Pharmaceutical Research, Vol. 4, No. 2, 1987

Report

Binding of Basic Drugs to Rat Lung Mitochondria

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Received July 1, 1986; accepted November 12, 1986

The role of the mitochondria in the accumulation of basic amine drugs in the rat lung was studied. Drug binding to the mitochondria was rapid and reached maximum levels after 2.5 min of incubation. Lipophilic basic drugs accumulated in the mitochondria more than nonlipophilic basic drugs and nonbasic drugs, and the accumulation was dose dependent. Schatchard plots revealed at least two independent sets of binding sites for basic drugs in the mitochondria. The binding was competitively inhibited by other basic drugs but not nonbasic drugs. The degree of inhibition by competing basic drugs was correlated with their lipid solubilities. These findings with isolated mitochondria agree with previous results obtained with the perfused lung preparation and indicate that the mitochondria play an important role in the accumulation of basic drugs in the lung.

KEY WORDS: basic drug; lipid solubility; lung; mitochondria; drug binding to lung mitochondria.

INTRODUCTION

Many basic drugs are accumulated in the lung after their administration to animals (1,2). Studies on the nature of drug accumulation in this organ have been performed in various fields (3,4). Previously (5–7), we examined the accumulation of drugs in the isolated perfused rat lung with artificial ventilation. A cationic group as well as a lipophilic group in the molecule was required for drug accumulation in the lung, and the mitochondrial fraction was shown to be the major accumulation site (7). Thus, investigating the binding specificity of basic drugs in the lung mitochondria is important for understanding drug action and toxicity. The binding of drugs to the liver mitochondrial membrane was reported by Huunan-Seppala (8), and that in each subcellular fraction by Bickel and Steele (9). Schneck *et al.* (10) found that propranolol accumulated in the lung mitochondrial and microsomal fractions. They reported that the mitochondria accumulate basic drugs but provided no details of the binding property. We report here the binding characteristics of various drugs to mitochondria and the importance of mitochondria in the accumulation of basic drugs in the lung.

MATERIALS AND METHODS

Materials. ^{14}C-Imipramine, quinine, diphenhydramine, and *N*-methylnicotinamide were purchased from commercially available sources. Phenylbutazone, metoclopramide, ^{14}C-metoclopramide, procainamide, and imipramine were kindly supplied by Fujisawa Pharmaceutical Co. Ltd., Osaka. All other materials were of analytical reagent grade. Imipramine, diphenhydramine, quinine, metoclopramide, procainamide, and *N*-methylnicotinamide represent basic drugs, sulfanilamide is considered to be largely neutral, and phenylbutazone is acidic.

Animals. Male Wistar rats weighing 170 to 220 g were used. They were housed in a constant environment (temperature, 23 ± 1°C; humidity, 55 ± 5%) and allowed water and food ad libitum.

Preparation of Rat Lung Mitochondria. The lung homogenate was prepared with a Potter–Elvehjem homogenizer in 9 parts of a medium consisting of 0.25 *M* sucrose and 3.4 m*M* Tris buffer, pH 7.4 at 4°C. The mitochondrial fraction was obtained by centrifuging the 600*g* (10-min) supernatant at 3300*g* for 20 min (Hitachi Centrifuge RPR 18-3 rotor). The precipitate was resuspended in the same buffered medium and centrifuged again for a further 20 min at 3300*g*. This washing procedure was repeated four times, and the final mitochondrial pellet was suspended in the same buffer and stored at 4°C. Monoamine oxidase and cytochrome oxidase, marker enzymes for mitochondria, were concentrated approximately eightfold in the mitochondrial pellet.

Binding Experiments. The rat lung mitochondria (0.5 mg protein) were incubated in the drug solution (2 ml) at various concentrations at 37°C. After centrifugation at 40,000*g* for 5 min, the supernatant was used to determine free drug (M, nanomoles of drug per milliliter of supernatant), and the mitochondrial pellet for bound drug (P; nanomoles of drug per milligram of protein in the packed pellet).

