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# DIFFERENTIAL EFFECTS OF CHLOROQUINE ON THE PHOSPHOLIPID METABOLISM OF *PLASMODIUM*-INFECTED ERYTHROCYTES*

HENRI J. VIAL,† MARIE-LAURE ANCELIN,† MONIQUE J. THUET‡ and JEAN R. PHILIPPOT†

† CNRS UA 530, INSERM U 58, 60 rue de Navacelles, 34090 Montpellier, and ‡ Laboratoire de Physique Biologique, Institut de Biologie, Boulevard Henri IV, 34000 Montpellier, France

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**Abstract**—The effect of the antimalarial drug chloroquine (CQ) on the phospholipid metabolism in *Plasmodium knowlesi*-infected simian erythrocytes has been studied by incubating cells with different labeled precursors and various concentrations of CQ. The drug induced considerable modifications of this metabolism but at the same time decreased nucleic acid and protein synthesis as well as the output of $^{14}CO_2$ from radioactive glucose. Phosphatidylcholine biosynthesis was severely reduced. However, under these conditions, CQ had the early effect of markedly increasing phosphatidylinositol labeling from radioactive inositol, fatty acids, 1-($^{14}$C)palmitoyl-lysophosphatidylcholine, but not from glycerol. Synthesis of phosphatidylserine from ($^{14}$C)serine and of phosphatidylethanolamine from labeled glycerol, ethanolamine, and serine was increased, especially at high CQ concentrations when the whole metabolism of the parasite was severely reduced. These effects reflect a deep differential effect of CQ on the intense phospholipid metabolism of the *Plasmodium*-infected erythrocytes, which might involve a redirecting of phospholipid metabolism similar to that induced by other cationic amphiphilic drugs, and a compensatory synthesis resulting from the severe blockage of phosphatidylcholine synthesis.

Chloroquine (CQ)§ is one of the most active phospholipidosis-inducing agents known. It belongs to the group of amphiphilic, weakly cationic drugs that penetrate biomembranes by non-ionic diffusion and accumulate in acidic compartments (such as in lysosomes) in their protonated form. Cells exposed to CQ readily take up the compound, accumulating it in a matter of minutes at concentrations many times higher than in the surrounding medium [1]. The intralysosomal accumulation of CQ has been correlated to a reduction of several lysosomal enzyme activities. In several species, including man, CQ treatment also causes an abnormal accumulation of lipids, mostly of phospholipids (PL), in organs with a high content and/or rapid turnover of PL [2, 3]. The reason for this phenomenon might be either an interaction between the drug and lipid-metabolizing enzymes or between the drug and PL as a substrate. A polar lipid gradient from cytosol into lysosomes has also been reported [2, 4–6]. As soon as the concentration of the polar lipids has attained a certain level, they arrange themselves in a typical order giving rise to a lamellated or crystalloid structure that is ultrastructural hallmark of numerous CQ-treated tissues [1, 2].

CQ is the most widely used drug in the treatment of the asexual erythrocytic stage in the plasmodial life cycle. The drug is taken up by erythrocytes infected with sensitive plasmodia but not by normal erythrocytes [7, 8]. The drug is concentrated mainly in the acidic food vacuole of the parasite, where host cell digestion takes place [9, 10]. Since the CQ-resistant *Plasmodia* accumulate significantly less CQ than susceptible parasites, it has been speculated that this drug acts by means of its lysosomotropism [1, 10–12].

The membrane biogenesis accompanying parasite growth requires an adapted PL biosynthesis [13, 14]. Our previous results have shown that parasitized erythrocytes have a considerable capacity for building new PL molecules [15] and can increase the PL content of erythrocytes by about 500% during the 24 hr *P. knowlesi* cycle [16].

Although the interaction between amphiphilic drugs and polar lipid metabolism is a generalized phenomenon not restricted to certain tissues [2], the effect of CQ, which is one of the most investigated lysosomotropic drugs, on the PL metabolism of *Plasmodium*-infected erythrocytes has not been reported. Nevertheless, interference by CQ with the intensive PL metabolism present in these cells is probable, and considering the above observations, it is possible that this interference contributes to the drug's action on parasite growth. In the present paper, we describe changes in the PL metabolism of *P. knowlesi*-infected simian erythrocytes induced by the CQ.

## MATERIALS AND METHODS

*Chemicals.* The labeled compounds (U-$^{14}$C)glucose, (1(3)-$^{3}$H) glycerol, ($^{14}$C)isoleucine, (9,10(n)-

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§ Abbreviations used: CQ, chloroquine; FA, fatty acid; PC, PE, PI, PS, phosphatidylcholine, -ethanolamine, -inositol, -serine; PL, phospholipid.

$^3$H)oleic acid, ($^{14}$C)stearic acid, (2-$^{14}$C)ethanolamine, ($^{14}$C-methyl)choline, and myo-(2-$^3$H)inositol were obtained from Amersham (Bucks., U.K.), ($^3$H(G)hypoxanthine and L-1-(1-$^{14}$C)palmitoyl-lyso-PC from New England Nuclear (Boston, MA) and L-(U-$^{14}$C)serine from CEA (France). RPMI 1640 and modified RPMI 1640 was provided by Eurobio (Paris, France). Chloroquine diphosphate was purchased from Sigma (St. Louis, MO).

