Biochimica et Biophysica Acta, 686 (1982) 197–203
Elsevier Biomedical Press

BBA 71091

THE TRANSPORT OF CHLOROQUINE ACROSS HUMAN ERYTHROCYTE MEMBRANES IS MEDIATED BY A SIMPLE SYMMETRIC CARRIER

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(Received August 11th, 1981)

Key words: Chloroquine transport; Symmetric carrier; Kinetics; Erythrocyte membrane; (Human)

The kinetic properties of the mediated transport of chloroquine in human erythrocytes are investigated. The high rates of translocation across the cell membrane and high adsorbance properties to glass surfaces have led to the development of new techniques for measuring initial rates of transport. Three different methodological procedures are used to accomplish a complete kinetic characterization of the system. All measurements were done at 25°C. Under zero-*trans* conditions the system displays complete symmetry, the Michaelis constants being 39.2 ± 2.4 μM for influx and 36.6 ± 5.6 μM for efflux. The respective maximal velocities are 206.4 ± 36.0 $\mu M \cdot min^{-1}$ and 190.0 ± 7.8 $\mu M \cdot min^{-1}$. Under equilibrium-exchange conditions the Michaelis constant is 108.6 ± 15.6 μM and the maximal velocity is 630.3 ± 50.4 $\mu M \cdot min^{-1}$. This 3-fold increase in both K and V over the zero-*trans* values indicates that the rate-limiting step in the transport of chloroquine is the movement of the unloaded carrier. The kinetic data are consistent with the prediction of a simple carrier model.

Introduction

Chloroquine (7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline) is mostly used in the chemotherapy of malaria [1] but is also known to exert a variety of effects in mammalian (see, for example, Refs. 2–5) and bacterial [6] cells. The exact mode of action of the drug is not known, but the fact that it accumulates and raises the pH inside lysosomes [7] has led Homewood et al. [8] to suggest that the parasite growth is inhibited by chloroquine inactivation of acid hydrolases in the digestive vacuoles.

The emergence of chloroquine resistance of malarial parasites in the early sixties and the consequent resurgence of malaria in areas where it was previously controlled or eradicated [9], on the one hand, and the implication of the erythrocyte host cell membrane in the drug resistance, on the other hand [10] (but see also Ref. 11), underscore the urgent need for better understanding of the hitherto undefined mode of transport of the drug across the red blood cell membrane.

Since preliminary experiments [12] indicated that chloroquine uptake by human erythrocytes displayed saturation kinetics, which are indicative of a mediated process, we chose to characterize the transport of chloroquine according to the formalism developed by Stein and Lieb [13]. In the present work we show that the kinetics of chloroquine transport in human erythrocytes are consistent with a model of a simple symmetric carrier, where the rate-limiting step is the translocation of the unloaded carrier across the membrane.

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Materials and Methods

Preparation of cells. Recently outdated human blood, type O, was obtained from Hadassah Hospital Blood Bank, Jerusalem. Erythrocytes were washed four times with phosphate-buffered saline (pH 7.4, 4°C) after the removal of the buffy coat. These cells were used directly for influx measurements, as described below. For efflux experiments, cell suspensions of known hematocrits (usually 9 to 18%) were equilibrated with various concentrations of labeled chloroquine ([*ring* 3-^{14}C]-chloroquine diphosphate, 30 mCi/mmole, New England Nuclear) at 37°C for 40 min. This equilibration period is at least 40-times the half-time of equilibration of chloroquine at the highest concentration used in these experiments.

Since chloroquine binds avidly to many materials, including various kinds of plasticware, one must estimate the free extracellular concentration of chloroquine, which should be equal to the intracellular concentration at equilibrium. Thus, at the end of the equilibration, the cell suspension is centrifuged and the supernate is sampled for its ^{14}C content. The extracellular chloroquine concentration can be calculated based on the specific activity of chloroquine in the loading solution. Packed loaded cells were kept at 0°C until use for efflux measurements. Unlike Macomber et al. [14] and Fitch et al. [10], we did not find any accumulation of chloroquine in normal erythrocytes.

Influx measurements. Influx of chloroquine into red blood cells was assayed by measuring the appearance with time of label in the intracellular space, using the controlled pore-glass-column techniques [12]. Columns were prepared from the barrels of 1 ml disposable syringes filled with 300 mg of controlled-pore-glass (CPG-10, 170 Å pore size, 200 to 400 mesh, Serva-Feinbiochemica, Heidelberg). These columns retain more than 98% of the chloroquine filtered through them. The columns were fitted into the cover of a vacuum chamber, each one on top of a collecting vial.

