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DIFFERENTIAL EFFECTS OF MEPACRINE, CHLOROQUINE AND HYDROXYCHLOROQUINE ON SUPEROXIDE ANION GENERATION, PHOSPHOLIPID METHYLATION AND ARACHIDONIC ACID RELEASE BY HUMAN BLOOD MONOCYTES

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Abstract—The 4-aminoquinolines chloroquine (CQ) and hydroxychloroquine (HCQ), and previously the 9-aminoacridine mepacrine (quinacrine) (MP), have been widely used in the treatment of inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus. Their effects are believed to be mediated through phagocytic cells but the precise biochemical basis remains uncertain. We have investigated the effects of these drugs on monocyte superoxide anion (SO) generation elicited by 5 different stimuli—opsonised zymosan (STZ), FMLP, A23187, TPA and fluoride—and sought correlations with effects on two processes which have been linked with monocyte activation, namely arachidonate (AA) release and transmethylation of phosphatidyl ethanolamine (PE) to phosphatidylcholine (PC). In all experiments conditions were adjusted to achieve leucocyte concentrations of drug comparable to those found during *in vivo* therapy. Neither CQ nor HCQ had any marked effect on SO release induced by TPA, A23187 or fluoride ion, excluding a significant effect on protein kinase C (PKC), calmodulin-dependent kinase(s) or the membrane-bound, superoxide generating NADP(H) oxidase. In contrast MP inhibited the response to TPA and A23187. Each drug also had different effects on surface receptor-dependent responses; thus HCQ inhibited FMLP- but not STZ-induced SO release, whereas CQ and MP inhibited the response to both stimuli. Each drug also displayed different effects on AA release and phospholipid (PL)-methylation; MP and HCQ, but not CQ, inhibited STZ-stimulated AA release while MP and CQ but not HCQ inhibited basal rates of PL-methylation in mononuclear cells (MNC). However, only MP inhibited PL-methylation in an enriched monocyte population.

We conclude that despite their close structural similarity, MP, CQ and HCQ each have different metabolic effects and their actions cannot simply be attributed to inhibition of lysosomal functions. Other possible mechanisms of action are discussed. The selective effects of each drug also provide further evidence for multiple pathways of monocyte activation.

CQ,§ HCQ and previously MP have been used widely in the treatment of rheumatoid arthritis and systemic lupus erythematosus, and have both anti-inflammatory and immunosuppressive actions [1, 2]. They have been shown to inhibit a variety of phagocyte functions such as Interleukin-1 release [2], chemotaxis and the secretion of lysosomal enzymes [1] but the biochemical basis remains uncertain. Many of these effects have been attributed to the lysosomotropic actions of antimalarials which result in inhibition of lysosomal functions including receptor recycling, lysosomal digestion and metabolism of lipids [1]. At high concentrations, which occur in lysosomes, these agents inhibit lysosomal enzymes such as phospholipase A_2 (PLA_2) [3]. Relatively little attention, however, has been paid to possible non-lysosomal actions of these agents in phagocytes. We have investigated this question further by studying the effects of the three structurally related compounds MP, CQ, HCQ on different pathways leading to monocyte superoxide generation and on processes linked with this function.

The precise molecular events which follow phagocyte membrane receptor binding by appropriate ligands and result in superoxide generation by a

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§ Abbreviations used: CQ, chloroquine; HCQ, hydroxychloroquine; MP, mepacrine; PE, PC, PS, PI, phosphatidyl ethanolamine, choline, serine and inositol respectively; Pi, phosphate; PA, phosphatidic acid; PL, phospholipid; DG, diacylglycerol; IP3, inositol trisphosphate; AA, arachidonic acid; PLA_2, phospholipase A_2; PLC, phospholipase C; CM, calmodulin; PKC, protein kinase C; MNC, mononuclear cells; Mo, monocytes; DBSS, Dulbeccos balanced salt solution; HBSS, Indicator free Hanks balanced salt solution; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; STZ, serum treated zymosan; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; DMSO, dimethyl sulphoxide; HPLC, high performance liquid chromatography.

plasma membrane bound NADPH-oxidase remain uncertain. Although there is evidence that receptor affinity for ligand, and linkage to subsequent events such as chemotaxis [4] and superoxide generation [5] may require phospholipid (PL)-methylation, recent studies suggest that phagocyte activation is accompanied by inhibition of PL-methylation [6] and its role in phagocyte activation remains uncertain. Receptor–ligand interaction results in hydrolysis of membrane phosphatidyl inositol bisphosphate to liberate diacylglycerol (DG) and inositol-1,4,5-trisphosphate (IP3) [7], transient Ca^{2+} fluxes [8], arachidonic acid (AA) release and activation of protein kinases [8, 9]. The exact linkage of these events to activation of the terminal NADPH oxidase remains unknown.