*Biological model.* We used splenectomized *Macaca fascicularis* monkeys (SANOFI, Montpellier, France) and cryopreserved [17] Washington strain, variant 1 of *P. knowlesi* (Dr G. Mitchell, Guy's Hospital, London). Levels of parasitemia were determined at appropriate intervals by microscopic examination of a Giemsa-stained film of tail blood. High infected blood was collected aseptically by venipuncture in citric acid–dextrose after the monkey had been anesthetized by an intramuscular injection of ketamine (Iffa-Credo, France). The monkeys were cured by intramuscular injection of CQ (150 mg) on three consecutive days. After 6 weeks, they could be reinfected. Cells were collected by centrifugation at 700 *g* for 10 min and washed once with basic medium (RPMI 1640 without choline, inositol and serine, supplemented with 35 mM Hepes, pH 7.4). White cells were removed by passage through a cellulose powder column (CF 11 Whatman) [18]. After two washes with basic medium, the infected erythrocytes were ready for experimentation.

*Assay of labeled precursor incorporations.* *P. knowlesi*-infected erythrocytes were preincubated at 10–20% hematocrit in basic medium containing the indicated concentrations of CQ. After 1 hr at 37°, cells were pelleted and the medium was removed. They were then incubated with radioactive precursors of lipid metabolism (glycerol, fatty acids, lysoPC, choline, ethanolamine, serine or inositol) or of basic metabolism (glucose, hypoxanthine, isoleucine). Incubations were carried out at a hematocrit of 7–15% in enriched medium composed of modified RPMI 1640 (20 μM glycerol, choline, ethanolamine, serine, 10 μM myo-inositol) supplemented with 35 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4), 1 mM ATP, 30 μM CoA, and 0.5 mM of the essential plasmatic fatty acids (FA), namely palmitic, stearic, oleic and linoleic acids in a molar ratio of 1.7/0.7/1.3/1.3, respectively, bound to fat-free bovine serum albumin (17–20 mg/ml). ATP and CoA facilitate the formation of the fatty acid derivatives (e.g. oleyl-CoA) involved in their incorporation into PL [19]. When lysoPC was the radioactive precursor, concentrations of the FA as well as bovine serum albumin were reduced by a factor of 10. After incubation at 37° in stoppered test tubes for the indicated time, reactions were stopped at 4°. The cells were washed once with 5 ml of basic medium and twice with 5 ml of 0.9% NaCl.

*Phospholipid isolation and determination.* For incubations performed with labeled precursors of PL, the cellular lipid material was extracted and then fractionated as described previously [15, 20]. When ($^{14}$C)ethanolamine was the precursor, thin layer chromatography on silica gel in $CHCl_3/CH_3OH/CH_3COOH/10\%$ $NaHSO_3$ (50:20:6:1.5) clearly separated phosphatidylcholine (PC), phosphatidylethanolamine (PE) and *N,N*-dimethyl-PE. After visualization with iodine vapor, the silica gel of the lipid spots was transferred to vials and radioactivities were determined. No measurable radioactivity was found apart from spots corresponding to the lipids mentioned in text. The amounts of labeled precursors incorporated into cellular lipids (nmol. $10^7$ cells$^{-1}$. time$^{-1}$) were computed on the basis of radioactivity incorporated into lipids and the specific activity of the precursor in the incubation medium. In experiments where (U-$^{14}$C) serine was incorporated into PE or PC, radioactivities were corrected for the loss of one radioactive carbon unit.

*Non-lipid metabolism studies.* To measure labeled isoleucine or hypoxanthine incorporation, incubated cells were washed twice with 0.9% NaCl at 4°, then mixed with 4 ml of cold 10% trichloroacetic acid. The precipitates were washed twice with 4 ml of 10% trichloroacetic acid and the final residues were solubilized in 0.8 ml NCS (Amersham) for 12 hr at 37°. 1.5 ml of decolorizing $H_2O_2$ (110 vol.) was added and radioactivities were counted in 20 ml of scintillation fluid. The release of $^{14}CO_2$ from (U-$^{14}$C)glucose metabolism was determined by incubating erythrocytes in glass tubes closed with rubber stoppers allowing the passage of a syringe needle, and containing a suspended fiberglass filter (1.6 cm$^2$ GF-D Whatman) moistened with 2 N NaOH. At the end of the incubation time, reactions were stopped by acidifying the medium with 0.5 ml of 2 N $H_2SO_4$ and the tubes were shaken for 2 hr to assure complete trapping of $^{14}CO_2$. Radioactivity of the filter was determined in a 15 ml scintillation cocktail.

*Measurement of choline and ethanolamine transport into infected erythrocytes.* This was performed on pure *P. knowlesi*-infected erythrocyte suspension (96–100% parasitemia) to eliminate the contribution of uninfected erythrocytes. These were obtained by washing infected red blood cells (10–25% parasitemia) and subjecting them to a Percoll-sorbitol fractionation, as recently described [21].

