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INHIBITION OF HUMAN NEUTROPHIL PROTEIN KINASE C ACTIVITY BY THE ANTIMALARIAL DRUG MEFLOQUINE

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Abstract—Mefloquine (α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol), an antimalarial drug, has been shown to inhibit human neutrophil functions, particularly oxygen-dependent bactericidal activity. Since calcium- and phospholipid-dependent protein kinase C (PKC) has a central role in the regulation of this function, we hypothesized that its activity might be altered by mefloquine. We found that mefloquine directly inhibited PKC in a dose-dependent manner, with an IC_{50} of 45 μM. This inhibition appeared to be non-competitive with respect to ATP, histone and phosphatidylserine. In addition, mefloquine inhibited the binding of [^{3}H]phorbol 12,13 dibutyrate to PKC, indicating that it interacts with the regulatory domain of PKC. By contrast, mefloquine had little or no effect on neutrophil cAMP-dependent protein kinase or its catalytic subunit. Phorbol myristate acetate-induced protein phosphorylation in intact neutrophils was also inhibited by preincubation with mefloquine at concentrations similar to those inhibiting superoxide anion production. These data suggest that inhibition of neutrophil functions by mefloquine may be due to the inhibition of cellular PKC and that mefloquine could have further biological effects in situations in which PKC is involved.

Calcium- and phospholipid-dependent protein kinase C (PKC†) plays a crucial role in cell proliferation and differentiation, as well as in signal transduction [1]. Studies on the biological functions of PKC have been stimulated by the discovery that tumor-promoting phorbol esters activate the enzyme [2].

Neutrophils possess a superoxide-producing enzyme system (NADPH oxidase) which is involved in the killing of microorganisms [3]. Binding of opsonized microorganisms to the phagocyte plasma membrane results in the activation of this enzymatic activity which is reflected by an oxidative burst associated with a rapid increase in oxygen consumption and production of superoxide anion and hydrogen peroxide. Many data suggest a role for protein phosphorylation and, in particular, the involvement of PKC in controlling this oxidative burst. Indeed, PKC is the intracellular receptor for PMA [2]; PMA activates both the respiratory burst and PKC [2, 4, 5]; activation of neutrophils by several agents including phorbol esters, results in the phosphorylation of PKC-dependent proteins that are involved in superoxide anion production, in particular a 47 kDa cytosolic protein [6, 7]; and PKC inhibitors have been reported to inhibit superoxide anion production and protein phosphorylation [8, 9]. Inhibitors of PKC have been very useful for studying its role in the regulation of various cell functions and might have potential as therapeutic tools. In this regard mefloquine, a 4-quinoline-methanol antimalarial drug widely used in the treatment and prophylaxis of *Plasmodium falciparum* malaria, has been reported to inhibit strongly some neutrophil functions, including luminol-dependent chemiluminescence and iodination [10, 11]. We demonstrate here that mefloquine inhibits not only superoxide anion production induced by PMA and PDBu but also the activity of PKC from the neutrophil cytosol and the phosphorylation of proteins in stimulated neutrophils. Mefloquine may prove to be a useful tool for cell function analysis.

MATERIALS AND METHODS

Materials. The following reagents were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.): PMA, PDBu, cytochrome *c*, PS, histone III-S, H-7 and bovine heart PKA. [γ-^{32}P]ATP (30–40 Ci/mmol), ^{32}P-labeled phosphoric acid (carrier-free, 8500–9120 Ci/mmol) and [^{3}H]PDBu (13.2 Ci/mmol) were from Du Pont New England Nuclear (Boston, MA, U.S.A.). DEAE-cellulose (DE-52) was from Whatman. Mefloquine was a gift from Laboratoire Produits Roche (Paris, France). Mefloquine was dissolved in distilled water at 1 mg/mL and diluted further in Hanks buffer for cell assays and distilled water for PKC assays.

