Effects of Free Fatty Acids on Release of Superoxide and on Change of Shape by Human Neutrophils

REVERSIBILITY BY ALBUMIN*

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Release of superoxide from human neutrophils was stimulated by cis-unsaturated fatty acids (e.g., arachidonate, linoleate). All the saturated and trans-unsaturated fatty acids tested were ineffective in this context. Binding of linoleate to neutrophils was biphasic and could be resolved into a linear and a saturable component. The extent of linoleate binding to the saturable component correlated strongly with the amount of O\textsuperscript{2-} released (r = 0.96). Palmitate, a saturated fatty acid, exhibited only linear binding to neutrophils and the binding was similar to the linear component of linoleate binding. All cis-unsaturated fatty acids tested decreased the fluorescence polarization of cis- and trans-parinaric acid used as membrane probes in suspensions of neutrophils, whereas the saturated fatty acid, myristate, increased the polarization. Fatty acids which stimulated O\textsuperscript{2-} release induced morphological changes (i.e., evaginations) in neutrophils, whereas the inactive fatty acids did not affect the cellular morphology. Effects on both the cell morphology and superoxide release were reversed by treatment of the cells with delipidated albumin. These data are discussed in relation to recent reports concerning the effects of free fatty acids on various biochemical systems.

Neutrophils produce substantial quantities of superoxide O\textsuperscript{2-} and hydrogen peroxide upon perturbation of their plasma membrane during phagocytosis or by a variety of agents (for review, see Refs. 4–6). The superoxide and hydrogen peroxide produced by neutrophils may interact to form the hydroxyl radical (OH·) and singlet oxygen (\( ^{1}O_{2} \)). These products of oxygen reduction (i.e., O\textsuperscript{2-}, H\textsubscript{2}O\textsubscript{2}, OH·) and excitation (\( ^{1}O_{2} \)) constitute key components in the oxygen-dependent antimicrobial mechanisms of phagocytes (1–3). The overall mechanism(s) by which the O\textsuperscript{2-}-producing system of neutrophils is activated remains largely obscure.

We and others (7–9) have reported that cis-unsaturated fatty acids stimulate the release of O\textsuperscript{2-} from neutrophils and macrophages. Trans-unsaturated and saturated fatty acids were inactive in this regard with human neutrophils (7) and guinea pig macrophages (9). Different effects of exogenous cis-unsaturated versus saturated fatty acids have been noted previously on a variety of cellular and subcellular phenomena (e.g., inhibition of lymphocyte capping (10); membrane transport (11)) (for review, see Ref. 12). Fatty acids may alter the physical properties of membranes (e.g. Refs. 13–15), induce membrane fusion (16), activate enzymes (e.g. Refs. 17 and 18), bind covalently to proteins (e.g. Ref. 19), and induce alterations of the cytoskeleton and contractile proteins (20).

In this paper, we have expanded our survey of the ability of fatty acids to stimulate O\textsuperscript{2-} release from human neutrophils and extended the study to compare binding to neutrophils of an active (linoleate) and an inactive (palmitate) fatty acid. Superoxide release and binding are correlated. The fluorescent probes cis- and trans-PnA were used to assess changes in the fraction of neutrophil membrane which is gel or which is liquid crystalline. In addition, we describe some remarkable morphological changes in neutrophils induced by cis-unsaturated fatty acids and show that both the morphological changes and fatty acid-stimulated O\textsuperscript{2-} release are reversed by albumin. A preliminary report on some of the data presented in this paper has appeared elsewhere (21).

**EXPERIMENTAL PROCEDURES**

**Materials**

Distilled-deionized water used throughout all experiments was supplied by a Barnstead water system (Barnstead Co., Boston, MA). Fatty acids were the highest grade available from Sigma or were obtained from NuChek, Elyssian, MN. Ferricytochrome c (Type VI), superoxide dismutase (Type I), human albumin (essentially fatty acid free), trypsin (Type IX), and inulin (from Dahlia tubers) were purchased from Sigma. [1-\( ^{14}C \)]Linoleic acid, [1-\( ^{14}C \)]palmitic acid, [methoxy-\( ^{14}C \)]inulin, and Aquaold scintillation fluid were products of New England Nuclear. cis- and trans-PnA were obtained from Molecular Probes, Flano, TX and also were the generous gift of Dr. Robert Simoni, Stanford University. Purity of cis- and trans-PnA was confirmed by thin layer chromatography and absorption coefficients and spectral ratios which were in agreement with those reported previously (22). Macrodex (dextran 70) and Ficoll-Paque were products of Pharmacia. Electron microscopic grade glutaraldehyde was obtained.