Transport of choline and ethanolamine into infected erythrocytes after CQ treatment was measured after preincubation with various concentrations of CQ, and a change of medium to produce similar conditions for the assay of labeled precursor incorporations. Transport measurements were initiated by rapidly mixing the cell suspensions with 100 μl of modified medium containing either 5 μM ($^3$H)choline (0.4 Ci/mmol) or 5 μM ($^3$H)-ethanolamine (0.90 Ci/mmol) to give a final hematocrit of 2.8%. After 7 min at 37°, the flux was stopped by adding ice-cold basic medium. Triplicate aliquots of the cell suspension were immediately placed on 300 μl of dibutyl phthalate (Kodak) in polyethylene tubes (containing 100 μl basic medium in the bottom) and centrifuged in a Beckman II microfuge at 10,000 *g* for 10 sec at 4°. Supernatants were discarded and cells were washed once with basic medium at 4°, and then lyzed and precipitated with 500 μl of 10% perchloric acid. After centrifugation, 400 μl of the supernatant was taken for radioactive determination.

All experiments were done at least twice with

each precursor, at different parasitemias, and with different monkeys. Within a single experiment, differences between data from control and CQ-treated cells were considered to be statistically significant, using Student's *t*-test only for $P \leq 0.05$.

## RESULTS

### *Effect of chloroquine on the* de novo *biosynthesis of lipids*

In the absence of CQ, as already described [15], ($^3$H)glycerol incorporation, which represents the *de novo* synthesis of lipids, is essentially recovered in PE, PC, phosphatidylinositol (PI) and phosphatidylserine (PS) as well as in diacylglycerols and triacylglycerols (42.3, 38.0, 6.1, 4.3, 7.0, 2.2% of the total lipid radioactivity respectively). Figure 1 shows that CQ significantly decreased (by 26%) the incorporation of ($^3$H)glycerol into PC when 50 $\mu$M was added to the incubation medium. However, no modification of PI radioactivity was observed over the whole range of CQ concentrations and incorporation into PS was significantly decreased only at the highest concentration, i.e. 0.5 mM. The most conspicuous aspect of this figure is the marked increase in ($^3$H)-glycerol incorporation into PE, which was present at 20 $\mu$M CQ (+38%) and lasted up to at least 0.5 mM. Radioactivities recovered in the neutral lipids, diacylglycerols and triacylglycerols were not significantly different from controls in the same experiment (not shown).

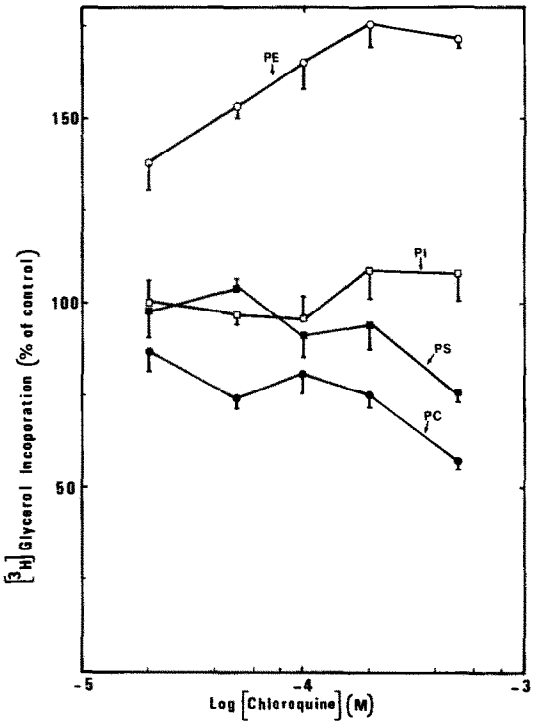


Fig. 1. ($^3$H)-glycerol incorporation into the lipids of *P. knowlesi*-infected erythrocytes as a function of chloroquine concentration. *P. knowlesi*-infected erythrocytes (55% parasitized, ring form) were incubated at 10% hematocrit in basic medium containing the indicated concentrations of CQ for 1 hr at 37°. The cells were then pelleted, the supernatant was removed and $8 \times 10^8$ cells were further incubated for 90 min at 37° in 1.2 ml of RPMI 1640-enriched medium containing 20 $\mu$M ($^3$H)glycerol (540 Ci/mol) and the various concentrations of CQ. Each point represents the mean of triplicate experiments expressed as a percentage of the control (cells incubated in the absence of CQ). Vertical bars correspond to 1 SE. 100% glycerol incorporation into phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) amounted to $25.6 \pm 1.6$, $2.9 \pm 0.3$, $4.1 \pm 0.4$, $28.5 \pm 1.2$ nmol/$10^{10}$ infected cells/90 min ± SE respectively. The incorporation of ($^3$H)glycerol into diacylglycerols and triacylglycerols of control cells corresponded to $4.7 \pm 0.4$ and $1.5 \pm 0.1$ nmol/$10^{10}$ infected cells/90 min ± SE respectively. None of the CQ concentrations modified the incorporation of the precursor into neutral lipids.