Activation of the NADPH oxidase can be effected both by particulate (e.g. STZ) or soluble (e.g. FMLP) ligands which bind to surface membrane receptors or by agents which act at more distal intracellular points in the pathway. For example, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and other synthetic analogues of DG directly activate protein kinase C (PKC) and superoxide generation, independently of PI turnover or Ca^{2+} fluxes [7, 10]. Ca^{2+} ionophore A23187 stimulates a rise in cytosolic Ca^{2+} which activates calmodulin (CM)-dependent kinases, but not PKC, and also results in generation of superoxide [8]. Fluoride also triggers superoxide release via a Ca^{2+} dependent mechanism [11].

In these experiments we have investigated further the mode of action of these drugs by seeking differential effects of MP, CQ and HCQ on (1) SO generation triggered by different stimuli, (2) basal rates of PL-methylation and (3) STZ-stimulated AA release.

MATERIALS AND METHODS

Preparation of cell suspensions. Venous blood was drawn from healthy human volunteers, anticoagulated with 4.5% EDTA in DBSS (pH 7.4) and mononuclear cells (MNC) separated on a Ficoll-Hypaque gradient [12]; the percentage of monocytes (Mo) was determined by size distribution on a Coulter counter (Model ZBI) [13]. Final cell suspensions were adjusted to between 0.3 and 0.8×10^6 monocytes/ml in indicator free HBSS. Where required, purified Mo (70–90% purity) were prepared on a Percoll gradient adjusted with saline to $d = 1.064$ using a modification of a previously described method [14].

Preparation of solutions of drugs. Stock solutions of CQ, HCQ and MP were made in DBSS and pH adjusted slowly to 7.4 with 0.1 M NaOH. Auranofin was dissolved in DMSO at 0.1 M and diluted to the required concentration in DBSS immediately prior to use. Piroxicam was dissolved in methanol at 0.1 M and diluted in DBSS immediately prior to use. The final concentration of DMSO or methanol did not exceed 0.1%; these concentrations had no effect on PL-methylation or SO release (not shown).

Preparation of stimuli. Zymosan (STZ) was opsonised in fresh human serum (2:1 w/v) at 37° for 30 min, washed twice, resuspended in HBSS to 20 mg/ml and stored frozen in aliquots. TPA (Sigma) was dissolved in DMSO (1 mg/ml), stored at −10° and diluted to 10 μg/ml in Ca^{2+}/Mg^{2+} free DBSS (pH 7.4) immediately prior to use. FMLP (Sigma) was dissolved in DMSO (2 mg/ml), stored at 4° and diluted with DBSS to 2 μg/ml immediately prior to use. Calcium ionophore A23187 (Sigma) was dissolved in methanol (2 mM) stored at −10° and diluted with DBSS to 100 μM immediately prior to use. The final concentrations of methanol or DMSO did not exceed 0.1% and had no effect on release or detection of SO. Sodium fluoride was dissolved in DBSS to 200 mM and pH adjusted to 7.4.

Measurement of SO production. SO production was measured by reduction of cytochrome *c* [15]. MNC or Mo (700 μl) were preincubated at 37° for specified times with each concentration of drug before addition of 100 μl cytochrome *c* (Sigma Type III) (100 μM final) and appropriate stimulus (100 μl). Cells were stimulated with FMLP (0.2 μg/ml) for 2 or 60 min or with STZ (2 mg/ml), TPA (1 μg/ml), A23187 (10 μM) or fluoride (20 mM) for 60 min. Each experiment was performed in quadruplicate on MNC from a minimum of 3 separate blood samples. Results of inhibitory studies are expressed as % of control value (no drug/inhibitor added). Cell viability (>95%) was confirmed by trypan blue exclusion. Control studies confirmed that contaminating lymphocytes did not affect the performance of the assay (not shown).