The influx experiment was initiated by injecting 300 μl of packed washed erythrocytes into a thermostatically controlled (25 ± 1°C) plastic beaker, containing 14.7 ml of vigorously stirred loading solution of various concentrations of labeled chloroquine. The final hematocrit of 2% was achieved within 1 to 1.5 s. At the desired time intervals, samples of 1 ml were transferred from the reaction vessel onto the columns through which they were filtered within 1 to 2 s. To the filtrate 15 μl of sodium dodecyl sulfate were added to achieve cell lysis. Part of the filtrate was taken for scintillation counting. Another part was taken for measurement of absorbance of hemoglobin at 540 nm. (Sodium dodecyl sulfate does not interfere with this measurement.) From these measurements and the known specific activity of the loading solution, the flux was calculated in mol/l cell H_2O per min [15].

Efflux measurements. In efflux experiments it is much more accurate to monitor with time the appearance of the label in the extracellular space. The controlled-pore-glass column could not be used for these experiments and an alternative method was developed based on the filter technique [15,16].

Since this technique uses glass-fiber pre-filters, steps had to be taken to prevent the adsorption of the extracellular free chloroquine to the pre-filters. This was achieved (as suggested to us by Dr. H. Rubin) by treating the pre-filters with poly(L-lysine) ($(C_2H_{13}N_2OBr)_n$, mol. wt. 3000, degree of polymerization 15, Miles-Yeda Ltd., Kiryat Weizmann, Rehovot, Israel) as follows: Pre-filters (Sartorius GmbH) were soaked for 2 h at room temperature in 200 μg/ml poly(L-lysine) in phosphate-buffered saline solution, and then allowed to dry on thick blotting paper. These treated pre-filters in series with 1.2 μm filters mounted in a filter holder (Sartorius GmbH) would retain less than 10% of the free chloroquine. Each batch of treated pre-filters was tested for chloroquine retention prior to use.

The efflux experiment was initiated by rapid injection of about 0.4 ml packed cells pre-loaded with various concentrations of labeled chloroquine into a plastic beaker containing 20 ml of vigorously stirred washout medium. Homogeneous mixing was achieved within less than 1 s. The washout medium contained phosphate-buffered saline for zero-*trans* efflux measurements, or phosphate-buffered saline and various concentrations of non-labeled chloroquine for equilibrium exchange, adjusted according to the determined free extracellular chloroquine concentration at the end of the

loading period (see above, Preparation of cells). At various intervals and at infinity (>40 min), samples of 0.5 to 1.0 ml were filtered rapidly through a poly(L-lysine)-treated pre-filter and 1.2 μm filter by vacuum suction. Samples of the filtrates were mixed with scintillation fluid and counted. The infinity values were used to calculate the exact volume of packed cells injected into the efflux medium.

Since very short sampling times were required for determination of initial rates of equilibrium-exchange conditions, a fully automatic sampling and filtration system has been constructed: five filters were connected in parallel to the washout vessel through electronically controlled solenoid valves. The filter outlets were connected to a vacuum box and positioned on top of collecting vials placed inside the box. The true times of solenoid activation were recorded on a storage oscilloscope.

Calculations. The flux is obtained from the following relation:

$$\text{Flux(mol/l cell } H_2O \text{ per min)} = \frac{\Delta C/\Delta t}{C_\infty} \cdot \frac{1000 \cdot 60}{SA \cdot 0.7}$$

$\Delta C/\Delta t$ is the rate of appearance of label in the extracellular fluids. C_∞ is the total radioactivity in the extracellular medium after more than 40 min of equilibration in the efflux medium. Since the extracellular volume is much larger than the cellular one, C_∞ equals the total radioactivity injected into the medium at $t = 0$; hence, it is proportional to the number of cells present in the efflux system. SA is the specific activity of the loading system in cpm/mol. Since isotonic solutions were used throughout the experiments, 0.7 was taken as the fractional water volume in erythrocytes. The factors 1000 and 60 are used to transform the results into appropriate dimensions (e.g., liter cells and minute).