### *Effect of CQ on non-lipid metabolism in the infected cells*

For comparative purposes, the effects of CQ on DNA, protein, and glucose metabolism were also investigated. Hypoxanthine, which is the purine preferred by intraerythrocytic plasmodia [14] and isoleucine, which undergoes high level of incorporation into parasite proteins and is absent from Rhesus monkey hemoglobin [22], were chosen to label, respectively, the nucleic acids and proteins of *P. knowlesi*-infected erythrocytes. The output of radioactive $^{14}CO_2$ from (U-$^{14}$C)glucose served as an index of glucose metabolism, which essentially represent the percentage of glucose metabolized via the parasitic pentose phosphate pathway [14, 23]. Figure 2 shows thtat the biosynthesis of nucleic acids and proteins was severely affected by CQ. Incorporation into the macromolecules was already significantly decreased by 25 and 31% at 20 $\mu$M and dropped rapidly at higher concentrations. Thus, protein synthesis was completely blocked after 1 hr of contact between infected cells and 0.5 mM CQ. Experiments carried out with (U-$^{14}$C)glucose showed a similar but less drastic decrease in the output of ($^{14}CO_2$) only when CQ concentrations reached 50 $\mu$M or higher.

### *Effect of CQ on fatty acid incorporation into phospholipids*

Plasma FA and lysoPC both serve as sources of the FA required for cellular PL biosynthesis [15]. Consequently, we assayed for saturated ($^{14}$C)-stearate, unsaturated ($^3$H)oleate, and L-1-($^{14}$C)palmitoyl-lysoPC. Since plasma FA are strongly bound to albumin and their incorporations into red blood cells is a function of the molar ratio FA/albumin [24, 25], a 0.5 mM total final concentration of the major plasmatic FA and a molar ratio FA/albumin equal to 2 were used in our experiments. Figure 3A shows that under these conditions, oleate incorporation into PC was slightly but significantly decreased at CQ concentrations of 20 $\mu$M and higher. Although the curves for the incorporation of this precursor into PS and PE recall that for PC, the results were significantly different from the control only from 0.2 mM CQ upward. By contrast, PI radioactivity increased significantly from 10 to 50 $\mu$M CQ, and then returned to control values. Similar results were obtained with ($^{14}$C)stearate as the radioactive

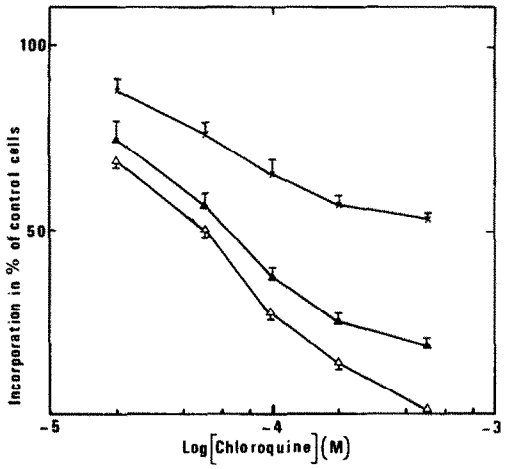


Fig. 2. Effect of chloroquine on the incorporation of labeled hypoxanthine (▲) and isoleucine (△) into acid-insoluble material and on the output of $^{14}CO_2$ from ($^{14}$C)glucose (×). ($^3$H)hypoxanthine (6 μCi/ml) and ($^{14}$C)isoleucine (0.8 μCi/ml) were added to a 0.6 ml suspension containing $3.8 \times 10^8$ ring-infected cells with 55% parasitemia. The output of $^{14}CO_2$ was measured after 2 hr contact between 100 μl of basic medium containing $2 \times 10^8$ infected cells (same parasitemia) and 100 μl of 0.9% NaCl containing radioactive glucose. The final glucose concentration was 7.4 mM, 0.34 Ci/mole. The incubations and processing of the cells were carried out as described in Fig. 1 and the text. Results are means of three separate experiments and are expressed as percentages of the radioactivity in control cells, which contained $1.12 \pm 0.06 \times 10^6$ ($^3$H) dpm and $49.5 \pm 1.8 \times 10^3$ ($^{14}$C) dpm. The release of $^{14}CO_2$ from control cells corresponded to $183 \pm 9$ nmol/$2 \times 10^8$ total cells. Vertical bars correspond to 1 SE.

precursor except that decreased incorporation into PE was more pronounced (−38% at 20 μM).

Since we have shown that extracellular FA and lysoPC compete for entry into infected erythrocytes [15], L-1-($^{14}$C)palmitoyl-lysoPC metabolism was assayed in the presence of only 50 μM of the mixture of major plasmatic FA, and bovine serine albumin was consequently decreased. The concentration of exogenous lysoPC used in this experiment (8 μM, i.e. a molar ratio of lysoPC/albumin equal to 0.3) was far lower than haemolytic concentrations. Figure 3B shows a significant decrease in ($^{14}$C)lysoPC recovery in cellular PC at low doses of CQ (−19% at 50 μM) whereas the decreases observed in the case of PE and PS started only at 0.2 mM (−22 and −28% respectively). On the other hand, an accumulation of ($^{14}$C palmitoyl)lysoPC in PI occurred at low doses of CQ, but the incorporation into this PL returned to the control value at 0.1 mM and above.

The incorporation of ($^3$H)oleate and ($^{14}$C)lysoPC into neutral lipids remained unchanged relative to control values at all CQ concentrations except 0.5 mM, at which radioactivities recovered in diacylglycerols and triacylglycerols were decreased by 20–53% with either precursor (Data not shown).