In additional control studies the effect of two other lipophilic antirheumatic drugs on STZ-stimulated SP release was examined. Briefly, MNC were preincubated with each drug for 15 min before stimulation with STZ.

Measurement of phospholipid methylation. To measure the effect of drugs on basal rates of methionine-dependent PL-methylation [3], 450 μl of Mo or MNC (2×10^6/ml) were preincubated at 37° with drugs at specified concentrations in quadruplicate for 60 min, then 10 μCi of L-[methyl-^{3}H]-methionine (New England Nuclear 80 Ci/mmol) added and a further 60 min incubation carried out. The cells were washed twice, resuspended in 2 ml of DBSS and phospholipids extracted with 5 ml of chloroform/methanol (2:1 v/v). Aliquots of the lipid extract were transferred to scintillation vials and evaporated before counting in 10 ml scintillation fluid (0.4% PPO; 70% toluene; 30% ethanol). Incorporation of ^{3}H-methyl from L-methionine into PC was confirmed by chromatography of phospholipids on a Waters HPLC (5 μ spherical silica column; isocratic mobile phase 100 ml acetonitrile/1.5 ml methanol/0.05 ml H_2SO_4); flow rate 1 ml/min; u.v. detection at 206 nm) [16].

In control studies, the effect of auranofin and piroxicam on PL-methylation was also measured. MNC were preincubated with each drug for 15 min before measurement of PL-methylation.

Measurement of release of radiolabelled AA-metabolites from ^{3}H-AA-labelled MNC. MNC (2×10^6/ml) in 10 ml of HBSS (+0.5% delipidated albumin) were labelled for 30 min with 5 μCi of AA-[5,6,8,9,11,12,14,15-^{3}H(N)] (New England Nuclear; specific activity 60 Ci/mmol) [3] then washed twice and resuspended in 10 ml of the same medium. Incorporation of ^{3}H-AA into PC was confirmed by HPLC

as described for methylation studies above. Aliquots (400 μl) were dispensed in duplicate into plastic tubes and incubated for 60 min with the specified doses of CQ or mepacrine. Cells were stimulated for 30 min with STZ (2 mg/ml) and then centrifuged rapidly. Two hundred millilitres of supernatant from each tube was transferred to scintillation vials containing 10 ml of scintillation fluid and radioactivity counted.

In vitro *accumulation of CQ and HCQ in mononuclear cells.* MNC (2×10^6/ml) in 1 ml of HBSS were preincubated in the presence of concentrations of CQ and HCQ up to 0.1 mM for periods up to 60 min at 37°. The cells were then centrifuged and washed twice with excess HBSS.

Extraction of CQ and HCQ from MNC. Two millilitres of water were added to the MNC pellet followed by 250 μl of 1M NaOH/0.6 M borate and 150 μl of internal standard and 5 ml of chloroform [17]. The internal standards for HCQ and CQ were CQ and HCQ respectively. The tubes were shaken for 10 mins, centrifuged for 10 min at 2000 rpm and the chloroform layer transferred to clean glass tubes and evaporated under N_2 at 37°. The residue was reconstituted in 100 μl of mobile phase (85% H_3PO_4 (0.015%)/15% CH_3CN).

HPLC measurement of CQ and HCQ. HPLC was performed on a Waters system (Waters Bondapak phenyl column; isocratic mobile phase, as above; flow rate 2 ml/min; u.v. detection at 330 nM) [17]. Standard curves were linear between 0.001 mM and 0.1 mM.

Effect of CQ, HCQ and MP on total phospholipid synthesis. To determine whether these drugs inhibit total PL synthesis, their effect on ^{32}P incorporation into total PL was examined. MNC (2×10^6/ml) were incubated for 60 min with 10 μC/ml of ^{32}P in phosphate free buffer at pH 7.4 in the presence or absence of each drug (0.1 mM). After incubation, PL were extracted with chloroform/methanol and ^{32}P incorporation into PL measured by scintillation counting as described above.

