Dissociation of human neutrophil membrane depolarization, respiratory burst stimulation and phospholipid metabolism by quinacrine

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Human neutrophils generate a respiratory burst with the elaboration of toxic oxygen metabolites upon appropriate stimulation. Subsequent to receptor–ligand interaction, the activation pathway of this burst is unknown. Here, attempts to correlate phospholipid turnover have demonstrated dissociation of lipid flux and burst activation. Quinacrine inhibited membrane depolarization, superoxide (O_2^-) generation, and net phosphatidylserine production with ID_{50}-values of 16 μM, 73 μM and >500 μM, respectively. The inhibitory profiles of these neutrophil activation parameters demonstrate a dissociation between membrane depolarization, respiratory burst stimulation, and phospholipid turnover.

Neutrophil *Depolarization* *Phospholipids* *Quinacrine* *Respiratory burst*

1. INTRODUCTION

The generation of the respiratory burst in the human neutrophil by either phagocytosable particles or soluble agonists results in the elaboration of toxic oxygen metabolites [1]. The metabolic apparatus responsible for this activity includes the activation of a partially characterized NADPH-oxidase [2], whose activation pathway is unknown [3]. Associative parameters of neutrophil stimulation include the dose-dependent depolarization of the neutrophil plasma membrane potential [4–6], and a rapid flux in lipid metabolism [7–12]. Mobilization of arachidonic acid by the activation of phospholipases, and the extensive turnover of membrane phospholipids during phagocytosis, are associative metabolic activities, which have not as yet been integrated into a coherent schema for a neutrophil activation sequence. Phospholipid methylation and phospholipase A_2 activation appear requisite for chemotaxis, but the relationship of these activities to membrane depolarization and the activation of the burst is unclear [9,10].

We have examined the inhibition of human neutrophil activation by quinacrine, an agent thought capable of blocking release of fatty acids from phospholipids as a consequence of phospholipase A_2 activation (reviewed in [13]). Quinacrine, however, may bind directly to phospholipids, forming less polar derivatives and thus perturbing membrane structure and function, rather than directly inhibiting phospholipase [13]. Here, we confirm that quinacrine alters membrane function as measured by the depolarization of the plasma membrane, and the activation of the respiratory burst, but without inhibiting phospholipid turnover, suggesting a dissociation of phospholipid turnover from these other parameters of neutrophil stimulation.

2. MATERIALS AND METHODS

Ferricytochrome *c*, cytochalasin B, phorbol myristate acetate (PMA), superoxide dismutase (SOD), NADPH, concanavalin A, and quinacrine were purchased from Sigma (St Louis MO). The fluorescent probe, di-S-C_3(5) was the generous gift of Dr Alan Waggoner, Carnegie-Mellon University (Pittsburgh MA). Organic solvents for HPLC were purchased from Waters Assoc. (Milford MA) and

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analytic aqueous solvents (Mallinckrodt, St Louis MO) were filtered on a Millipore 0.22 μm filter. [^{3}H]Phorbol-dibutyrate (PDBu) was purchased from New England Nuclear (Boston MA).

Human neutrophils were harvested from normal donors and prepared to >95% homogeneity as in [2]. Oxygen consumption was assessed with a Yellow Springs Clark electrode oxygen monitor [2] and O_2^- production was quantitated by the continuous assay of SOD-inhibitable cytochrome *c* reduction, in a thermostated Perkin-Elmer Model 557 spectrophotometer, for intact cells [14] and for a 27000 × *g* particulate preparation used to measure NADPH-oxidase activity [2]. In experiments employing quinacrine, both sample and reference cuvettes contained equal quantities of the agent, which was preincubated with the cells for 3 min at 37°C before stimulation with either PMA (1 μg/ml) alone or concanavalin A (50 μg/ml) (after 3 min preincubation with cytochalasin B (5 μg/ml)). Binding assays of [^{3}H]PDBu were performed by described methods [14].