*Effect of CQ on the incorporation of PL polar heads into* Plasmodium *infected erythrocytes*

Figure 4A shows that ($^{14}$C)choline entry into PC was inhibited by 32% at 50 μM and fell progressively with increasing concentrations. In contrast, an accumulation of ($^3$H)inositol in PI occurred at low doses of CQ, leveling off at 50 μM. However, this accumulation was slow at the highest dose of CQ.

Chloroquine effects on ($^{14}$C)ethanolamine metabolism, which labels both PE and PC [15] differed according to the PL. An increased labeling of PE was observed at 50–200 μM whereas a slight but significant decrease in PC labeling was observed at 200 μM CQ (Fig. 4A). Since this model, i.e. increased formation of PE coupled with a decreased formation of PC by PE *N*-methylation could be favorable to an accumulation of the methylated-intermediate, we tested for this compound. However, the thin layer chromatographic analysis showed no significant formation of *N,N*-dimethyl-PE, even at the highest dose of CQ.

The metabolism of ($^{14}$C)serine which is simultaneously incorporated into PS, PE and PC [15, 26], was also modified. The experiments in Fig. 4B, carried out with a suspension of erythrocytes, 10% parasitized by the early stage of the parasite showed that incorporation of this precursor into PS and PE was considerably increased by CQ, but only at high doses. Radioactivity in PC remained at the control value at all CQ concentrations.

We checked the effect of CQ on the entry of radioactive choline and ethanolamine into *P. knowlesi* infected erythrocyte, since the changes observed in their incorporation into PL molecules could have been due to a change in the availability of these precursors for the parasite. The experiments were carried out under the same conditions as above except that the infected cells were isolated by the recently developed method of percoll-sorbitol gradient fractionation [21]. This was done to allow a precise measurement of the entry of these precursors into infected erythrocytes alone since choline and ethanolamine can enter uninfected erythrocytes by facilitated transport and passive diffusion, respectively (Ancelin and Vial, in preparation). Figure 5 shows that after 60 min of contact with various concentrations of CQ, the rate of ethanolamine entry into infected cells was not altered. Similarly, the entry of choline into the infected cells was not modified by CQ except at its highest concentration (0.4 mM), in which case entry was reduced by 44%.

## DISCUSSION

This work shows that CQ induces a considerable modification of PL metabolism in *P. knowlesi*-infected erythrocytes. Glycerol and polar heads are not incorporated into normal erythrocyte glycerolipids and we have shown that the incorporation of FA and lysoPL into infected erythrocyte PL is 70–500-fold higher than in normal erythrocytes [15, 19]. Moreover, we never observed a significant reticulocyte response as reported in other similar systems [27, 28]. Consequently, the PL incorporation measured can be attributed without possible confusion to the infected erythrocytes.

At the same time, however, hypoxanthine and isoleucine incorporations into their respective macromolecules were also severely reduced, along

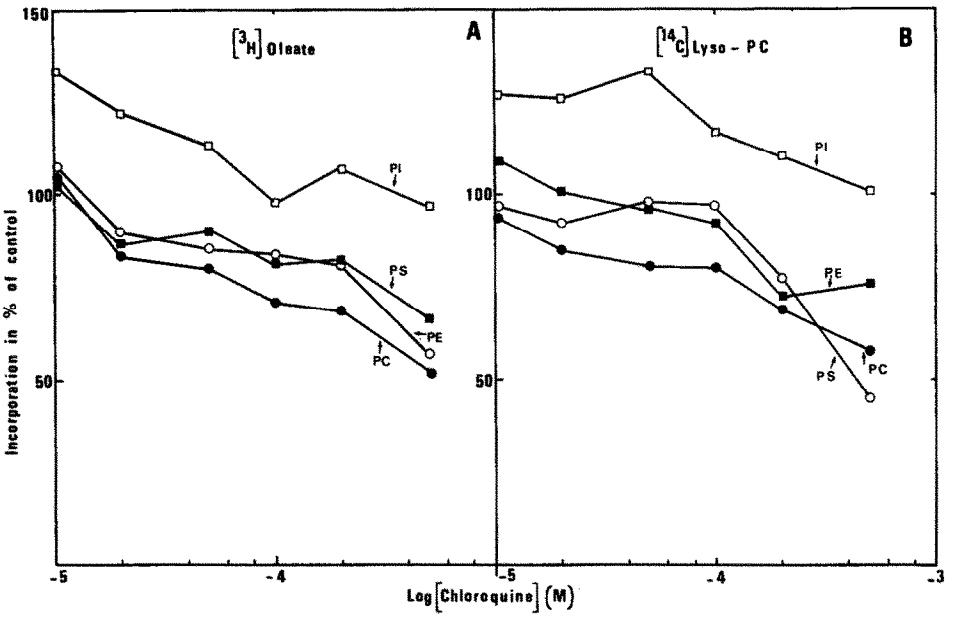


Fig. 3. Effect of increasing concentrations of chloroquine on the incorporation of ($^3$H)oleate and L-1-($^{14}$C)palmitoyl-lysoPC into the lipids of infected erythrocytes. Preincubation, incubation, and processing of the cells were carried out as described in the text and Fig. 1. $1.6 \times 10^9$ infected erythrocytes (10% ring parasitized) were incubated in the presence of: (A) 130 $\mu$M ($^3$H)oleate, 22 Ci/mol; (B) 8 $\mu$M ($^{14}$C)lysoPC, 57 Ci/mol. The incorporations are means of three experiments and are expressed as percentages of incorporation in the absence of CQ. In control cells, the incorporations (nmol/$10^{10}$ infected cells/90 min ± SE) into PC, PS, PI and PE were respectively: (A) 40 ± 1.2, 8.2 ± 0.4, 9.9 ± 0.3 and 75 ± 5; (B) 102 ± 3, 4.5 ± 0.2, 7.5 ± 0.8 and 58.5 ± 2.7. The standard errors of the mean for the data points ranged from 2 to 12% of the plotted mean values from three observations.