Isolation of neutrophils. Human neutrophils were separated from the venous blood of healthy volunteers by 2% Dextran sedimentation followed by centrifugation on Ficoll-Paque (Eurobio, Paris, France) and subsequent lysis of erythrocytes. Neutrophils were then washed and resuspended in

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† Abbreviations: mefloquine, α-(2-piperidyl)-2,8-bis-(trifluoromethyl)-4-quinolinemethanol; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PMA phorbol myristate acetate; PDBu, phorbol 12,13 dibutyrate; PS, phosphatidylserine; EGTA, ethyleneglycolbis-(aminoethylether)tetraacetate; TCA, trichoroacetic acid; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; DMSO, dimethyl sulfoxide.

Hanks buffer. The purity of neutrophil preparations was greater than 96%.

Superoxide anion production. This was measured in terms of superoxide dismutase-inhibitable reduction of cytochrome *c* [12] using a Uvikon 860 spectrophotometer. Aliquots of 5×10^5 cells were preincubated in the absence (control) or presence of mefloquine at various concentrations for 5 min at 37° before stimulation with PMA (100 ng/mL) or PDBu (250 ng/mL).

Preparation and assay of PKC. Neutrophils were resuspended at 1×10^7 cells/mL in lysis buffer (20 mM Tris–HCl pH 7.5, 0.25 M sucrose, 2 mM EDTA, 5 mM EGTA, 0.01% leupeptin, 2 mM phenylmethylsulfonyl fluoride and 50 mM 2-mercaptoethanol), sonicated for 4×10 sec at 4° and then centrifuged at 100,000 *g* for 1 hr at 4° in a TL100 Beckman ultracentrifuge. The supernatant is referred to as the cytosolic fraction. PKC was then partially purified from the cytosol by means of DE-52 column chromatography [13, 14]. PKC activity was assayed in a reaction mixture (0.1 mL) containing 20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 20 μg of histone III-S, 4 μg PS, 0.9 μg diolein, 0.5 mM $CaCl_2$, 10 μM ATP (containing 0.5 μCi of [γ-^{32}P]ATP) and 2 μg of partially purified enzyme, in the absence (control) or presence of the indicated concentrations of mefloquine. The reaction mixture was incubated for 10 min at 30°. The reaction was stopped by the addition of 400 μL of ice-cold 20% TCA and 100 μL of bovine serum albumin (2.5 mg/mL) as carrier. Tubes were centifuged for 10 min at 10,000 *g* and the supernatant discarded; the pellet was dissolved in 0.5 N NaOH. Ice-cold TCA was added and the mixture was again centrifuged. The pellet was dissolved in 0.5 N NaOH and 4 mL of dimilume was added as scintillation fluid. PKC activity was corrected for non-specific activity assayed in the absence of PS/diolein and calcium.

Binding assay. The binding of [^{3}H]PDBu to PKC was determined in a reaction mixture (0.25 mL) containing 0.05 M Tris–HCl pH 7.4, 0.5 mM $CaCl_2$, 100 μg/mL PS, 25 μg/mL protein or 5 μg of purified PKC, mefloquine, and 20 nM [^{3}H]PDBu (1–3×10^4 cpm/pmol). Non-specific binding was defined as the portion of total binding not displaceable by a 1000-fold excess of unlabeled PDBu. After 30 min at 30°, PKC–PDBu were collected on polyethyleneimine-treated Whatman GF/C glass-fiber filter and washed with 3×3 mL of 0.5% ice-cold DMSO according to Tanaka *et al.* [15].

PKA activity. PKA (from neutrophils) and its catalytic subunit (from bovine heart) were assayed under the same conditions as PKC except that 0.5 mM EGTA and 2 μM cAMP were added instead of PS/diolein and calcium in the presence of 0.5 mg/mL of histone III-S.

Protein phosphorylation in intact cells. The experiments were carried out using a modification of previously described methods [16]. Neutrophils were washed and resuspended in the loading buffer (20 mM HEPES/Tris pH 7.4 containing 0.15 M NaCl, 5 mM KCl and 10 mM glucose) in the presence of ^{32}P-labeled phosphoric acid (0.5 mCi/50×10^6 cells/mL). The suspension was incubated for 1 hr at 37° with gentle agitation every 15 min.

