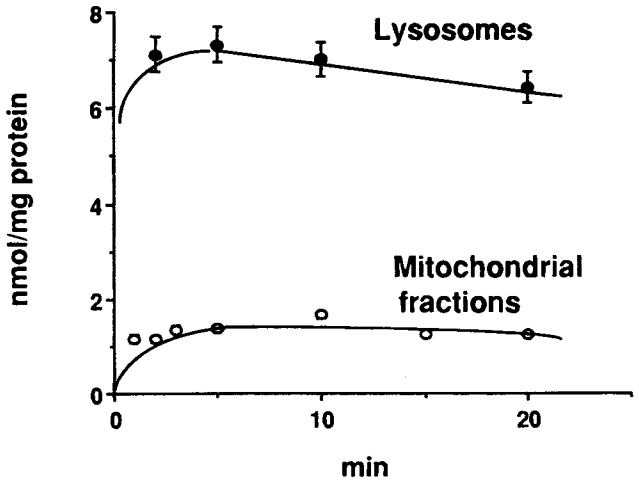

Fig. 3. Time courses of IMP uptake into Lys and Mit fractions. Lys and Mit fractions were incubated with 1 μM IMP at 37°C as described under Materials and Methods. Values represent the mean ± SEM of three experiments.

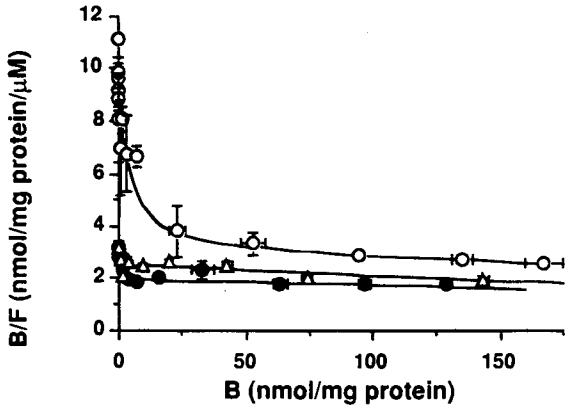

Fig. 4. Scatchard plots of IMP uptake into Lys and Mit fractions. Lys and Mit fractions were incubated at 37°C for 10 min with IMP in the presence (△, Lys) or absence (○, Lys; ●, Mit) of 50 mM NH_4Cl. Each point represents the mean binding value ± SEM of three to six experiments at one IMP concentration.

almost identical, with a preferential affinity for Lys over any other organella including Mit (Fig. 2). Mit plays an important role as a reservoir for basic drugs (4). However the relative specific contents (over the total homogenate) of the drugs used in this study were about 1.5 in the Mit fractions, which were 1/3~1/6 of those bound in Lys.

When Lys and Mit fractions were isolated and their binding activities to IMP were compared in vitro, the affinity of Lys for IMP was 5 times higher than that of the Mit fractions on a protein basis (Fig. 3). The results also showed that Lys has at least two binding sites, one with high affinity/low capacity and the other with low affinity/high capacity, whereas Mit fractions have only one, low affinity binding site. Our results differ from another report showing that the binding of basic drugs to lung Mit fractions was biphasic (13). This could be due to the presence of contaminating Lys in the Mit fractions.

The high affinity site of Lys disappeared upon adding NH_4Cl and CPZ, both of which increased the pH in acidic