Results

Zero-trans influx

Fig. 1 demonstrates the uptake of chloroquine into human erythrocytes as a function of time from solutions containing various concentrations of chloroquine. The common intercept for all the uptake isotherms on the ordinate reflects the efficiency of trapping of extracellular chloroquine by the controlled-pore-glass mini-columns at all chloroquine concentrations used, since constant total

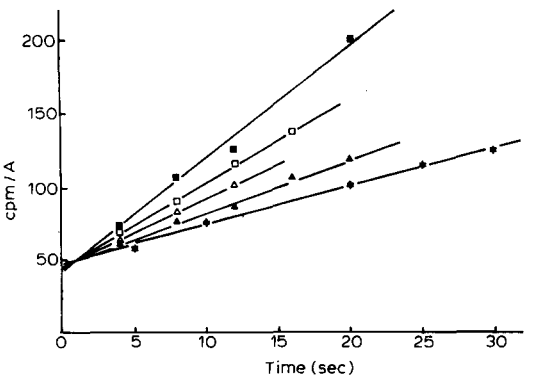

Fig. 1. Zero-*trans* influx of chloroquine in human erythrocytes. Washed erythrocytes were injected into loading solution containing various concentrations of [*ring*-3-^{14}C]chloroquine-labeled solutions. At the indicated times, aliquots of 1 ml were filtered through controlled pore glass mini-columns (see Materials and Methods). The filtrates were sampled after lysis for radioactivity and for hemoglobin content. The concentrations of chloroquine in the loading solutions were (in μM): ■, 7; □, 27; △, 52; ▲, 102; ∗, 202.

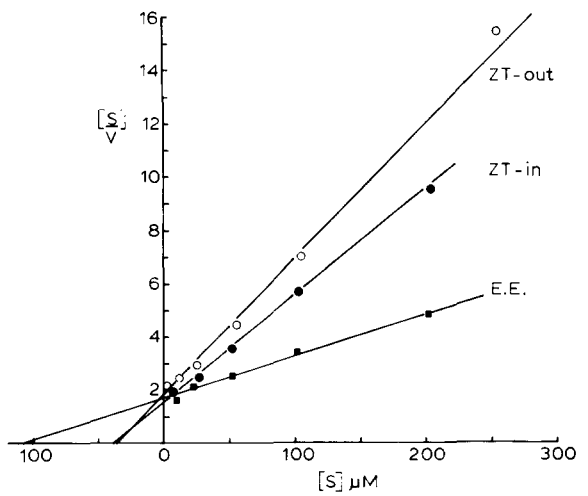

Fig. 2. Zero-*trans* efflux and influx and equilibrium-exchange efflux of chloroquine in human erythrocytes. Results are given according to the Haldane linearization, $[S]/v$ vs. [S]. The slopes are equal to $1/V$ and the intercepts on the X-axis give the Michaelis constants. The derived kinetic parameters and the correlation coefficients of the linear-regression analysis are given in Table I.

TABLE I

KINETIC PARAMETERS OF CHLOROQUINE TRANSPORT ACROSS HUMAN ERYTHROCYTE MEMBRANES

Compilation of kinetic parameters (mean ± S.D.) and the coefficient of correlation of linear regression (r) of [S]/v vs. [S].

Procedure	V (10^{-6} mol/l cell H_2O per min)	K_m (μM)	r	V/K_m (min^{-1})
Zero-*trans* influx (2→1)	206.4 ± 3.6	39.2 ± 2.4	0.999	5.26 ± 0.33
Zero-*trans* efflux (1→2)	190.0 ± 7.8	36.6 ± 5.8	0.997	5.19 ± 0.85
Equilibrium-exchange (ee)	630.3 ± 50.4	108.6 ± 15.6	0.991	5.80 ± 0.95

radioactivities were used throughout these experiments.

The determination of the kinetic parameters of uptake, i.e., K_m and V_{max}, was carried out by linear regression of S/v vs. S, where S is the chloroquine concentration and v is the rate of uptake at this concentration, determined from the slopes of the uptake isotherms. The linearized graphs are shown in Fig. 2 and the derived values are given in Table I.