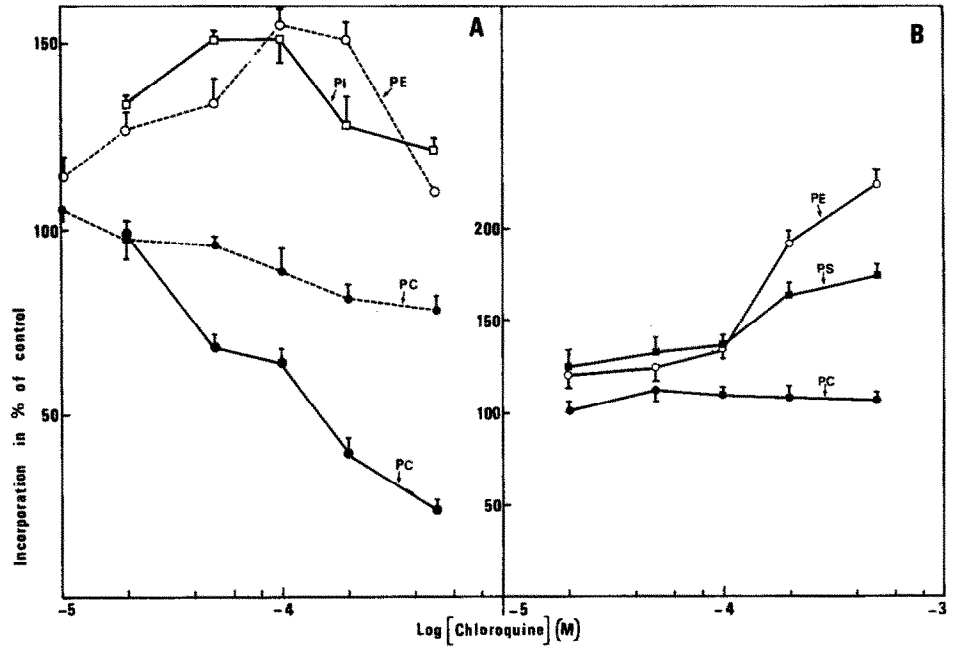


Fig. 4. Incorporation of labeled choline, inositol, ethanolamine, and serine into the phospholipids of *P. knowlesi*-infected erythrocytes as a function of choloroquine concentration. Incorporations were measured as in Fig. 1 except that the radioactive precursors were: (A) 20 $\mu$M ($^{14}$C)choline, 20 Ci/mol, and 10 $\mu$M ($^3$H)inositol, 183 Ci/mol or 20 $\mu$M ($^{14}$C)ethanolamine, 9 Ci/mol; (B) 20 $\mu$M ($^{14}$C)serine (83 Ci/mol). The experiments with ($^{14}$C)ethanolamine used $1.6 \times 10^9$ infected erythrocytes, 10% parasitized. Data are the means of three independent experiments and are expressed as percentages of the incorporations observed in the absence of CQ. Vertical bars correspond to 1 SE. In control cells, the incorporations (nmol/$10^{10}$ infected cells/90 min ± SE) were: (A) choline into PC, 17.0 ± 0.6 (●); inositol into PI, 0.24 ± 0.01 (□); ethanolamine into PE, (○——○) and PC (●——●): 100 ± 7 and 26.0 ± 1.7; (B) serine into PS (■), PE (○) and PC (●) 8.5 ± 0.6, 11.6 ± 0.5, 1.5 ± 0.2, respectively.

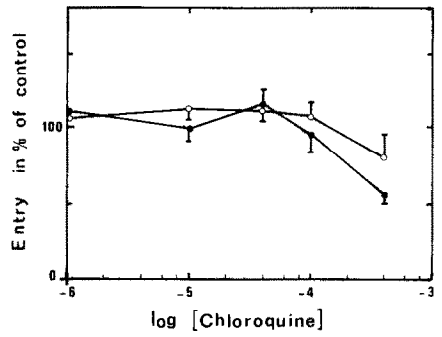


Fig. 5. Effect of chloroquine on labeled choline and ethanolamine entry into *P. knowlesi* infected erythrocytes. Entry of 5 μM ($^3$H) choline (●) or 5 μM ($^3$H) ethanolamine (○) into $3.5 \times 10^7$ *P. knowlesi* infected erythocytes (100% parasitemia) was measured for 7 min at 37°. Cells were first preincubated for 60 min at 37° in the presence of various CQ concentrations as described in Methods. Entry rates into untreated infected erythrocytes were 4.5 ± 0.5 nmol and 11.0 ± 0.7 nmol/$10^{10}$ infected cells/7 min for choline and ethanolamine, respectively. Entry is expressed as a percentage of that in control cells without contact with CQ. Data are means of the results of a representative experiment done in triplicate. Vertical bars corresponds to ± SE.

with the output of $^{14}CO_2$ from glucose metabolism. The first modifications of these metabolisms appeared at 10–20 μM. Although there are no data indicating how crucial each of these metabolisms is, the decrease in the metabolism of nucleic acid and protein, and in the metabolism of glucose via the pentose shunt pathways, under our experimental conditions, probably followed the lethal effect of CQ on the parasite [29].