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The abbreviations used are: O\textsuperscript{2-}, superoxide; cis-PnA, cis-parinaric acid (9,11,13,15-cis, trans, trans, cis-octadecatetraenoic acid); trans-PnA, trans-parinaric acid (9,11,13,15-all-trans-octadecatetraenoic acid).

1 The abbreviations used are: O\textsuperscript{2-}, superoxide; cis-PnA, cis-parinaric acid (9,11,13,15-cis, trans, trans, cis-octadecatetraenoic acid); trans-PnA, trans-parinaric acid (9,11,13,15-all-trans-octadecatetraenoic acid).

2 The following fatty acids are referred to in the text by their common names: arachidonate, 5,8,11,14-all-cis-eicosatetraenoate; linoleate, cis-9-cis-12-octadecadienoate; petroselinate, cis-6-octadecenoate; palmitate, n-hexadeccanoate; and myristate, n-tetradecanoate.
from Polysciences, Warrington, PA. Osmium tetroxide was obtained from Alfa, Danvers, MA. Epon 812 was obtained from Balser Union, Hudson, NH.

Methods

Preparations of Neutrophils—Human neutrophils were purified from whole blood by combining the dextran sedimentation method of Skoog and Beck (23) with a Ficoll-Paque gradient procedure that is outlined in detail elsewhere (24). This procedure resulted in cell preparations that contained ≥98% neutrophils with a viability always ≥90%.

Superoxide Release—Superoxide release from neutrophils was measured continuously at 37 °C by following the superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm (25, 26). The standard assay mixture (1.0 ml) consisted of a modified Dulbecco’s (27) medium (138 mM NaCl, 2.7 mM KCl, 16.2 mM Na2HP04, 1.47 mM KH2PO4, 0.315 mM CaCl2, 0.315 mM MgCl2, 7.5 mM d-glucose, pH 7.35) containing 0.075 mM ferricytochrome c. All other conditions were identical to those previously reported (7).

Stock solutions of fatty acids were prepared immediately prior to use, as described earlier (7).

Binding Studies—Neutrophils (3 × 106/ml) were incubated in 15 ml of the modified Dulbecco’s medium described above, but without cytochrome c, for 3 min at 37 °C in siliconized flasks. Just prior to the addition of the fatty acid, [3H]inulin was added to the reaction mixture so that the final specific activity was 0.5 μCi/ml. The [3H]-labeled fatty acid was then added in a fashion which yielded a constant specific activity of 0.09 μCi/ml for this substance. Since maximum binding of fatty acids to neutrophils occurred within 30 s (data not shown), the reaction mixture was incubated for 60 s at which time 1.4-cc aliquots were removed in triplicate and spun for 5 s at 15,600 × g in a microcentrifuge at room temperature. The supernatant was removed, the pellet resuspended in 200 μl of the incubation medium, and the amount of 14C and 3H in each pellet determined with an Aquasol scintillation fluid, which promoted the solubilization of the cells. The [3H]inulin counts were used to calculate the number of 14C counts that were not cell-associated (i.e. counts trapped in the extracellular medium of the pellet). The total number of molecules of fatty acid bound per cell was calculated from the specific activity of the original fatty acid.

Spectroscopic Methods—Fluorescence polarization measurements of cis- and trans-PnA in suspensions of neutrophils were made on a Perkin-Elmer MPF 2A fluorescence spectrophotometer equipped with Glan-Thomson polarizers. A 390-nm cutoff filter was used to diminish interference from scattering. The excitation wavelength was 520 nm, and the emission was recorded at 510 nm (28, 29). Excitation slit width was 3 to 6 mm. Emission slit widths were 6 to 12 mm. A lever arm permitted convenient switching of the emission polarizer from 0 to 90°. Typical polarization experiments were as follows. The cells (5 × 106/ml) were incubated in 3-ml quartz cuvettes (1-cm optical path) at 37 °C for 2 min. cis- or trans-PnA was added from an anhydrous stock solution to a final concentration of 1.6 μM, and fluorescence intensities in the parallel orientation (I1) and the perpendicular orientation (I2) were recorded every 10 s until the steady state value was reached. The polarization ratio (P = I1/I2) was calculated. An aliquot of the test fatty acid was then added, and the new values of the fluorescence in each orientation were recorded. The change in the polarization ratio (ΔP) was calculated.