RESULTS

Effect of drugs on SO release

Preincubation of MNC for 60 min with 0.0001 to 0.1 mM CQ resulted in dose-dependent inhibition of STZ- or FMLP-stimulated SO production, but had no inhibitory effect on the response to TPA, or fluoride ion (Table 1a). Enhancement of the response to fluoride was observed which was maximal at 0.01 mM CQ and a similar less marked effect also occurred with TPA. Slight non-dose-dependent inhibition of the response to A23187 was seen. Analysis of this effect revealed that inhibition occurred only during the first 10 min of incubation; thereafter rates of SO release were unaffected by CQ. The effect of CQ on zymosan-stimulated SO release was time dependent (Table 1b). In contrast to CQ, HCQ had only minor effects on STZ-stimulated SO release but still caused marked inhibition of the response to FMLP. MP inhibited the response to STZ, FMLP, TPA and A23187 but not to fluoride (Table 1a).

Both auranofin and to a lesser extent piroxicam inhibited STZ-stimulated SO release (Table 1a).

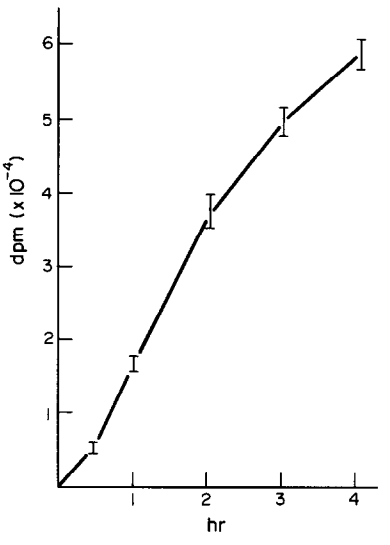

Fig. 1. Time course of methionine-dependent incorporation of ^3H-methyl into phospholipid in mononuclear cells Fig. 2.

Effect of drugs on phospholipid methylation

^3H-methyl incorporation into PL in unstimulated MNC was approximately linear for up to 4 hr (Fig. 1); radiolabel was incorporated into PC but not PS or PI. Preincubation of MNC with CQ or MP for 60 min caused inhibition of PL-methylation while HQ had little effect (Table 2). However, the actions of CQ and MP on an enriched Mo population were different and, although MP again caused inhibition, the effect of CQ was much less marked and non-dose-dependent (Table 2). In view of this latter result the action of CQ on STZ-induced SO production was reexamined using enriched Mo; CQ caused identical inhibition of SO release to that found in mixed MNC preparations (data not shown). It should also be noted that mean rates of PL-methylation in enriched Mo preparations were approximately double the rates of methylation in MNC.

Control studies showed that inhibition of PL-methylation was not due to non-specific global inhibition of PL synthesis. Thus, total ^{32}P-incorporation into PL in the presence of 0.1 mM CQ, HCQ and MP were 128 (±1.0)%, 123 (±7.7)% and 145 (±9)% of control (no drug added) respectively.

The two other lipophilic drugs we have examined, auranofin and piroxicam, had no significant effect on PL-methylation at non-toxic doses. Piroxicam caused 15% inhibition at 0.1 mM while auranofin produced 9% inhibition at 0.01 mM. At concentrations of auranofin >0.01 mM there was significant toxicity and loss of cell viability (>50%) and effects on PL-methylation above 0.01 mM are therefore non-specific.

Effect of drugs on release of ^3H-radiolabel from ^3H-AA labelled MNC

It has been suggested that the basis for some of the cellular effects of CQ, HCQ and MP is their ability to inhibit PLA_2-dependent release of AA. Indeed MP has been widely used as a "specific"

Table 1. Effect of drugs on monocyte SO generation
(a) Effect of drugs on SO response to different stimuli