Isolated Lung Perfusion. The perfusion method of isolated lung was performed as described previously (5). Namely, the lung was ventilated with carbogen gas (95% O_2 and 5% CO_2) at a rate of 60 times/min by applying alternating negative pressure to the chamber. The perfusate (10 ml) consisted of a mixture of rat fresh blood and Krebs–Ringer bicarbonate buffer (1:1), equilibrated with carbogen gas before perfusion. The isolated lung was perfused at a rate of 8 ml/min using a peristaltic pump. In the drug accu-

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0724-8741/87/0400-0142$05.00/0

mulation study, drug solutions of various concentrations were added to the perfusate. After 60 min of perfusion, the concentration ratio of drug in the lung (L; nanomoles of drug per milligram of protein in the lung tissue) to unbound drug in the perfusate (Pf; nanomoles of drug per milliliter) was obtained.

Partition Coefficients. The partition coefficient of each drug was obtained by our previous method (7).

Analytical Methods. Quinine was analyzed by the fluorometric method of Brodie *et al.* (11). ^{14}C-Imipramine and ^{14}C-metoclopramide were determined with a Tri-Carb liquid scintilation spectrometer (Model 3330, Packard Instrument Co.). *N*-Methylnicotinamide was estimated according to Carpenter and Kodicek (12). Procainamide and sulfanilamide were analyzed with a high-pressure liquid chromatograph (LC-6A, Shimadzu Co.). Protein was quantitated by Lowry's method (13).

Data Analysis. Saturation drug binding curves were analyzed by the method of Schatchard (14), in which the regression lines were drawn by the least-squares method to determine the association constant (K_a) of each drug and the binding capacity (B_{max}). The inhibition constant (K_i) for each competitive inhibitor was calculated by the method of Lineweaver and Burk (15).

RESULTS

Incubation Conditions

The time course of drug accumulation was studied at 37°C to establish when equilibrium is reached. Figure 1 shows the time courses of the mitochondrial pellet-to-medium (P/M) drug concentration ratio of imipramine, quinine, and metoclopramide (1 μM). The P/M ratio gradually increased, reached the maximum level at 2.5 min, and remained at a plateau or decreased slightly. Each drug bound rapidly to the mitochondria, and the form of the binding curves was similar, only the magnitude varying. Thus, the drug accumulation experiment was performed at the incubation time of 2.5 min.

Binding experiments were performed with mitochondria prepared immediately or 1, 2, or 3 days earlier to check the stability of the mitochondria. The binding affinity of the drug was highest in the pellet prepared immediately before the experiment and gradually decreased with increasing storage periods. Thus, mitochondria were prepared immediately before the binding experiment.

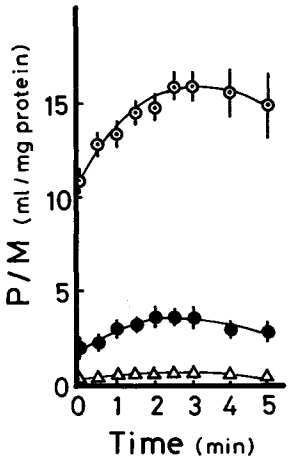

Fig. 1. Time course of drug binding to rat lung mitochondria. Mitochondrial pellets (0.5 mg protein) were incubated at 37°C for different time intervals with imipramine (⊙), quinine (●), or metoclopramide (△) (1 μM). P/M is the concentration ratio of drug in the mitochondrial pellet (P) over drug in the medium (M). Each point represents the mean ± SE of four to six experiments.

Drug Accumulation in the Mitochondria

To clarify the characteristics of the drug binding to rat lung mitochondria, the accumulation of various drugs was monitored. The binding amount and the P/M concentration ratios of each drug are shown in Figs. 2 and 5. Imipramine and quinine accumulated more readily than other drugs, while nonbasic drugs (sulfanilamide and phenylbutazone) did not accumulate in the mitochondria. The order of basic drug binding and of their P/M ratio was imipramine > quinine > metoclopramide > procainamide > *N*-methylnicotinamide.

The relationship between lipid solubility and drug binding to the mitochondria is shown in Fig. 2. Lipid solubilities are expressed as the partition coefficient between chloroform and isotonic phosphate buffer (pH 7.4). The binding of basic drugs correlated well with their respective partition coefficients (r = 0.957), and the P/M ratio increased as the lipid solubility of the basic drug increased.

