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The pH-dependence of chloroquine uptake by phosphatidylcholine vesicles

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The pH-dependence of chloroquine uptake by phosphatidylcholine bilayered vesicles was investigated to examine the relative affinity of ionized and un-ionized chloroquine species to membrane bilayers. The results suggest that the neutral species and monocation are taken up by the bilayers but only the un-ionized species interacts significantly with the hydrocarbon interior.

The action of chloroquine as an antimalarial appears to depend on accumulation of the drug in the food vacuole of the parasite (Yayon & Ginsberg 1983). This requires passage of chloroquine across at least four membranes: the host erythrocyte membrane, the membrane of the parasitophosphorus vacuole, the external parasite membrane and the membrane of the food vacuole. Clearly, the action of chloroquine depends critically on its ability to cross membranes.

The drug (CQ) is a base with two sites of ionization (pK_a values of 10·1 and 8·1 (Irvin & Irvin 1947)). At physiological pH it is present largely as the dication (CQ^{2+}; about 83%), with some monocation (CQ^+; about 17%), while the neutral species (CQ°) is present only in very small quantities (less than 0·05%). Such observations led Homewood et al (1972) to propose that it is CQ^+ which passes through cell membranes at physiological pH.

Phospholipid bilayered vesicles have been widely used as model membranes. Calorimetric studies by Chawla et al (1979) on the interaction of chloroquine with phosphatidylcholine (PC) bilayers, at relatively high chloroquine concentrations (10^{-3} to 10^{-1} M), showed competitive binding between chloroquine and ionic lipids. Those workers also provided evidence, from proton NMR studies, that chloroquine interacts with the methylene groups of the hydrocarbon chain rather than with the methyl groups of the quaternary nitrogen of the choline group of PC. Lullman & Wehling (1979) studied the binding of a range of cationic drugs, including chloroquine, to various types of phospholipid vesicles, including PC, and demonstrated that chloroquine binding to PC was low when compared with the other drugs, though particularly high compared with phosphatidylserine, an anionic phospholipid. The evidence therefore suggests that both ionic and hydrophobic interactions are important.

Our study aimed to investigate these matters further by studying the pH dependence of chloroquine uptake by PC bilayered vesicles to determine the relative affinity of CQ°, CQ^+ and CQ^{2+} for membrane bilayers and provide information on the likely contribution of these species to passive diffusion across membranes.

PC was chosen because it is the most prevalent phospholipid in erythrocyte membranes (Rouser et al 1968) and its charge is pH-independent over the pH range used in the study (Bruni & Palatini 1982), which avoids the complication of possible ion binding being dependent on the variable charge of the phospholipid.

Materials and methods

Chloroquine as the diphosphate salt, was supplied by Sigma Chemical Co. (St. Louis, MO, USA). All glassware coming into contact with solutions of the drug was silanized using Aquasil silanizing liquid (Pierce, Rockford, IL, USA). The buffer used contained 20 mM Na_2HPO_4 and 125 mM NaCl and was adjusted to the required pH using 1 M HCl or NaOH.

PC vesicles were prepared by evaporating the solvent from a solution of PC (Type XI-E, Sigma Chemical Co. St Louis, MO, USA) in chloroform, weighing the dried PC (~100 mg), then adding 20 ml of buffer. The mixture was then sonicated for 3 min using a Heat-System Ultrasonic W-375 sonicator. The presence of multibilayered vesicles was verified by electron microscopy.

Binding of chloroquine to the PC vesicles was determined by equilibrium dialysis using glass dialysis cells and a cellophane semipermeable membrane (Type

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20, Union Carbide Corp., Chicago, IL, USA). Dialysis was conducted in a shaking water bath at 37 °C. It was established, using molybdenum blue (Rosenberg 1969), that no PC molecules crossed the cellophane membrane. The equilibration time was not more than 5 h. The buffer and phospholipid compartments were assayed (the latter after acid extraction of the PC) by HPLC using a fluorimetric detector (Tett et al 1985). The pH was measured at the end of dialysis.

The hexane–water partition coefficient of CQ° and the apparent chloroquine partition coefficient (at pH 7·4) into hexane were also determined: in the former case, 20 ml 0·05 M NaOH solution, containing 1 mM chloroquine, was added to 1 ml n-hexane; in the latter case, 10 ml of buffer adjusted to pH 7·4 was added to 10 ml n-hexane (in both cases the aqueous and organic phases were pre-equilibrated). After vortexing, the mixtures were placed in a water bath at 37 °C for 4 and 2 h, respectively, with periodic vigorous shaking. The aqueous phase was then assayed and the amount of chloroquine in the organic phase determined by difference.

Results and discussion

Fig. 1 shows the amount of chloroquine bound per kg of PC at different unbound chloroquine concentrations at various pH values. The almost linear binding isotherms indicate an absence of significant saturation (at a free drug concentration of 6 μM about 1 molecule is bound per 1700 molecules of PC at pH 10·7) and that an increase in buffer pH leads to an increase in drug uptake by PC vesicles. CQ^{2+} can be excluded as a major binding species since the fraction of total drug in this form declines sharply over the pH range studied while the extent of binding increases. Up to pH 9·1, the fraction in the form CQ^+ increases with increasing pH (assuming pK_a values of 10·1 and 8·1), then declines, while the neutral fraction increases with pH over the whole pH range. The continued increase in binding with increasing pH above pH 9·1 suggests that CQ° is the major binding species over this pH range. However, it is not clear from inspection of Fig. 1 whether there is a significant contribution to binding due to CQ^+, particularly at low pH.