On the other hand, PL synthesis either increased or decreased depending on the PL species. The results presented here show that CQ generally decreased lipid precursor incorporation into PC and increased it into PI, PS and PE. For instance, ($^3$H)glycerol incorporation increased selectively in PE but decreased in PC; the acceleration of labeled oleate, stearate and lysoPC incorporation was limited to PI but first decreased in PC. The incorporation of the labeled polar head groups, inositol, ethanolamine and serine into their respective PL was clearly stimulated in the presence of CQ, but choline incorporation decreased considerably. ($^3$H)glycerol as well as FA are precursors common to all glycerolipids. This is also true of the FA released from lysoPC [15]. Hence, an effect of CQ on the uptake of these precursors would be parallel in all glycerolipid classes. However, a modification of polar head incorporations, since each of them is specific for a particular PL, could reflect an effect of CQ on precursor uptake. We checked that CQ does not alter the entry of the cations choline and ethanolamine under our conditions, except at the highest CQ concentrations (Fig. 5). Thus, the modifications of PL precursor incorporations by CQ cannot be explained by a change in the entry of so various precursors into the infected cells. This is strengthened by the observed differential effect of CQ on glycerol and FA metabolism depending on the PL.

The decreased biosynthesis of PC observed with labeled glycerol, FA, lysoPC and choline could be the result of a generalized and nonspecific toxic effect of CQ on the infected cells, as already observed in the case of nucleic acid, protein and glucose metabolisms, or may be part of a specific alteration of PL metabolism by CQ in the infected erythrocytes. However, a generalized disturbance of parasite metabolism does not take into account the fact that PI, PS and PE metabolisms were stimulated. In this context, the most striking results concerning the effect of CQ were the increased biosynthesis of these three PL. These data demonstrate that CQ redirects PL metabolism, an action reminiscent of the cationic amphiphilic drugs, in that its net effect is to decrease the synthesis of PC and to increase that of PI and PS.

CQ and other amphiphilic drugs have in common two main biochemical effects which lead to the accumulation of PL, principally in lysosomes. They cause an impairment of PL degradation and a redirection of PL synthesis toward acidic PL, resulting from an inhibition of phosphatidate phosphohydrolase (EC 3.1.3.4). This second change involves a diversion of glycerolipid synthesis away from PL synthesized by the Kennedy pathway, toward CDP-diacylglycerol-derived PL [2, 4, 6, 30].

Thus, a stimulation of PI metabolism is compatible with an inhibition of phosphatidate phosphohydrolase. However, the present study also shows large differences between labeled precursor incorporation into PI. The drugs stimulated only myo-($^3$H)inositol, FA and lysoPC incorporation into PI but did not stimulate ($^3$H)glycerol incorporation. The incorporation of the latter precursor is a consequence only of the *de novo* synthesis of PI. Thus, the absence of a concomitant increase in the incorporation of ($^3$H) glycerol into PI could be seen as a renewal of the phosphorylinositol and FA moieties of PI, conserving the glycerol backbone of the molecule. The conservation of the glycerol backbone is supported by a recent study showing that cationic amphiphilic drugs stimulate the $Mn^{2+}$-dependent inositol exchange reaction [31]. However, other observations indicate that PI synthesis via a *de novo* pathway is also activated in the PI response, probably because the diacylglycerols generated by the breakdown of PI do not enter the metabolic cycle for resynthesizing PL but are used as FA donors [32, 33]. Apart from a specific PI response in infected erythrocytes (we have just demonstrated the presence of a PPI cycle in *Plasmodium*, Vial *et al.*, in preparation), the lack of a concomitant stimulation of glycerol entry into PI by CQ could also indicate that PI is synthesized via a glycero-3-phosphate pool which is not affected by CQ.

The second group of stimulated incorporations concerned PS and PE. The decrease in ($^3$H)glycerol entry into PS and of labeled FA and lysoPC into PS and PE occurred only at high CQ concentrations and were probably related to the considerable alteration of parasite viability by these drug concentrations (see Fig. 2). In this context, the increased PS labeling from ($^{14}$C)serine and increased PE labeling from

radioactive glycerol, ethanolamine and serine can only be more significant since they are present only at high CQ concentrations.

PC is synthesized via the CDP-choline pathway but can also be formed from PE via successive PE *N*-methylations. The parasite metabolizes ethanolamine into PE, but PS decarboxylation can provide a large proportion of the PE requirements of infected cells. PS is the most abundantly synthesized PL, but only low levels are present since it is only a precursor of PE and PC, the two most abundant PL in infected erythrocytes [15, 26]. The increase in PS labeling by ($^{14}$C)serine but not by glycerol recalls the effect of CQ on PI metabolism and could well reflect the redirection of PL synthesis by the amphiphilic cationic drugs at the main branchpoint in the pathway leading to the various glycerolipids. Since PS functions as a precursor of PE, PE labeling is logically increased.