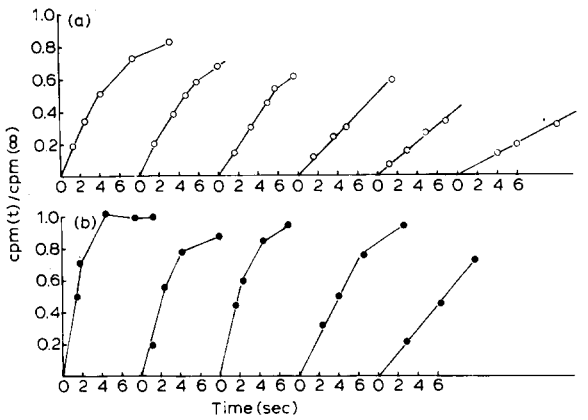

Fig. 3. Zero-*trans* and equilibrium-exchange efflux of chloroquine from human erythrocytes. Cells were loaded in solution containing various concentrations of labeled chloroquine at 37°C for 40 min. At time $t=0$, packed loaded cells were injected into thermostatically controlled solutions containing either phosphate-buffered saline alone (zero-*trans* upper panel) or phosphate-buffered saline + various chloroquine concentrations, identical to the intracellular ones (lower panel). At the times indicated, samples were withdrawn through 1.2 μm filters, and the label content was determined by scintillation counting. Results were normalized with counts present in the extracellular medium at $t=\infty$ (25 to 40 min) which are indicative of the volume of cells injected into the efflux medium. The rates of efflux were calculated from the linear parts of the efflux isotherms. The chloroquine concentrations (in μM) were, from left to right: (A) zero-*trans*, 3, 10.6, 25.6, 55, 105; (B) equilibrium exchange, 8.9, 16.2, 79, 160.

Zero-trans and equilibrium-exchange efflux

Fig. 3 demonstrates a typical experiment where zero-*trans* and equilibrium-exchange effluxes of labeled chloroquine from human erythrocytes are measured against time. Since C_∞ is a measure for the number of cells present in the efflux medium, results were normalized with the appropriate values of C_∞. The rate of equilibrium-exchange (i.e., the slope of the linear part of the egress curve) at all concentrations is significantly larger than that of zero-*trans* efflux. This is a clear demonstration of *trans*-acceleration, a feature common to many carrier-mediated transport systems. Here, again, the data were analyzed by linear regression of S/v vs. S, where S is the calculated free intracellular chloroquine concentration (see Materials and Methods). The linearized plots are shown in Fig. 2 and the derived kinetic values are given in Table I.

Discussion

Chloroquine is an important antimalarial drug, which acts on the intraerythrocytic stage of the malarial parasite. In order to reach its site of action, it has first to cross the permeability barrier of the host cell, i.e., the erythrocyte membrane. The ionic nature of this compound ($pK_1 = 10.2$, $pK_2 = 8.3$ [17]) suggests that at the physiological pH it cannot translocate across the erythrocyte membrane by simple diffusion and, therefore, its

transport might be mediated by one of the transport systems known to be present in the red blood cell membrane.

The experimental testing of the mode of transport of chloroquine is hampered by lack of specific inhibitors which would efficiently stop its translocation across the membrane and thus allow a precise determination of the initial rates. A further experimental complication arises from the high rate of translocation of chloroquine across the red cell membrane, which precludes the possibility of rapid cooling as a means for stopping transport. We were therefore forced to rely on methods based on fast separation of cells from either loading or efflux medium. For influx measurements, where the most accurate results are obtained by measuring the intracellular content of the tested compound, we developed a method based on the high affinity of chloroquine for glass surfaces [12]. For efflux measurements we reverted to the filtration technique [15,16,18], after the problem of chloroquine adsorption to fiberglass pre-filters had been solved. The results shown in Figs. 1 and 3 clearly demonstrate that initial rates of transport were accurately determined.

The three experimental procedures used in the present study, i.e., zero-*trans* influx and efflux as well as equilibrium exchange, are sufficient for testing and characterizing a simple carrier mechanism [19] and for the rejection of a pore-mediated transport system [13]. Previous applications of this methodology were used to demonstrate by kinetic criteria that uridine [15] and leucine [20] are translocated across the erythrocyte membrane by simple asymmetric carriers, while tryptophan translocation is mediated by symmetric carrier [20]. The same methodology has been used to demonstrate that galactose is transported across the red cell membranes by two antiparallel asymmetric carriers [18].

We shall now attempt to demonstrate that the transport of chloroquine across the red cell membrane is mediated by a simple symmetric carrier.