Stimulus	Control—no drug added nmol red. cyt C/ 10^6 monocytes/ min Mean (+SEM)	SO generation % (± SEM) of control 0.0001 mM	0.001 mM	0.01 mM	0.1 mM	N
			after 60 min preincubation with CQ			
STZ	0.79 (0.5)	91 (5)	87 (3)	80 (5)	54 (5)	4
FMLP (2′)	1.8 (0.2)	—	100 (6)	109 (20)	41 (4)	3
FMLP (60′)	0.5 (0.03)	—	111 (2)	86 (6)	37 (3)	3
Fluoride	0.46 (0.04)	102 (10)	109 (12)	120 (4)	108 (8)	4
TPA	0.63 (0.2)	—	100 (4)	110 (2)	104 (2)	3
A23187	0.95 (0.2)	—	89 (5)	91 (3)	85 (2)	3
			after 60 min preincubation with mepacrine			
STZ	0.96 (0.2)	100 (14)	102 (18)	96 (16)	26 (12)	3
FMLP (2′)	2.1 (0.5)	—	109 (13)	95 (7)	31 (4)	3
FMLP (60′)	0.59 (0.1)	—	99 (7)	67 (9)	18 (5)	3
Fluoride	0.39 (0.06)	99 (9)	93 (5)	110 (9)	91 (8)	5
TPA	1.6 (0.5)	—	94 (3)	74 (8)	31 (14)	3
A23187	0.8 (0.3)	—	92 (6)	67 (5)	32 (10)	3
			after 60 min preincubation with HCQ			
STZ	0.9 (0.2)	—	109 (3)	100 (3)	86 (4)	3
FMLP (2′)	2.3 (0.6)	—	104 (10)	64 (9)	23 (4)	3
FMLP (60′)	0.66 (0.12)	—	97 (11)	83 (11)	46 (5)	3
Fluoride	0.4 (0.12)	—	102 (5)	103 (1)	78 (2)	3
TPA	1.1 (0.3)	—	98 (0.4)	98 (4)	98 (10)	3
A23187	0.9 (0.3)	—	98 (3)	98 (4)	93 (5)	3
			after 15 min preincubation with (i) auranofin (ii) piroxicam			
(i) STZ	0.8 (0.3)	109 (1)	102 (4)	39 (5)	—	3
(ii) STZ	1.2 (0.6)	—	91 (3)	77 (6)	59 (6)	4

(b) Time dependence of inhibitory effect of CQ on STZ-stimulated SO release

Preincubation (min)	Concentration of CQ 0.001 mM	0.01 mM	0.1 mM
0	100	100	100
30	95 (3)	90 (4)	72 (3)
60	87 (2)	80 (4)	55 (4)

inhibitor of PLA_2 during studies of phagocyte function [15, 16] and CQ has been shown to inhibit lysosomal PLA_2 [17]. Our data cast doubt on this "specificity" and led us to reexamine the effect of these agents on release of phospholipid-derived AA from STZ-stimulated monocytes. MNC were labelled with ^{3}H-AA and then the effect of each drug on STZ-stimulated ^{3}H-radiolabelled AA-metabolite release was measured. The results with each drug were clearly different: while MP, and to a lesser extent HCQ, caused inhibition of release of radiolabel, CQ had no effect (Table 3).

Table 2. Effects of drugs on phospholipid methylation in (a) mononuclear cells (b) purified monocytes

Drug	Methyl-group incorporation Mean (+SEM) fmoles methyl per 10^6 MNC per 60 min	Mean % (±SEM) of control after preincubation with drug 0.001 mM	0.01 mM	0.1 mM	N
(a) MP	84 (9)	99 (8)	87 (9)	21 (5)	4
CQ	91 (2)	82 (4)	70 (9)	46 (7)	4
HCQ	97 (6)	95 (2)	106 (3)	90 (5)	4
Auranofin	113 (7)	93 (10)	91 (6)	—	3
Piroxicam	118 (4)	86 (5)	100 (5)	86 (3)	6
(b) CQ	170 (30)	86 (8)	90 (5)	84 (7)	3
MP	160 (25)	106 (28)	87 (19)	32 (6)	3

Table 3. Effect of CQ, HCQ and MP on STZ-stimulated release of ^{3}H-AA metabolites from ^{3}H-AA labelled monocytes

Inhibitor	N	Inhibition of ^{3}H-AA release, CQ, HCQ or MP % (±SEM) of STZ-stimulated control) 0.001	0.01	0.1 mM CQ or MP
CQ	4	96 (3)	95 (8)	100 (9)
MP	4	85 (3)	82 (8)	48 (6)
HCQ	4	111 (9)	93 (4)	54 (7)

Table 4. *In vitro* accumulation of CQ and HCQ in mononuclear cells

Concentration of drug (mM)	Mean (± SD) nmoles/10^6 MNC CQ	HCQ
0.001	0.09 (0.02)	0.05 (0.02)
0.01	0.34 (0.4)	0.65 (0.1)
0.1	1.39 (0.09)	1.89 (0.2)

MNC were incubated with the indicated concentrations of CQ or HCQ for 60 min at 37°, prior to extraction of drug as described ($n = 3$).