Intriguing and more difficult to explain is the large increase in the entry of labeled glycerol and ethanolamine into PE, indicating a stimulated *de novo* synthesis. The redirection of glycerolipid synthesis by cationic amphiphilic drugs in fact involves a decreased synthesis of all PL originating from the Kennedy pathway by an action at the phosphatidate phosphohydrolase step [4]. This should include ($^{14}$C)ethanolamine-labeled PE. However Leli and Hauser [31] have recently suggested an inhibition of CTP:phosphocholine cytidyltransferase (2.7.7.15) by cationic amphiphilic drugs and we have recently demonstrated that ($^{14}$C)ethanolamine is incorporated into PE by a Kennedy pathway distinct from that of PC biosynthesis in infected erythrocytes [34, 35]. Hence, PC biosynthesis could be selectively decreased, and PE biosynthesis could escape this phenomenon either because it is localized in another compartment or due to specific enzymes [34, 35] not altered by CQ. The increased synthesis of PE could occur by a compensatory mode as it does when infected erythrocytes are deprived of choline (personal observations). Molecular mechanisms leading to this effect remain to be determined. This will require a better enzymatic characterization and localization of PL metabolism.

The action of the first antimalarial compound on PL metabolism in infected erythrocytes was not known even though the biosynthesis of these molecules is plethoric in infected erythrocytes and that the interactions between CQ and phospholipid metabolism are largely documented. The present work shows that CQ induces various modifications of PL metabolism in *P. knowlesi*-infected erythrocytes, which can be summarized as follows:

(1) A general disturbance of the whole parasite metabolism. The decrease in PC biosynthesis, which is concomitant with a decrease in nucleic acid, protein and glucose metabolism, could result from the toxic and generalized effect of CQ on the infected erythrocytes.

(2) An early effect, the increase in PI labeling from inositol, FA and lysoPC, but not from glycerol, could result from the redirection of glycerolipid metabolism caused in various tissue by CQ and other amphiphilic cationic drugs. The decrease in PC biosynthesis could also reflect this phenomenon.

(3) PE biosynthesis is clearly increased by the Kennedy pathway as well as by PS decarboxylation. This increase, which occurs at high CQ concentrations, when other metabolic pathways are severely affected could be due to a compensatory mode resulting from the severe blockage of PC biosynthesis.

CQ, which was discovered in 1934, is the leading antimalarial drug, but its mechanism of action remains obscure. At present, two conflicting theories are used to explain its mechanism of action: the lysosomotropic weak base hypothesis and the ferriprotoporphyrin IX complexation hypothesis [12, 36–38]. Nevertheless, as reported in the above mentioned reviews, neither hypothesis is sufficient to explain the available data. Ginsburg and Geary [38] argue that the well known CQ-phospholipid interaction could offer an intriguing explanation for CQ action and the phenomenon of reversible CQ-resistance.

CQ concentration of 0.4 $\mu$M is usually observed in human plasma [39]. It has clearly been demonstrated that around 0.1 $\mu$M of CQ is needed to produce 50% inhibition of *Plasmodium* growth but only over a period of 48 hr of drug action [12, 38, 39]. It is well known that the length of exposure to CQ, the number of parasites in the cultures, and frequent changes of medium influence the $IC_{50}$ of CQ, since the drug is quickly removed by the susceptible stages. Moreover, at 1 $\mu$M external CQ concentration, the most sensitive stages of *Plasmodium* (trophozoites and schizonts) only begin to show toxicity after 2 hr [38]. In our experiments, significant effects were observed from 10–20 $\mu$M, after 60 min of preincubation with CQ, i.e. 200 times its $IC_{50}$ (or 50 times its plasmatic level). But the duration of exposure was 48 times shorter than in experiments aimed at determining the $IC_{50}$. It should also be noted that CQ accumulates in *Plasmodium*-infected red cells, where the concentration of the drug can be as much as 1000 times higher than in plasma [7] leading in our experiments to a large drug depletion in the medium after cell incubation. A high hematocrit is a necessity when working with expensive radioactive precursors. It is reasonable to assume that the infected erythrocytes can concentrate CQ 250 times (i.e. a cell/medium ratio of 250 for CQ concentration) [7, 11]. In this context, incubation of cells at 20 $\mu$M, a concentration at which effects on PL are clearly observed, would correspond to a CQ concentration of 1.4 $\mu$M after 1 hr of incubation under the conditions of Fig. 1, i.e. only one order of magnitude above the $IC_{50}$ after 48 hr of incubation.

The present study shows that CQ directly interferes with the PL metabolism of infected cells, leading notably to an increased synthesis of acidic PL. A marked increase in anionic PL, especially PI, is a characteristic phenomenon of CQ treatment, resulting in altered surface charges of membranes and phospholipidosis [2, 40]. Due to the considerable biosynthesis of PL, which accumulate in the newly synthesized membranes of the malarial parasite [13, 15, 16], we cannot exclude the possibility that PL are involved in the CQ antimalarial action, notably by altering fundamental physiological cell functions such as membrane homeostasis.

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