If both CQ° and CQ^+ contribute significantly to PC binding of the drug, the expected relationship between the amount bound per kg of PC (B) and the free concentrations of CQ° and CQ^+ is

$$B = K^+[CQ^+] + K°[CQ°] \quad (1)$$

where K^+ and K° are binding constants or partition coefficients for CQ^+ and CQ°. Rearranging equation 1 gives

$$B/[CQ^+] = K^+ + K°[CQ°]/[CQ^+] \quad (2)$$

Equation 2, with K^+ and K° independent of drug concentration, predicts a linear relationship between $B/[CQ^+]$ and $[CQ°]/[CQ^+]$. Linear regression, applied to the transformed data, provided a slope of K° = 188 ± 28 (2 s.e.) litre (kg PC)$^{-1}$ and intercept of K^+ = 14 ± 3 (2 s.e.) litre (kg PC)$^{-1}$. These parameters were used to calculate the lines shown in Fig. 1. The non-zero intercept indicates that the contribution of ion binding is significant. Estimating the relative contributions to binding of the monocation and neutral species by calculating the relevant terms in equation 1, shows that about 98% of binding at pH 7·4 is due to the CQ^+, but its relative contribution decreases sharply with increasing pH.

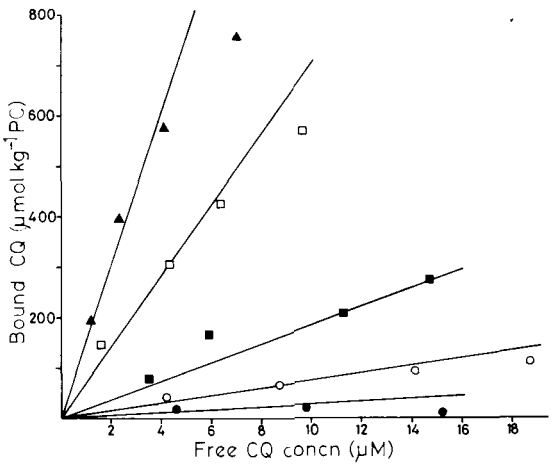

FIG. 1. Binding isotherms showing the amount of chloroquine (CQ) bound per kg of PC at different free CQ concentrations at various pH values: pH 7·5 (●), pH 8·1 (○), pH 8·8 (■), pH 9·8 (□), pH 10·7 (▲). The lines shown at corresponding pH values are derived from the calculated K° and K^+ values (eqn 1).

The partition coefficient of CQ° for PC vesicles, K°, is smaller by two orders of magnitude than that for octanol ($10^{4.63}$; Lullman & Wehling 1979) and chloroform (about 10^4, estimated in preliminary work in this study). Sonication of the PC mixture after addition of chloroquine did not alter binding, indicating that access of drug to the bilayers was not a limiting factor. Partition coefficients were measured using hexane, at pH 12·5 (where CQ° predominates) and at pH 7·4 (where CQ^+ predominates over CQ°, although CQ^{2+} is the major species). A hexane–water partition coefficient for CQ° of 241 ± 16 (2 s.e.) was calculated. Assuming that hexane provides a model for the membrane interior, the CQ° hexane-water partition coefficient overestimates K° for the bilayers because the hydrocarbon chains occupy a fraction of the bilayer volume. The values are in agreement if the hydrocarbon phase is about 80% of the volume of the bilayer.

The partition coefficient of CQ^+ in hexane was too small to be measured. The apparent partition coefficient of chloroquine in hexane at pH 7·4, 0·04 ± 0·01 (2 s.e.), can be accounted for by the small fraction of CQ° present.

These results indicate that both CQ° and CQ^{+} interact with PC bilayers, with their relative contributions determined by the pH of the medium. The reasonable agreement between partition coefficients for hexane and PC vesicles suggests that CQ° partitions into the hydrocarbon interior of the bilayer. CQ^{+} appears to bind to the polar head groups of the phospholipid, with the hydrophobic aromatic portion of the molecule situated within the hydrocarbon interior of the bilayer. This is supported by the observation that no significant binding could be attributed to CQ^{2+}; the second charge on CQ^{2+} is located on the nitrogen adjacent to the aromatic nucleus (Rosenberg & Schulman 1978), which would require the charged nitrogen to be situated within the hydrocarbon interior of the bilayer. The data of Chawla et al (1979) were obtained at much higher concentrations than those of the present study but provide some support for the proposed binding mechanism. The pH of their study was not reported, but uncontrolled pH is most likely to produce CQ^{2+} or CQ^{+} as the dominant species. Their proton NMR data, indicating a preferential interaction of chloroquine with the methylene groups of the hydrocarbon chain, is consistent with an interaction of the hydrophobic portion of CQ^{+} with the hydrocarbon interior, while the charged nitrogen interacts with the anionic phosphate groups. The proposed mechanism is also consistent with the observation of Lullman & Wehling (1979) who found chloroquine binding to PC to be much less than to phosphatidylserine, with a net negative charge at the pH values studied, which suggests a surface ionic interaction. PC is zwitterionic, with a net zero charge and can be expected to be arranged in a bilayer such that local electrical neutrality is maintained with the anionic phosphate of one PC molecule close to the cationic nitrogen of an adjacent PC molecule. The ion binding observed in this study suggests that this pairing of surface charges is imperfect, with local unpaired charges providing a site for interaction with CQ^{+}.

Our observations indicate that CQ° should diffuse freely across membranes, whereas CQ^{+} transport would appear to require a carrier mechanism (as suggested by Yayon & Ginsberg 1982). The low apparent partition coefficient for chloroquine at physiological pH for both PC vesicles and hexane suggests that passive diffusion of CQ° is likely to be a minor route of passage across membranes. The large contribution of ion binding at physiological pH indicates that caution is needed in interpreting membrane transport data, to distinguish between transport across a membrane and surface ion binding.

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