Concentration of CQ and HCQ in leucocytes

To determine the pharmacological relevance of the concentrations of drugs we have used *in vitro* HCQ and CQ levels were measured in MNC after incubation with HCQ or CQ *in vitro* and compared with levels of HCQ in MNC from four patients receiving regular therapy (400 mg/day). The mean level of HCQ in MNC from these four patients was 1.57 nmoles HCQ/10^6 cells (range 0.42–3.85). Leucocyte accumulation of HCQ or CQ *in vitro* was time and dose dependent. Achievement of levels comparable to those found in patients required incubation of MNC (1 ml of 2×10^6/ml) for 60 min with up to 0.1 mM CQ or HCQ (Table 4). The *in vitro* concentrations of CQ and HCQ used in our experiments therefore paralleled closely those found *in vivo*.

Effect of drugs on leucocyte viability

Leucocyte viability (>95%) was unaffected by incubation of MNC with up to 0.1 mM CQ, HCQ or MP for up to 2 hr at 37° as assessed by trypan blue exclusion.

DISCUSSION

The precise metabolic pathways which lead to SO generation by stimulated phagocytes remain uncertain but a number of events have been implicated. These include PLC-dependent hydrolysis of membrane PI-bisphosphate to liberate DG and IP3 [7], transient calcium fluxes [8], AA release from PL and phosphorylation of proteins [8, 9]. A number of studies on various cell types have suggested that PL-methylation may be linked to transduction of plasma membrane receptor mediated signals [18, 19, 20]. For example in adipocytes PL methyltransferase activity is rapidly stimulated by ACTH but inhibited by insulin [19]. Similarly in hepatocytes, glucagon activates the PL-methyltransferase and the effect can be mimicked by cAMP or fluoride [20]. Several studies have reported that methylation inhibitors selectively block surface receptor dependent functions of phagocytes including chemotaxis and SO release [4, 5]. In contrast, macrophage phagocytosis does not have a requirement for methylation [21]. Other studies have also argued against a role for PL-methylation during signal transduction on the basis that phagocyte activation is accompanied by sulphoxidation of methionine and a resultant inhibition of PL-methylation [6]. It is possible that this represents a regulatory mechanism analogous to sulphoxidation of the chemotactic stimulus FMLP during phagocyte activation [22] and provides a means of limiting the respiratory burst. This hypothesis would reconcile the apparently conflicting views on the role of methylation during the respiratory burst.

In our experiments we first examined the effect of CQ, HCQ and MP on SO generation by mononuclear phagocytes stimulated by different agents. Neither CQ nor HCQ had any marked effect on SO release induced by TPA, A23187 or fluoride ion, excluding a significant effect on PKC, calmodulin-dependent kinase(s) or the membrane bound SO-generating NADP(H)-oxidase. The weak effect of CQ on the response to A23187 may reflect a minor inhibitory action either on calmodulin or on calcium fluxes. In contrast MP inhibited the response to TPA and A23187 but not to fluoride suggesting it acts on PKC and calmodulin-dependent events but not the terminal NADP(H) oxidase. Other studies have also shown that MP is capable of inhibiting calmodulin [23]. The lack of effect of MP on the response to fluoride suggests that fluoride activates a Ca^{2+} dependent pathway [11] independent of calmodulin or PKC.

CQ inhibited SO release in response to STZ and FMLP (i.e. surface membrane receptor dependent stimuli) suggesting either that surface receptors or metabolic events linked to these receptors might be affected by CQ. In contrast, HCQ caused marked inhibition of FMLP-induced SO release but had only a minor effect on the response to STZ. Possible mechanisms include direct inhibition of PLC or inhibition of the linkage between activated receptors and PLC. The different effects of CQ and HCQ suggest that sequestration of receptors within lysosomes, while possibly being a contributory factor, cannot be the sole explanation for the action of these drugs. MP also inhibited FMLP- and STZ-mediated SO release; although this may be due to its action on calmodulin or PKC, another action on receptor function, similar to the effect of CQ and HCQ, cannot be excluded.