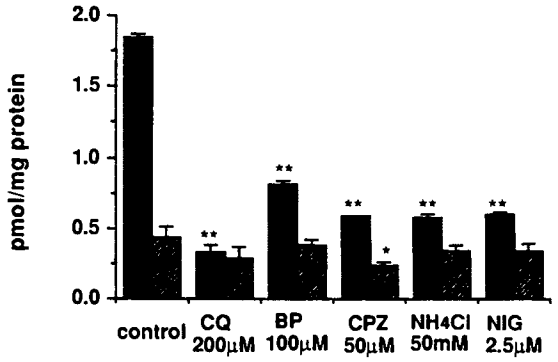

Fig. 5. Effect of basic drugs and ionophore on the uptake of IMP into Lys and Mit fractions. Lys and Mit fractions were incubated at 37°C with IMP (1 nM) in the presence and absence of CQ, BP, CPZ and NIG, and the amounts of IMP taken up into each organella were measured as described under Materials and Methods. Bars represent the mean ± SEM of three experiments. *, significantly different to each of the control value ($p < 0.1$). **, significantly different to each of the control value ($p < 0.001$). ■, Lys; ▨, Mit fractions. CQ; chloroquine, BP; bipenden, CPZ; chlorpromazine, NIG; nigericin.

organella (intralysosomal pH was measured by using anti-fluorescein rabbit IgG according to Poole et al. (5)), while low affinity sites of neither Lys nor Mit fractions disappeared with these compounds. These results indicated that the binding of basic drugs to high affinity sites of Lys is dependent on the intralysosomal pH. The value of K_d for low affinity sites of Lys was significantly smaller than that of Mit fractions. This issue needs further consideration including functions other than increasing intralysosomal pH. We have also shown the inhibitory effects of some other basic drugs and an ionophore on the uptake of IMP into Lys. The uptake of IMP into Lys was inhibited about 70% by NH_4Cl and NIG (Fig. 5). All of these agents raised the intralysosomal pH to 6.5~7.0 without destroying lysosomal membranes below the concentrations used in this study (data not shown). Therefore, the results suggested that at least 70% of the IMP taken up into Lys was pH-dependent and that it was to the high affinity site. The slight (<20%) decrease of drug uptake in the Mit fractions induced by these agents probably reflected the effect of contaminating acidic vesicles (such as low density Lys, Golgi or endosomes). That 4% of NAGA was found in the low-density fractions of Percoll density gradients is consistent with this interpretation. BP and CPZ were essentially as inhibitory as CQ and NH_4Cl on the uptake of IMP in Lys, but the required concentrations were lower.

CPZ (50 μM) also inhibited most of the uptake of IMP into Mit fractions (Fig. 5). It has been reported that basic drugs bind to mitochondrial monoamine oxidase depending on their lipid solubility (4). The lipid solubilities of CPZ, BP and CQ are 2.6, 0.3 and 0.05 times higher, respectively, than that of IMP (2), and CPZ was the most soluble among them. These values combined with the pH-perturbing effect of CPZ described above may explain why the inhibition of the IMP uptake in the Mit fractions was the highest with CPZ of the drugs tested in this study.

This study showed that Lys in spite of being only 1% of total liver cell volume (14) contributed about 10% to the subcellular distribution of basic drugs in rat liver at 10 min after iv administration. Clinically, the effective plasma concentration of IMP is 100~300 ng/ml. Then, the liver concentration is 14.9~44.6 pmol/mg protein estimated by using the apparent liver-to-plasma concentration ratio (7.5) and 18% protein per tissue (15). This value was 110~329 times smaller than the B_{max} of Lys high affinity sites, 4.9 nmol/mg protein. Therefore, it was inferred that the IMP uptake into Lys within the clinical concentration is mostly due to the specific sites dependent on the intralysosomal pH. Moreover, since basic drugs may be accumulated in these sites after administration, it may be surmised that the contribution of Lys to the subcellular distribution increased with decreases in the plasma concentration over time after drug administration, though amounting to only about 10% in this experiment. Clinically, the effect of a drug during the withdrawal of basic drugs might be maintained through release of the drug accumulated in Lys. These effects may be especially important in combination therapy using basic drugs, which themselves affect the intralysosomal pH. Further investigation regarding the essential relations between the accumulation of basic drugs within Lys and time dependency is needed. However, these results suggested that the accumulation of basic drugs in Lys should not be clinically ignored.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research provided by the Ministry of Education, Science and Culture of Japan.

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