Experiments were designed to limit the contribution of scattering and background fluorescence to a small fraction of the total signal (typically less than 10%). All intensities used are corrected for scattering, inner and outer filter effects, and background fluorescence; in no cases did the corrections lead to qualitative changes in the results. Degradation of the probe was slight over the time scale of the measurements. Control experiments indicated that neither isomer of PnA was capable of stimulating O2− release at the concentration employed.

Ultrasound Studies—Morphological experiments were performed on human neutrophils stimulated with various fatty acids as described under “Methods.” A, 20-carbon fatty acids; arachidonate (O), 8,11,14-all-cis-eicosatetraenoate (X), 11-cis-14-cis-eicosadienoate (Δ), trans-11-trans-14-eicosadienoate (A), 8-cis-eicosanoate (O), 11-cis-eicosaenoate (X), and 13-cis-eicosenoate ( ). B, 18-carbon fatty acids; 6,9,12-all-cis-octadecatrienoate (Δ), 9,12,15-all-cis-octadecatrienoate (X), trans-9-trans-12-octadecadienoate (P), 6-cis-octadecenoate (C), trans-6-trans-9-octadecadienoate ( ), cis-11-octadecenoate (O), cis-9-octadecenoate (V), and cis-9-octadecenoate ( ). C, fatty acids of ≤16 carbon atoms; cis-9-hexadecenoate ( ), cis-9-hexadecenoate (O), trans-9-hexadecenoate ( ), 11-dodecanoate ( ), hexadecanoate ( ), tetradecenoate (X), dodecanoate (V), n-hexanoate (Δ), and n-octanoate ( ). The mean response ± S.D. at an optimum concentration for each fatty acid is indicated by error bars. After exposure of cells to fatty acids (~150 μM) that were inactive, the stimulating agent phorbol 12-myristate-13-acetate was added to the assay mixture and O2− was released at levels normal for this stimulus. Since viability is a prerequisite for O2− release by neutrophils, the inability of those fatty acids to elicit O2− release was shown not to be due to cell damage or death.

Fig. 1. Time course of superoxide release by human neutrophils stimulated with various cis-unaturated fatty acids. The curves show the initial portions of the progress curves for superoxide release from neutrophils (1.5 × 106/ml) stimulated with 82 μM arachidonate ( ), 45 μM linoleate ( ), 81,11,14-all-cis-eicosatetraenoate ( ), and 245 μM cis-11-cis-14-eicosa dienoate ( ) which were added to the assays last to initiate the reactions (bold arrow). All other assay conditions are described under “Methods.” The inset shows the effect of preincubating the stimulating agents (i.e. 82 μM arachidonate (O) and 3.2 μM 4β-phorbol-12-myristate-13-acetate ( ) for variable periods of time before the reactions were initiated by the addition of prewarmed (37 °C) cells (1.5 × 106/ml). The 100% values are those observed when the reactions were initiated by the respective stimulating agents.

Fig. 2. Ability of different concentrations of various fatty acids to stimulate superoxide release by human neutrophils. Superoxide release from human neutrophils was monitored after stimulation of the cells (1.5 × 106/ml) with different concentrations of fatty acids as described under “Methods.” A, 20-carbon fatty acids; arachidonate ( ), 8,11,14-all-cis-eicosatetraenoate (X), 11-cis-14-cis-eicosadienoate (Δ), trans-11-trans-14-eicosadienoate (A), 8-cis-eicosenoate (O), 11-cis-eicosaenoate (X), and 13-cis-eicosenoate ( ). B, 18-carbon fatty acids; 6,9,12-all-cis-octadecatrienoate (Δ), 9,12,15-all-cis-octadecatrienoate (X), trans-9-trans-12-octadecadienoate (P), 6-cis-octadecenoate (C), trans-6-trans-9-octadecadienoate ( ), cis-11-octadecenoate (O), cis-9-octadecenoate (V), and cis-9-octadecenoate ( ). C, fatty acids of ≤16 carbon atoms; cis-9-hexadecenoate ( ), cis-9-hexadecenoate (O), trans-9-hexadecenoate ( ), 11-dodecanoate ( ), hexadecanoate ( ), tetradecenoate (X), dodecanoate (V), n-hexanoate (Δ), and n-octanoate ( ). The mean response ± S.D. at an optimum concentration for each fatty acid is indicated by error bars. After exposure of cells to fatty acids (~150 μM) that were inactive, the stimulating agent phorbol 12-myristate-13-acetate was added to the assay mixture and O2− was released at levels normal for this stimulus. Since viability is a prerequisite for O2− release by neutrophils, the inability of those fatty acids to elicit O2− release was shown not to be due to cell damage or death.
Formed at a cell concentration of 3 × 10⁶/ml in 6 ml of the modified Dulbecco's medium described above. The cells were preincubated for 3 min at 37°C prior to the addition of the fatty acid. Cells were incubated with the fatty acids for 1 min and then fixed by the rapid addition of 0.66 ml of 25% glutaraldehyde. Fixation was carried out for 1 h at room temperature. The cells were then washed 3–4 times in the incubation medium and stored at 4°C overnight. Washed cells were postfixed in 2.0% OsO₄, 0.1 M phosphate buffer (pH 7.2) for 1 h at room temperature. They were subsequently dehydrated through a graded series of ethanol solutions and embedded as a pellet in Epon 812.