In view of the suggested links between PL-methylation and membrane receptor-dependent functions we explored the effect of CQ, HCQ and MP on this metabolic pathway. Initial control studies of the effect of up to 100 μM CQ, HCQ or MP on phospholipid synthesis as measured by ^{32}P incorporation into total phospholipid showed some enhancement of net synthesis rather than an inhibitory effect; thus inhibitory effects on methylation discussed below are not attributable to a global non-specific inhibition of PL synthesis by these drugs.

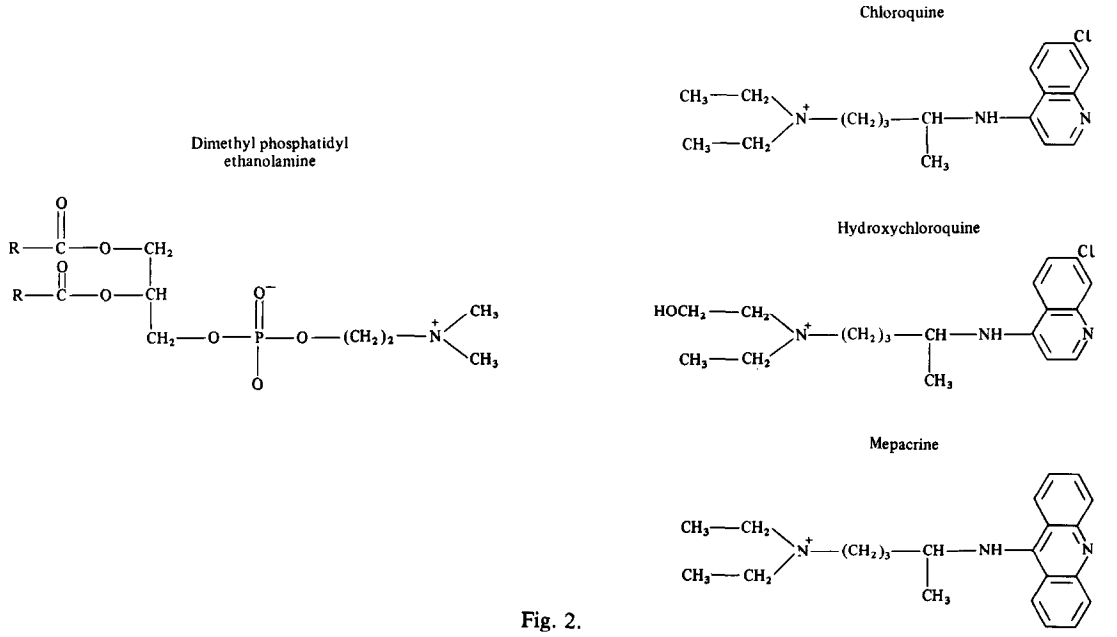

Fig. 2.

Both CQ and MP, but not HCQ, inhibited basal rates of PL-methylation in MNC. The differential effects of HCQ and CQ were surprising in view of their close structural similarity. However, comparison of the *N*-terminal moiety of each drug with the *N*-terminal head-group of intermediates of the PL-methylation pathways reveals structural homologies which may explain these differences. For example, MP and CQ resemble dimethyl-PE (Fig. 2) and therefore might act as PL-analogue inhibitors and compete for the active binding site of the methyltransferase. The failure of HCQ to inhibit PL-methylation could be attributable to the —OH substitution which might cause steric hindrance and reduce interaction of the *N*-terminal portion with the enzyme binding site. This hypothesis is being investigated by examining the effect of these drugs on methylation of each of the PL intermediates in this pathway.

The striking parallel between the dose response curves for the effect of each drug on STZ-induced SO release and inhibition of PL-methylation in MNC suggested a causal link. However, it was not clear from these studies on mixed MNC whether inhibition of PLM was occurring only in Ly or in Mo or equally in both cell populations. The effect of CQ and MP on methylation in an enriched Mo population was therefore examined. MP inhibited PL-methylation less strongly than before and CQ had no effect at all up to 0.1 mM. However the effect of CQ on STZ-induced SO release was the same on purified Mo, as on Mo in a mixed MNC population. Thus inhibition of PL-methylation cannot be the basis of inhibition of SO release by CQ. The greater sensitivity of MNC (predominantly Ly) than Mo to CQ-mediated inhibition of methylation remains unexplained but may reflect the greater ability of phagocytes to sequester CQ within lysosomes.