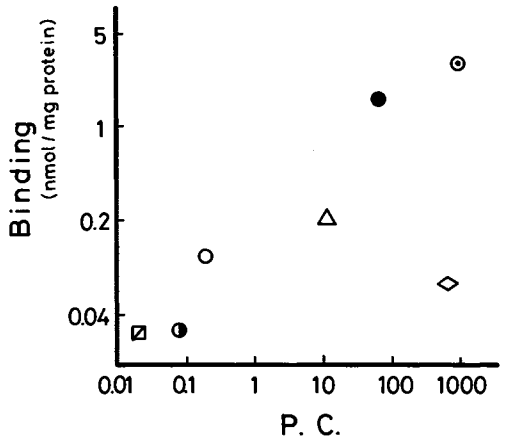

Fig. 2. Effect of lipid solubility on drug binding to lung mitochondria. Mitochondrial pellets were incubated at 37°C for 2.5 min with imipramine (⊙), quinine (●), metoclopramide (△), procainamide (○), *N*-methylnicotinamide (⊠), sulfanilamide (◑), or phenylbutazone (◇) (1 μM). Each point represents the mean ± SE of four to seven experiments. The SE of each drug is smaller than the symbol used.

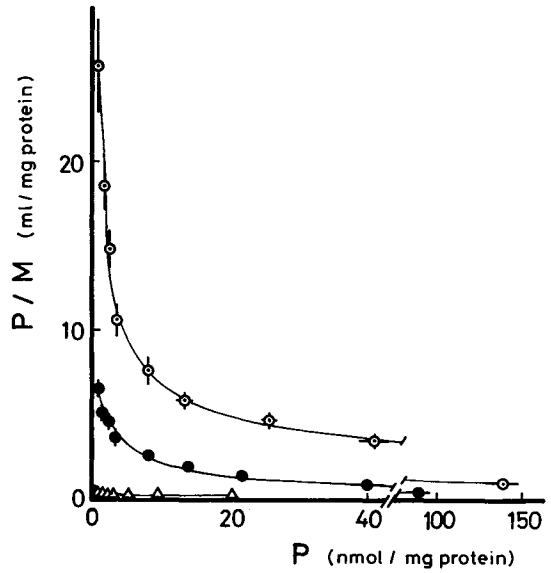

Fig. 3. Schatchard plot of specific binding of imipramine (⊙), quinine (●), and metoclopramide (△) to rat lung mitochondria. Each point represents the mean ± SE of four to seven experiments.

However, the lipophilic acidic drug phenylbutazone did not show any specific accumulation.

The effect of the drug concentration in the medium on binding to the mitochondria is shown in Fig. 3. It is evident that there are at least two types of mitochondrial binding sites for each drug, one high-affinity/low-density site and one low-affinity/high-density site. The binding affinity and maximum number of binding sites are provided in Table I. The binding capacity for each basic drug was similar, while the association constants correlated with their lipid solubility.

Competition for Mitochondrial Drug Binding

The effect of a second drug on the binding of basic drugs was studied to investigate further drug accumulation in the mitochondria. Table II shows the inhibition by various drugs on the binding of imipramine, quinine, or metoclopramide. Nonbasic drugs and poorly lipid-soluble basic drugs did not affect drug binding to any noticeable degree. On the other hand, a lipophilic basic drug strongly and competitively inhibited the binding. Diphenhydramine was found to be very potent in inhibiting the binding of each basic drug to the mitochondria. The inhibitory potency of competing basic drugs was correlated with their respective partition coefficients. Figures 4A and B show the Lineweaver–Burk plots of quinine binding to mitochondria with or without the addition of diphenhydramine and metoclopramide. The amount of quinine bound to the pellet decreased with increasing concentrations of the second drug. The binding regression lines clearly intercepted the ordinate at the same point in each case, indicative of competitive inhibition. The inhibiting potency of diphenhydramine was greater than that of metoclopramide, while *N*-methylnicotinamide (Fig. 4C) and phenylbutazone, a lipophilic acidic drug (Fig. 4D), were inactive. The same experiments were performed for the binding of imipramine and metoclopramide. Both diphenhydramine and metoclopramide competed with these drugs at the same site in the mitochondria, since the same intercept on the ordinate was obtained. The inhibition constants (K_i) of various drugs calculated by the Lineweaver–Burk method are shown in Table III. The inhibitory potency of diphenhydramine was higher than that of metoclopramide.