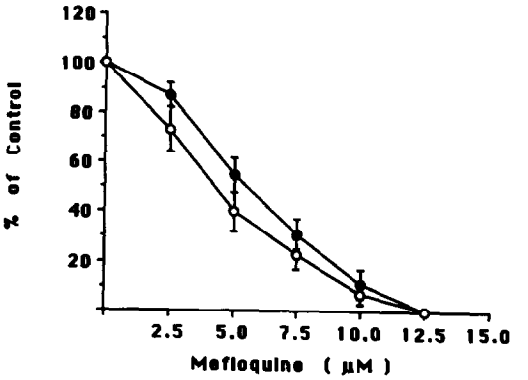

Fig. 1. Effect of mefloquine on superoxide anion production by neutrophils stimulated by 100 ng/mL of PMA (●) or 250 ng/mL of PDBu (○). Neutrophils were preincubated in the absence (control) or presence of mefloquine at the indicated concentrations for 5 min at 37°, and then stimulated with PMA or PDBu in the presence of 80 μM of cytochrome *c*, and reduction was recorded at 550 nm. The amount of superoxide anion generated is expressed as percentage of control values. Values represent the means ± SD of three to five experiments.

The cells were then washed twice in incubation buffer supplemented with 1 mM $MgCl_2$ and 0.25 mM $CaCl_2$ and resuspended at 10^7 cells/mL. For the assay 2×10^6 cells were preincubated with mefloquine for 5 min at 37° and stimulated with PMA (100 ng/mL) for 5 min at 37°. H-7 (100 μM) was used as a control for the inhibition of protein phosphorylation in intact neutrophils [8]. The reaction was stopped by addition of lysis buffer and neutrophils were sonicated as described above. Proteins were precipitated with TCA at 10%, washed in water and solubilized in SDS-buffer, boiled for 3 min, and subjected to 13% SDS–PAGE [17]. Gels were stained with Coomassie blue, destained, dried and autoradiographed for 48–72 hr with X-R or X-OMAT Kodak films.

Statistics. Data were analysed using Student's *t*-test for paired data; results are expressed as means ± SD on N experiments.

RESULTS

Effect of mefloquine on neutrophil superoxide anion production

The results for the effect of mefloquine on the neutrophil respiratory burst induced by PMA and PDBu are shown in Fig. 1. After a 5-min incubation at 37° the inhibitory effect of mefloquine was dose-dependent with IC_{50} values of 5.5 and 4.4 μM, respectively. Superoxide anion production in controls was 4.02 ± 0.32 nmol O_2/min/10^6 cells (N = 5) for PMA (100 ng/mL) stimulation and 2.8 ± 0.26 nmol O_2/min/10^6 cells (N = 5) for PDBu (250 ng/mL) stimulation. This inhibition was not due to an effect on cell viability, since lactate dehydrogenase activity and Trypan blue exclusion remained at 94–97% of control values at the concentrations tested.

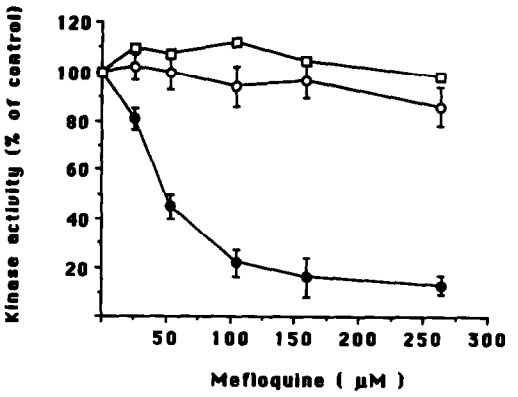

Fig. 2. Effect of mefloquine on neutrophil PKC (●), PKA (□) and its catalytic subunit activity (○). Assays were performed in the presence of 10 μM ATP, 0.2 mg/mL histone III-S, 0.5 mM $CaCl_2$, 40 μg/mL PS and 9 μg/mL diolein for PKC, or in the presence of 10 μM ATP, 0.5 mg/mL histone III-S, 0.5 mM EGTA and 2 μM cAMP for the activity of PKA and its catalytic subunit. Results are expressed as (protein kinase activity in the presence of mefloquine/protein kinase activity in the absence of mefloquine) × 100. Values are means ± SD of three to five experiments.