Since MP, CQ and HCQ have a hydrophobic aromatic nucleus the question arises as to whether this part of the molecule plays a role in the inhibitory actions of these drugs on monocyte function and PL-metabolism. To address this question we examined the effect of two non-amphipathic, hydrophobic drugs—auranofin and piroxicam—on monocyte function. Neither drug has a basic amine side chain, auranofin being neutral while piroxicam is acidic. Both of these drugs inhibited monocyte SO release in response to stimulation by STZ in a dose-dependent fashion but neither drug had a significant effect on PL-methylation at non-toxic doses. This suggests that the hydrophobic properties of CQ and MP may play a role in inhibition of STZ-stimulated SO release, but that hydrophobicity *per se* is not responsible for the actions of CQ and MP on PL-methylation.

To determine whether the effects of MP, CQ and HCQ are shared by other aromatic amines we have performed preliminary studies on the effect of trifluoperazine on monocyte function. This is a markedly hydrophobic drug with inhibitory actions on PKC, calmodulin and PL metabolism [24, 25]. Its hydrophobic properties are reflected by its distribution in lipid rich tissues and it has major clinical actions on the central nervous system. Phenothiazines and other aromatic amines are known to cause immunological abnormalities [26] and, after prolonged use, some cause phospholipidosis [25] similar to that caused by CQ and MP. Our preliminary results show that trifluoperazine inhibits STZ-induced SO release (ID_{50} 17 μM), phorbol ester-stimulated SO release (ID_{50} 5 μM) and PL-methylation (ID_{50} 23 μM). Thus although there is close correspondence between effects on PL-methylation and STZ-induced SO release, in contrast to CQ, HCQ and MP, the drug had greater effects on phorbol ester-stimulated SO release. This may reflect its greater hydrophobicity. These data suggest that other aromatic amines may share some of the properties of CQ, HCQ and MP and suggests that other drugs with comparable structures may have potentially useful suppressive actions in inflammatory

cells. To date the antiinflammatory effects of such compounds have not been seriously investigated because their lipophilicity and consequent major CNS actions preclude this application. None the less, the ability of aromatic and other amines to cause immunological disturbance suggests that such drugs have significant clinical actions on inflammatory and immune cells. However, in the absence of information concerning levels of phenothiazines in leucocytes *in vitro* or in leucocytes of patients receiving these drugs, it is difficult to relate our preliminary *in vitro* findings to *in vivo* effects.

Previous studies have suggested that CQ and MP inhibit cell activation by inhibiting PLA_2-dependent AA release from PC and that PC synthesised via methylation of PE is a principal source of this AA [18]. Thus inhibition of methylation in some cell systems results in inhibition of stimulated AA release [18]. We found that while MP, and to a lesser extent HCQ, inhibited STZ-induced release of radiolabel from ^{3}H-AA labelled cells, CQ did not. Also the inhibitory effects of MP and HCQ on AA release did not parallel their effects on methylation or SO release. Thus although we cannot exclude the possibility that inhibition of PLA_2 contributes to the effect of MP and HCQ, it cannot explain the action of CQ on surface receptor dependent SO release. Additionally, these data show that CQ inhibition of SO release is not simply due to loss of cell responsiveness to STZ which might be expected if surface receptors had been depleted. As we have not quantified surface receptors for mannose or complement (C3b/C3bi) [27, 28] we cannot entirely exclude this possibility.

In conclusion these data show that three structurally similar drugs CQ, HCQ and MP, have different and relatively selective effects on pathways of monocyte activation which cannot be attributed simply to lysosomotropism. Thus although all three are lysosomotropic, each drug shows different effects on PL metabolism and on different pathways leading to SO release. Comparisons with other hydrophobic drugs suggests that inhibition of surface-receptor-stimulated or phorbol ester-stimulated responses reflect the relative hydrophobicities of HCQ, CQ and MP. Actions on PL-methylation appear to reflect both hydrophobicity and the presence and structure of the amine side chain. These experiments therefore suggest that the selective effects of HCQ, CQ and MP on different aspects of monocyte function and metabolism are largely determined by their different physicochemical properties.

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