Quantitation of neutrophil phospholipids was performed by adaption of the high-pressure liquid chromatographic technique in [15]. Cells (5×10^7 cells/ml), preincubated at 37°C in phosphate-buffered saline (containing 0.9 mM $CaCl_2$ and 0.45 $MgCl_2$) [14] with or without quinacrine (100–500 μM) for 3 min were stimulated with PMA (1 μg/ml). After 1–3 min, the activation was terminated by adding the cell mixture to 7 vol. chloroform:methanol (2:1); this suspension was homogenized, filtered on Whatman no.4 filter paper (Whatman, England) and separated with 3 saline washes. The residual lower phase was dried under nitrogen and resuspended in 1.0 ml chloroform; 5–15 μl was chromatographed on a Waters Assoc. (Milford MA) liquid chromatographic system consisting of a Model 272 gradient system, Model 450 Variable wavelength absorbance detector (detection at 203 mM), and a Hewlett-Packard Model 3390A integrator. The Silica gel column, 30 cm × 4 mm i.d. Micro-Pak S I-10 column obtained from Varian Assoc. (Palo Alto CA), was developed with acetonitrile–methanol–85% phosphoric acid (130:5:15, by vol.) at 1.0 ml/min at 21°C. Calibration of the system and quantitation with standard compounds was performed daily with phosphatidylserine (PS), phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, phosphatidyldimethylethanolamine, lysophosphatidylcholine and lysophosphatidylethanolamine (LPE) (Sigma, St Louis MO); after use the column was washed successively with methanol–water (1:1, v/v), methanol, dichloromethane, hexane and stored in THF (Waters Assoc., Milford MA). Recovery was >85% as assessed by tracer experiments with [^{3}H]LPE (New England Nuclear, Boston MA).

Membrane potential changes were evaluated as in [4]; fluorescence of a 10^6 cells/ml KRP suspension equilibrated for ≤5 min with 2 μM di-SC_3-(5) was monitored on a Perkin Elmer 650 S10 spectrofluorimeter equipped with stirring and thermostating (37°C) devices. Quinacrine (5–200 μM) was added and 1.5 min incubation time were allowed to elapse before PMA (1 μg/ml) was added.

3. RESULTS AND DISCUSSION

The neutrophil respiratory burst is inhibited by quinacrine, as assessed by both O_2^- production and oxygen consumption. A comparable dose–response was obtained with either PMA or concanavalin A in both assays. PMA was used exclusively in subsequent studies (fig.1). (The ID_{50} for PMA was 73.7 ± 24.6 μM (mean ± SD, $n = 7$), in the O_2^- assay.) To determine if quinacrine's inhibitory effect was due to direct inhibition of the NADPH-oxidase, a 27000 × *g* particulate preparation from PMA stimulated cells was studied. The ID_{50} for the O_2^--generating NADPH-oxidase was >2 mM and could not be determined accurately. (At 500 μM quinacrine, O_2^- generation was inhibited <20%.) Thus the inhibitory effect of quinacrine appeared to occur at a step prior to NADPH-oxidase activation, rather than by inhibition of the enzyme itself. Trypan blue exclusion in the presence of 500 μM quinacrine was within normal limits (not shown), and thus direct cellular toxicity could not account for inhibited respiratory burst activity. That the inhibition was not related to interference of ligand binding was suggested by the similar inhibitory profiles for distinct receptors and by normal binding of PDBu (an analogue of PMA with high specific binding) to the phorbol receptor (not shown).

These findings inferred that the quinacrine inhibition of the respiratory burst was at a site in the

activation sequence between receptor–ligand binding and NADPH-oxidase stimulation. Since depolarization of the neutrophil's membrane potential precedes detectable O_2^- production, the effect of quinacrine upon the depolarization was examined. As shown in table 1, which gives data from one of 4 similar expt, quinacrine has no effect on the fluorescence of di-SC_3-(5) in a cell free system, nor does it affect the resting fluorescence of di-SC_3-(5) loaded human neutrophils which reflects their resting membrane potential. It does, however, markedly diminish the initial rate of depolarization (V_o), the most sensitive parameter measuring early stimulus response [4]. The compound also reduces the maximal attainable depolarization ($\Delta F/F_0$). The quinacrine ID_{50} dose (required to decrease the PMA-induced depolarization by 50%) was 16.2 ± 4.2 μM (mean ± SD, n = 4), which is ~1/5th that required to reduce the ensuant formation of O_2^- by 50% (fig.1). The membrane response is reduced by 75% with 50 μM quinacrine, and is not further decreased by higher concentrations of the drug (fig.2). The respective quinacrine inhibitory profiles of O_2^- generation and membrane depolarization are sufficiently disparate to suggest a complex association of these activation parameters, which are as yet to be defined, and further support the hypothesis that depolarization is a separate and dissociable event in the stimulus response [4–6].