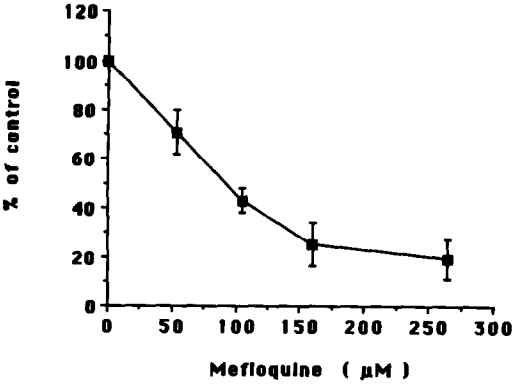

Fig. 4. Effect of mefloquine on [^{3}H]PDBu binding to PKC. After incubation of the reaction mixture for 30 min at 30°, the reaction was stopped by adding 3 mL of ice-cold 0.5% DMSO. This mixture was collected on a polyethyleneimine-treated GF/C glassfiber filter and washed with 0.5% DMSO; the radioactivity on the filter was then determined. Values are means ± SD of three experiments.

PKC inhibition by mefloquine

PKC was partially purified from neutrophil cytosol. Activity was assayed in the presence of sonicated PS/diolein, 10 μM ATP and histone III-S (0.2 mg/mL) as phosphate acceptor. Under these assay conditions mefloquine was found to inhibit PKC (Fig. 2) in a dose-dependent manner with an IC_{50} of 45 μM. Basal protein kinase activity (in the absence of calcium and PS/diolein) was not affected by the drug.

Mechanism of PKC inhibition by mefloquine

Inhibitors of PKC could interact with the substrate binding sites (protein or ATP) or with the regulatory site where activation occurs. Kinetic studies with Lineweaver–Burk analysis showed that the inhibition of PKC activity by mefloquine was non-competitive with respect to ATP (Fig. 3A), histone (Fig. 3B) and PS (Fig. 3C). Indeed inhibition was not overcome by high concentrations of these cofactors. The non-competitive character of the plots for ATP, histone and PS implies that mefloquine does not interact directly with the corresponding sites on PKC.

Effect of mefloquine on phorbol ester binding to PKC

Phorbol esters such as PDBu and PMA are known to activate PKC by binding directly to its regulatory domain. To further analyse the mechanism by which mefloquine inhibits PKC activity, we studied PDBu binding to the enzyme (Fig. 4). Mefloquine inhibited [^{3}H]PDBu binding to PKC with an IC_{50} of 90 μM when assayed with neutrophil cytosol, indicating that it may interact with the phorbol ester binding site. We verified that mefloquine at 90 μM inhibits PDBu binding to purified PKC (41.2 ± 6.2% of control in three experiments). This figure is similar to the IC_{50}, indicating that the binding of PDBu to proteins other than PKC is negligible.