Table I. Binding of Basic Drugs to the Rat Lung Mitochondrial Pellet[a]

Drug	Affinity site	B_{max}[b]	K_a[c]
Imipramine	High	4.7 ± 0.5	8.4 ± 2.5
	Low	155 ± 12	0.030 ± 0.005
Quinine	High	4.2 ± 0.6	1.2 ± 0.1
	Low	135 ± 7	0.014 ± 0.002
Metoclopramide	High	3.5 ± 1.0	0.013 ± 0.001
	Low	254 ± 48	0.0009 ± 0.0001

[a] Each value represents the mean ± SE of three to five experiments.
[b] Maximum number of binding sites (nmol ligand/mg protein).
[c] Association constants for each class of binding site (1/μ*M*).

Relation of Drug Accumulation in the Perfused Lung and the Lung Mitochondria

In order to clarify whether drug accumulation in the isolated perfused lung can be predicted with the binding characteristics to mitochondria, the drug uptake by the perfused lung was compared to that by the mitochondria. The

Table II. The Inhibition of Drug Binding to the Mitochondria by Various Competing Drugs[a]

Inhibitor	PC[b]	% inhibition		
		Imipramine	Quinine	Metoclopramide
Diphenhydramine	442	32.1 ± 2.9	37.4 ± 3.9	29.3 ± 2.0
Metoclopramide	12.0	19.6 ± 1.7	17.8 ± 2.3	15.0 ± 2.6
Procainamide	0.17	8.6 ± 5.4	6.5 ± 4.7	3.0 ± 4.0
N-Methylnicotinamide	0.02	−0.1 ± 3.1	−4.7 ± 2.6	0.4 ± 3.2
Sulfanilamide	0.08	3.2 ± 2.7	−5.0 ± 2.8	−2.4 ± 3.5
Phenylbutazone	770	−2.5 ± 3.0	2.8 ± 2.5	0.2 ± 2.8

[a] Each value represents the mean ± SE of three to six experiments. Mitochondrial pellets were incubated with a mixture of 1 μM of a basic drug and 50 μM of inhibitor.
[b] Partition coefficient: $CHCl_3$/pH 7.4 phosphate buffer.

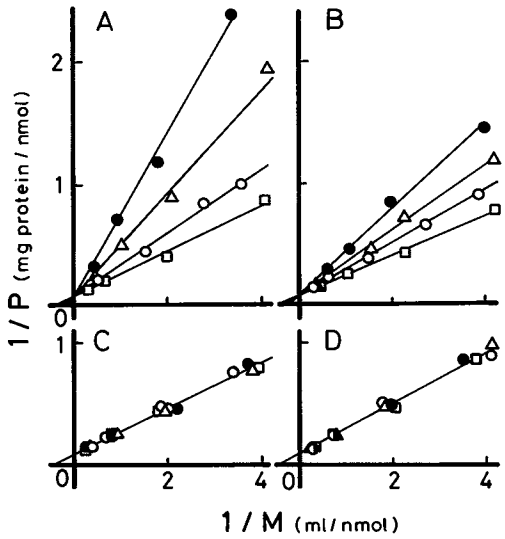

Fig. 4. Lineweaver–Burk plots of quinine binding to the rat lung mitochondrial pellet in the presence or absence of diphenhydramine (A), metoclopramide (B), *N*-methylnicotinamide (C), or phenylbutazone (D). Mitochondrial pellets were incubated at 37°C for 2.5 min with quinine and another drug (□, 0 μ*M*; ○, 5 μ*M*; △, 15 μ*M*; ●, 50 μ*M*).