In an attempt to relate phospholipid metabolism to the quinacrine effects on respiratory burst and depolarization, phospholipid turnover in resting and stimulated neutrophils was examined. We have adapted high-pressure liquid chromatographic analysis for these studies, yielding rapid and reproducible quantitation of several phospho- and lysophospholipids, whose metabolism accompanies the activation process and likely contributes to membrane fluidity changes and/or depolarization seen in the stimulated cell [7–12]. Chromatograms of lipid extracts demonstrated changes in total phospholipid composition which were both time- and stimulus-dependent. The most dramatic flux noted over the first 3 min of stimula-

Table 1

Representative effect of quinacrine upon membrane depolarization of resting and PMA-stimulated neutrophils

F_0[a]	[Quinacrine] (μM)	F_1[b]	V_0[c]	Delay[d] (min)	($\Delta F/F_0$)$_{max}$[e]
6.7	0	6.6	18.4	0.3	1.47
6.3	10	6.2	16.7	0.4	1.90
7.7	20	7.4	10.7	0.5	1.20
6.5	50	6.3	4.4	0.6	0.62
8.0	200	8.7	5.3	0.8	0.82

[a,b] The fluorescence ($\lambda_{exc.}$ 620, λ emission, 670) before (F_0) and after (F_1) addition of quinacrine (expressed in arbitrary units)

[c] V_o, slope of initial fluorescence changes (arbitrary units), an accurate measure of initial stimulus–response

[d] Delay before onset of fluorescence change upon PMA stimulation (indicated in minutes)

[e] ($\Delta F/F_0$)$_{max}$, maximum relative depolarization ($F_{final} - F_1$)/F_0

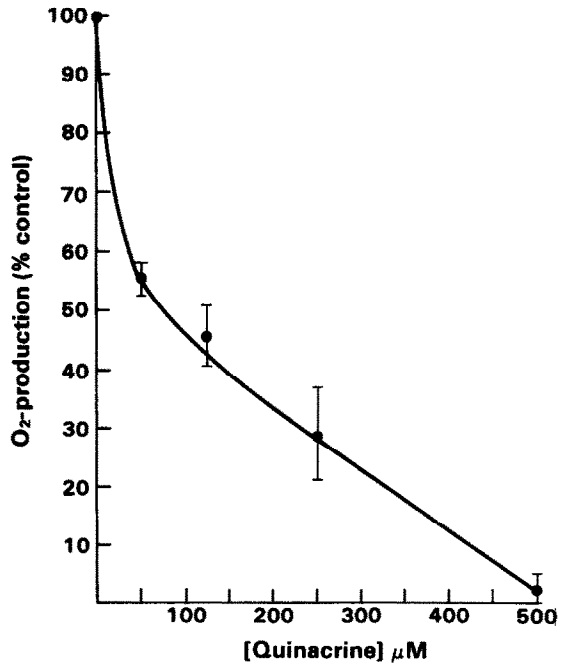

Fig.1. Quinacrine inhibition of O_2^- production by PMA-stimulated neutrophils. The data is the mean and standard deviation from 4 expt performed as in section 2. The 100% value for O_2^- generation was 7.92 ± 1.3 nmol.10^6 cells^{-1}.min^{-1} (mean ± SD) (rates measured at 2 min post-PMA addition).

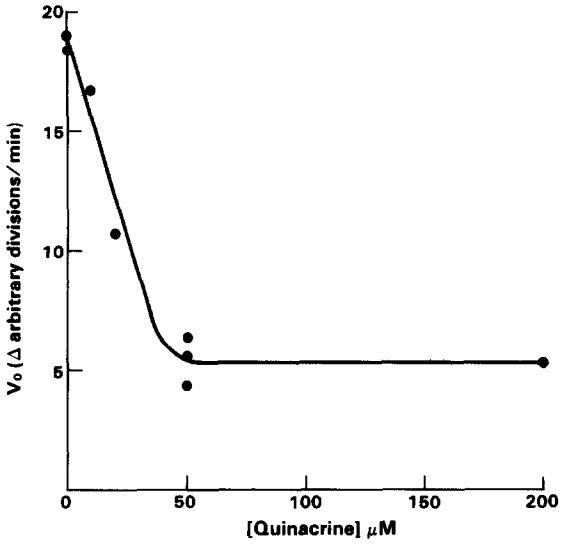

Fig.2. Rate of initial fluorescence change (V_0) of di-SC_3-(5) in PMA-stimulated cells. A representative experiment of quinacrine inhibition of plasma membrane depolarization, performed as in section 2.

tion was a rise in phosphatidylserine (PS) as noted by radioisotope incorporation techniques [15]. PS levels increased to 80% of maximum by 1 min stimulation and plateaued by 3 min, forming a net increase of 3.02 ± 1.21 ng/10^7 cells (mean ± SD, $n = 4$). Quinacrine at 500 μM inhibited PS formation to 64.6 ± 23.1% ($n = 4$) of controls, while at 100 μM, it did not inhibit PS generation at all. The minimal 7-fold difference in ID_{50}-values for phospholipid turnover and respiratory burst activation suggests that the burst inhibition by quinacrine is due to other unidentified intermediate activation steps between receptor–ligand binding and NADPH-oxidase expression, possibly through non-specific plasma membrane pertubation, as suggested in platelets and erythrocytes [13].

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