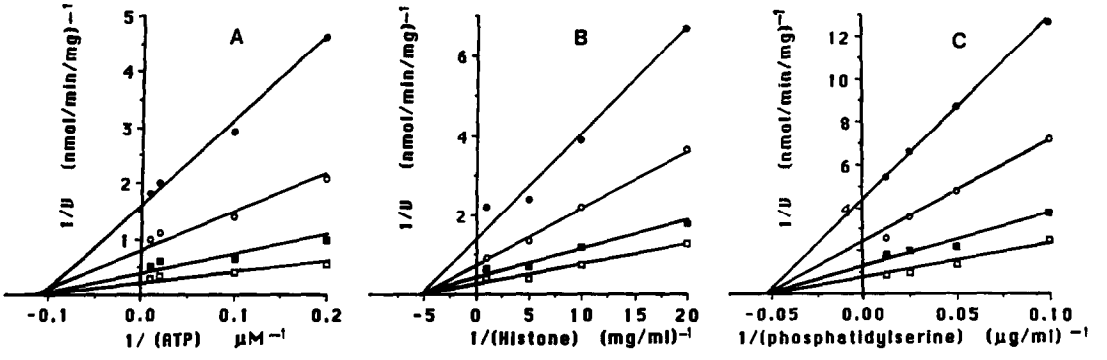

Fig. 3. Mefloquine inhibition of PKC with respect to ATP (A), histone (B) and PS (C). See legend to Fig. 2 and Material and Methods for experimental details. The final concentrations of mefloquine in the assay mixture were 0 (□), 53 (■), 132 (○) and 264 μM (●) (0, ~20, ~50 and ~100 μg/mL, respectively).

Effect of mefloquine on PKA

To investigate the selectivity of the inhibitory effect of mefloquine on PKC, we tested its effect on PKA, another protein kinase important in transmembrane signaling (Fig. 2). At concentrations as high as 260 μM mefloquine inhibited only slightly PKA from bovine heart and did not inhibit the activation or activity of PKA from neutrophil cytosol, suggesting that mefloquine inhibition of PKC is relatively specific.

Effect of mefloquine on PMA-induced phosphorylations in intact neutrophils

As shown in Fig. 5 and in agreement with the relevant literature [18], PMA induced the phosphorylation of a large number of proteins in intact human neutrophils, in particular those with a molecular mass of 47, 49 and 67 kDa. The inhibition was dose-dependent and occurred at concentrations within the range of those necessary to block the PMA-induced oxidative burst; mefloquine (6.25 μM) inhibited protein phosphorylation to an extent similar to that observed with 100 μM H-7.

DISCUSSION

It has been shown previously that the antimalarial drug mefloquine inhibits neutrophil functions related to the oxidative burst, e.g. chemiluminescence and iodination activity. In this report, we show that mefloquine strongly inhibits neutrophil superoxide anion production induced by the PKC agonists PMA and PDBu. As the neutrophil respiratory burst induced by PMA and PDBu is mediated most likely by PKC [19–21], we tested the effect of mefloquine on semi-purified PKC from neutrophil cytosol. Mefloquine was found to inhibit activity efficiently. It is noteworthy that mefloquine inhibited the neutrophil oxidative burst at concentrations 10 times lower than those that inhibited PKC in neutrophil homogenates. The reason for this difference is not clear. One explanation is that mefloquine, which has a high affinity for plasma membrane lipids [22], might accumulate in neutrophils, as suggested by Ferrante *et al.* [11]. Indeed, mefloquine inhibited the PMA-induced oxidative burst and protein phosphorylations in intact neutrophils over a similar range of concentrations (Figs 1 and 5). Since PKC is one of the major effectors involved in protein phosphorylation related to the oxidative metabolism of neutrophils, it is likely that low concentrations of mefloquine inhibit activity of this enzyme in intact cells. These data also suggest that mefloquine is concentrated (at least 10-fold) by neutrophils, thus, providing cellular concentrations required to inhibit PKC activity.

Our kinetic studies showed that the inhibition of PKC activity by mefloquine was non-competitive with respect to ATP, histone and PS, implying that the drug does not interact directly with the sites corresponding to these cofactors. Furthermore, mefloquine inhibited [^{3}H]PDBu binding to PKC, indicating that it may interact with the binding site of phorbol esters. Previous studies have established that the PDBu-binding site is on the regulatory domain of PKC [23] and other "specific" PKC inhibitors such as sphingosine [24], calphostin C [25] and dequalinium [26] have been shown to interact with this domain. Our data show that mefloquine inhibits PKC but not PKA or its catalytic subunit. The fact that the two protein kinases are closely related (sequence homology of their catalytic domain