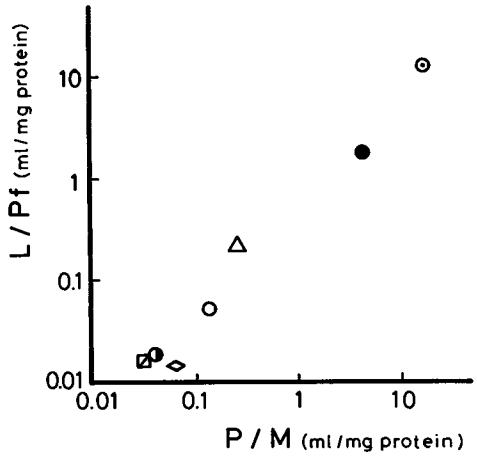

Fig. 5. Relationship of drug accumulation in the perfused lung and the lung mitochondria. L/Pf: the concentration ratio of drug in the perfused lung (nmol/mg protein) to unbound drug in the perfusate (nmol/ml) at 20 μ*M*. P/M: the concentration ratio of drug in the mitochondria (nmol/mg protein) to free drug in the medium (nmol/ml) at 1 μ*M*. Each point represents the mean ± SE of four to seven experiments. The SE of each drug is smaller than the symbol used. (⊙) Imipramine; (●) quinine; (△) metoclopramide; (○) procainamide; (▨) *N*-methylnicotinamide; (◑) sulfanilamide; (◇) phenylbutazone.

resultant plot (Fig. 5) yielded a straight line with a correlation coefficient of 0.982 ($P < 0.01$).

DISCUSSION

Examination of drug binding to rat lung mitochondria revealed specific binding sites for basic drugs. First, lipophilic basic drugs accumulated in the mitochondria more than nonlipophilic basic drug and nonbasic drugs (Fig. 2). Second, the accumulation was dose dependent (Fig. 3). And third, the binding of one basic drug was competitively inhibited by another basic lipophilic drug but not by nonbasic drugs (Fig. 4 and Table II). The binding sites consisted of at least two independent sets, and the binding affinity of each basic drug was regulated by its lipid solubility. We previously found that basic lipophilic drugs were accumulated in the isolated perfused lung in a dose-dependent binding manner and that the accumulation was competitively inhibited by other basic drugs (5,6). Moreover, the K_a and B_{max} for each basic drug in the lung mitochondrial pellet were in the same order as that in the perfused lung, with K_a and B_{max} values for the high-affinity site of 8.1 μM^{-1} and 2.4 nmol/mg protein for imipramine, 1.4 μM^{-1} and 2.3 nmol/mg protein for quinine, and 0.06 μM^{-1} and 2.0 nmol/mg protein for metoclopramide in the perfused lung (unpublished data). We document here that the drug accumulation in the perfused lung correlated well with the binding to the lung mitochondria (Fig 5). These results indicate that the mitochondria play an important role in the accumulation of basic drugs in the lung and that various basic drugs bind to the same sites in the lung mitochondria. Moreover, the lung accumulation of various drugs can be predicted on the basis of their mitochondrial binding.

Many investigators have previously studied drug binding to the mitochondria, mostly of the liver. Further, the binding properties of drugs with different physicochemical characteristics have not been studied under the same experimental conditions.

Minchin *et al.* (16) found that the K_a and B_{max} of chlorphentermine binding to the lung mitochondrial fraction (obtained at 15,000*g* for 15 min and using equilibrium dialysis at 37°C for 3 hr) were 2.01 mM^{-1} and 108 nmol/mg protein, respectively. The lipid solubility of chlorphentermine resembles that of metoclopramide, for which we report here similar binding parameters at its low-affinity site. It is not clear why the high-affinity site for chlorphentermine was not previously detected in the mitochondria, but it may be due to differences in the purity and viability of mitochondria and/or the experimental conditions.

Bickel and Steele (9) noted that basic lipophilic compounds bind to two independent types of sites in rat liver mitochondria and that the K_a and B_{max} for the high-affinity site were 0.022 μM^{-1} and 88 nmol/mg protein for imipramine and 0.058 μM^{-1} and 211 nmol/mg protein for chlorpromazine. These values differ markedly from the values we

Table III. Effect of a Second Basic Drug on the Accumulation of a Basic Drug in the Mitochondrial Pellet[a]

Inhibitor	K_i[b]		
	Imipramine	Quinine	Metoclopramide
Diphenhydramine	1.3 ± 0.1	5.3 ± 0.4	39.5 ± 10.1
Metoclopramide	3.5 ± 0.3	16.3 ± 2.3	54.5 ± 12.0

[a] Each value represents the mean ± SE of three or four experiments.
[b] Inhibition constant (μ*M*).

obtained for rat lung mitochondria. In a preliminary experiment with rat liver mitochondria, we found that the binding parameters of imipramine were similar to those obtained by Bickel and Steele. Thus the variation in the binding parameters seems to be due to the difference in organ tissue rather than one in the experimental conditions. Specific binding sites for basic drugs in the mitochondria appear to play a major role in determining drug accumulation in the lung.

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