Thin sections were cut with a diamond knife and collected on bare copper grids. Sections were stained with uranyl acetate and lead citrate. Specimens were examined and micrographs taken with a Philips 200 EM.

RESULTS

General Observations and Correlations—Upon exposure to most cis-unsaturated fatty acids, human neutrophils released O₂⁻ which was detected by the ability of this anion to reduce ferricytochrome c. Maximal rates were observed in most cases within 6 s of the addition of the stimulus (e.g. with arachidonate or linoleate), the major exceptions being 8,11,14-all-cis-eicosatetraenoate and cis-11-cis-14-eicosa dienoate, with which latency periods of about 30 and 70 s, respectively, were observed (Fig. 1). Rates in all cases were calculated from the linear phase of the progress curves.

To obtain the maximal rate of O₂⁻ release, it was necessary to initiate the reaction by adding the fatty acid to the assay mixture as the last component. If the fatty acid (e.g. arachidonate) was incubated in the assay system prior to initiation of the reaction by addition of warmed cells, a variable diminution in activity (25–90%) was always observed. Prior incubation of the fatty acid for periods >2 min, however, led to no further loss of activity (Fig. 1, inset). Controls reported previously (7) indicated that arachidonate itself, and not one of its oxidation products, was responsible for stimulating O₂⁻ release.

The abilities of different fatty acids to stimulate O₂⁻ release are compared in Fig. 2. The overall pattern is similar to that which we noted earlier (7) for a smaller number of compounds, i.e. cis-unsaturated are active, trans-unsaturated and saturated are inactive. The only exceptions were the cis-eicosamononoenoic acids (8-, 11-, or 13-) which were inactive. Comparison of the optimal rates of O₂⁻ released, for all of the cis-unsaturated fatty acids tested, with properties of these molecules, revealed correlations with the number of the carbon atom from which the first double bond after the carboxyl group arises (r = -0.758, 14 points), the total number of

**Fig. 3.** Correlation between the maximum rate of superoxide release by neutrophils and the melting point of the fatty acid utilized as the stimulating agent. The rate of superoxide release by neutrophils stimulated with the optimum concentration of the cis-unsaturated fatty acid is plotted versus the melting point for each of the fatty acids. The point for petroselinate (18:1 (6)) is shown but was omitted in calculating the correlation coefficient (r = 0.965) (see text).

**Fig. 4.** Binding of linoleate and palmitate to human neutrophils. The data of A show the binding curves for linoleate (●, curve a) and palmitate (○, curve b) to human neutrophils (3 × 10⁶/ml). Binding was measured as described under "Methods." The error bars represent the mean binding ± S.D. for linoleate (n = 7) and palmitate (n = 9) at the fixed concentrations indicated. The binding of linoleate to human neutrophils was resolved into the saturable ( ■) and linear components (Δ) shown in B. These curves were constructed by drawing line c (representing the linear component) through the origin and parallel to the region of curve a which exhibited a constant slope (i.e. between 50 and 175 μM). Subtracting line c from curve a yielded the saturable component (■, curve b). The data points (Δ) of line c are the differences between the data of curves a and c. The dose-response curve for O₂⁻ release from neutrophils (3 × 10⁶/ml) upon stimulation with linoleate (x, line o) is plotted as the percentage of the maximum rate (100% = 58 ± 9 S.D. nmol of O₂⁻/min/10⁶ cells, n = 9) for comparative purposes. The inset of B shows the correlation between the per cent of the maximum rate of O₂⁻ released versus the per cent of the maximum amount of linoleate bound to the saturable component (r = 0.96; 15 points from 4 separate experiments).
double bonds ($r = 0.788$, 14 points) and the melting points (for those compounds for which values are available) ($r = -0.734$, 10 points). If petroselinate (cis-6-octadecenoate) was omitted from these correlations, the $r$ values were −0.744, 0.860, and −0.965, respectively. The last mentioned correlation is shown in Fig. 3. Oleate has the highest melting point (16.2 °C) of the active fatty acids with the exception of petroselinate (30 °C). Whether petroselinate stimulated the cells by a different mechanism is not known.