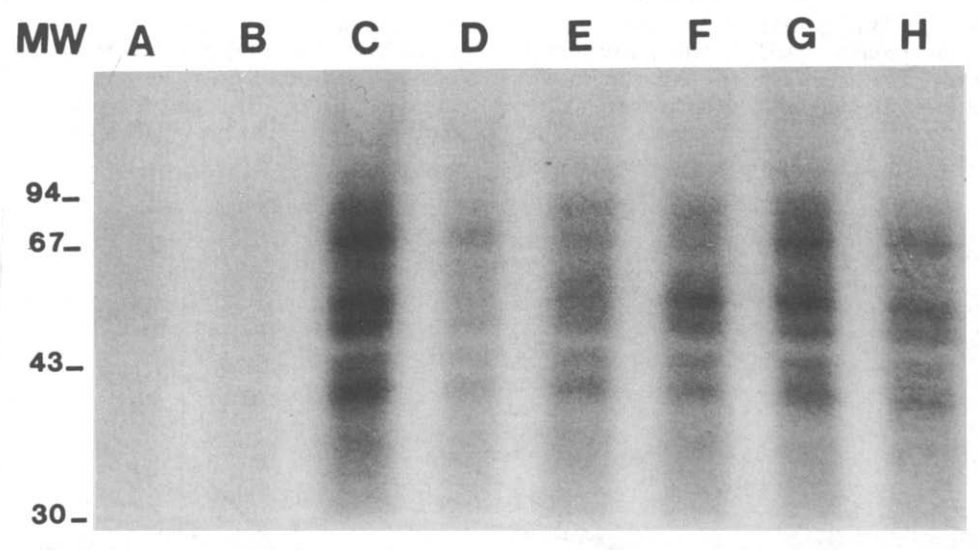

Fig. 5. Effect of mefloquine on PMA-induced phosphorylation of neutrophil proteins as demonstrated by ^{32}P autoradiography of SDS–PAGE. Cells were loaded with [^{32}P]phosphoric acid, preincubated with mefloquine and activated by PMA; extracts electrophoresed on 13% polyacrylamide gels as described in Materials and Methods. Lane A, control; lane B, 25 μM mefloquine; lane C, 100 ng/mL PMA; lane D, 25 μM mefloquine + PMA; lane E, 12.5 μM mefloquine + PMA; Lane F, 6.25 μM mefloquine + PMA; lane G, 3 μM mefloquine + PMA and lane H, 100 μM H-7 + PMA.

[27, 28]) favours further the assumption that mefloquine does not interact with the catalytic subunit of PKC, either. In contrast, other PKC inhibitors, such as H-7 [29], which act on the catalytic domain do not show a high specificity for PKC, given the amino acid sequence homology of this subunit with other protein kinases.

PKC is a key enzyme involved in the regulation and functions of many cell types. That mefloquine is able to inhibit PKC activity in intact cells further extends the relevance of our data to domains other than neutrophils. Although to our knowledge PKC activity has not been found in the cytosol of *P. falciparum* [30], mefloquine could inhibit other kinases important for the metabolism of this protozoon; this would provide an attractive hypothesis to explain the antimalarial activity and suggest possible efficacy against other protozoa such as *Entamoeba* [31].

PKC inhibition may also be involved in some of the side-effects observed during mefloquine therapy. In particular, mefloquine can induce severe impairment of the human central nervous system [32], where PKC is of major importance for signal transduction and neurotransmitter release [33, 34]. Finally, some oncogenes have been found to participate in protein phosphorylation and selective PKC inhibitors may provide a new class of antiproliferative agents [35, 36].

In conclusion, mefloquine appears to inhibit the neutrophil oxidative burst via its effect on PKC. Mefloquine interacts with the regulatory domain of PKC, as shown by its inhibition of PDBu binding to the enzyme. Whether or not the inhibition of PKC by the antimalarial agent mefloquine plays a role in the pharmacological action of this drug remains to be determined.

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