First, the observation that transport of chloroquine is a saturable function of substrate concentration is indicative of mediated transport. Mediation could occur either through carriers or through channels. As the maximal velocity of transport at equilibrium exchange is 3-fold larger than in either zero-*trans* procedure, it is clear that *trans*-acceleration occurs, so that a simple pore model cannot possibly apply [13]. Second, an inherent property of a simple carrier is that the V/K ratios of all three experimental procedures utilized in the present work must be equal. From Table I and from the equality of the intercepts on the ordinate ($=K/V$) of the linearized curves (Fig. 2), it is obvious that within the experimental errors this requirement is met in the chloroquine transport system. Third, following Heinz and Durbin [21], the general equation describing the unidirection flux (v) of substrate from one solution (1) across a membrane to the other solution (2) is given by a modification from Ref. 13:

$$v_{1\to 2} = \frac{(K+S_2)S_1}{K^2R_{00} + KR_{12}S_1 + KR_{21}S_2 + R_{ee}S_1S_2}$$

The reverse unidirectional flux, $v_{2\to 1}$, is obtained by interchanging subscripts 1 and 2. S_1 and S_2 are the substrate concentrations in solutions 1 and 2, respectively; K is an affinity parameter related to the measurable K_m values as shown below, and the R values are the reciprocals of the maximal velocities. Thus, if we define the intracellular space as 1 and the extracellular medium as 2, we obtain the following relations:

Equilibrium exchange: $R_{ee} = 1/V_{ee}$; $K_{ee} = K\dfrac{R_{00}}{R_{ee}}$

Zero-*trans* efflux: $R_{12} = 1/V_{12}$; $K_{12} = K\dfrac{R_{00}}{R_{12}}$

Zero-*trans* influx: $R_{21} = 1/V_{21}$; $K_{21} = K\dfrac{R_{00}}{R_{21}}$

As $R_{00} = R_{12} + R_{21} - R_{ee}$, hence there are four observable independent parameters which completely specify the simple carrier. The R terms are the reciprocals of the maximal velocities, and thus they represent 'resistance' [22] or the average time it takes a single transporter to complete a transport cycle under the determined experimental condition, R_{00} can be considered as the resistance afforded by the membrane to the unloaded carrier.

Using the values of V from Table I, we obtain the following 'resistances':

$R_{21} = (4.84 \pm 0.08) \cdot 10^{-3}$; $R_{12} = (5.26 \pm 0.22) \cdot 10^{-3}$; $R_{ee} = (1.59 \pm 0.13) \cdot 10^{-3}$; and $R_{00} = (8.51 \pm 1.19) \cdot 10^{-3}$. All values are in units of $\min \cdot \mu M^{-1}$. As R_{00} is larger than any other R, one can conclude that the rate-limiting step in the transport of chloroquine across the red cell membrane is the translocation of the unloaded carrier.

With the resistance parameters known, we proceed to estimate independently and redundantly the fourth observable independent parameter of the simple carrier, the affinity parameter K. This is done using the K_m values obtained by the three experimental procedures and the resistance parameters and their interrelations as shown above. Results are given in Table II. Obviously, all three estimates of K are identical to within the experimental error.

We can conclude and say that all of the available data are consistent with a model of a simple carrier for the transport of chloroquine across the red blood cell membrane. This transport system thus joins a set of other transport systems in red blood cells, which have been characterized as being mediated by a simple carrier according to the formalism of Lieb and Stein [15,20].

Most of the hitherto described carrier transport systems display a considerable degree of asymmetry in that their K_m and V values are substantially higher for efflux than influx. This phenomenon has been attributed to the uneven distribution of free carriers at the two membrane interfaces [23].

The carrier which mediates the translocation of chloroquine is obviously symmetrical, since both the V and the K_m values for zero-*trans* influx and efflux are practically equal. A similar symmetry has been shown for the transport of tryptophane. It now remains to find out which is the natural transport system of the red blood cell membrane that is mediating the transport of chloroquine. A most plausible candidate is the system that transports the dibasic amino acids such as lysine and arginine [24]. This system displays similar K_m values but markedly lower V values in comparison to chloroquine. Preliminary results obtained in our laboratory indicate that the uptake of chloroquine is inhibited by lysine and arginine but also by other amino acids such as histidine and tryptophan.

TABLE II

INDEPENDENT ESTIMATION OF K: THE SIMPLE CARRIER AFFINITY PARAMETER

Relations used	Value (μM)
$K = K^{zt}_{2\to1}(R_{21}/R_{00})$	22.2 ± 3.4
$K = K^{zt}_{1\to2}(R_{12}/R_{00})$	22.6 ± 4.9
$K = K^{cc}(R_{cc}/R_{00})$	20.2 ± 4.4
Mean ± S.D.	21.7 ± 1.3

Acknowledgements

This work was supported by WHO/UNDP/World Bank Special Program for Research and Training in Tropical Diseases. We thank Professor W.D. Stein for helpful discussions and critical reading of the manuscript, and Professor H. Rubin and Dr. Z.I. Cabantchik for helpful comments.

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