**Binding Studies**—The binding of palmitate and linoleate to human neutrophils was compared to attempt to account for the different effects of these compounds as representative models in stimulating $O_2^-$ release. Human neutrophils bound palmitate in a linear fashion in the concentration range examined, with no evidence of saturation (Fig. 4A). In contrast, the binding curve for linoleate was biphasic (Fig. 4A) and could be resolved into linear and saturable components (Fig. 4B). The former component was almost identical to the binding curve for palmitate.

The dose-response curve for $O_2^-$ release from neutrophils stimulated with linoleate closely paralleled the saturable binding component of linoleate (Fig. 4B). When the amount of linoleate bound to the saturable component and the amount of $O_2^-$ released were normalized with respect to the maximum levels of binding and $O_2^-$ released, respectively, the correlation (Fig. 4B, inset) between the saturable binding of linoleate and $O_2^-$ released was even more striking ($r = 0.96$; 15 points from 4 separate experiments).

The saturable binding of linoleate was analyzed by several graphical procedures (Fig. 5). The data exhibit an inflexion point (i.e., half-maximal binding occurred at about 5 μM linoleate) and approached a plateau when plotted in the form recommended by Klotz (30) (Fig. 5A). Scatchard analysis (Fig. 5A, inset) exhibited a pattern diagnostic for positive cooperativity (e.g., see Ref. 31). With both of these procedures the maximum number of binding sites was about $2 \times 10^8$ molecules/cell. Positive cooperativity in the binding of linoleate was most apparent in the Hill plot which had a slope of 1.7 (Fig. 5B).

**Reversibility of $O_2^-$ Release Stimulated by Fatty Acids**—One

![Fig. 5. Analysis of the saturable binding of linoleate to human neutrophils. The saturable binding component of linoleate to neutrophils is presented in the semilogarithmic form recommended by Klotz (30) as a Scatchard plot (inset) and as a Hill plot (B).](image)

![Fig. 6. Reversibility of superoxide release from human neutrophils stimulated with arachidonate or linoleate. Reaction progress curve of $A$ demonstrates the superoxide-dependent reduction of ferricytochrome c by human neutrophils (1.5 × 10⁶/ml) stimulated with arachidonate (41 μM) which was added last to initiate the reaction (arrow A). Assay conditions are described under "Methods." Addition of delipidated albumin (7.2 μM) to the stimulated cells (arrow B) caused a rapid cessation of the reaction. Superoxide release was subsequently reinitiated (arrow C) and again terminated (arrow D) by the addition of arachidonate (41 μM) and albumin (7.2 μM), respectively. The inset shows an analogous experiment in which linoleate (80 μM) was the stimulating agent (at arrows A', C', and D') and albumin (18 μM) was employed to inhibit $O_2^-$ release (arrows B' and D'). The effect of the concentration of albumin on the inhibition of $O_2^-$ release is shown in B. Albumin was added to the reaction mixtures approximately 30 s after stimulation of the cells (1.5 × 10⁶/ml) by either 41 (●) or 82 μM (▲) arachidonate. The per cent of inhibition was calculated from the equation \[ (v - v_{\text{lin}})/v \times 100 \], where $v$ and $v_{\text{lin}}$ are the rates in the absence and presence of albumin, respectively. The theoretical concentrations of albumin calculated to effect 50% inhibition are indicated by arrows. These values were calculated from the concentration of arachidonate which produces half-maximal stimulation (i.e., about 20 μM, Fig. 2) and the ability of 1 mol of albumin to bind 6 mol of fatty acid (e.g., Refs. 32 and 33). The lowest concentration of arachidonate which maximally stimulates $O_2^-$ release was 41 μM. The arrowhead indicates the concentration of albumin which binds this amount of arachidonate and should, therefore, produce 100% inhibition with curve (●) and 0% inhibition with curve (▲).](image)
molecule of albumin binds 6 molecules of fatty acids with binding constants ranging from $10^6$ to $10^8$ M$^{-1}$ (e.g., see Refs. 32 and 33). Addition of delipidated albumin (7.2 µM) to neutrophils stimulated with arachidonate (41 µM) caused a rapid cessation of O$_2^-$ release. Further addition of arachidonate and of albumin alternately stimulated and reversed the phenomenon (Fig. 6A). Similar results were observed with linoleate as the stimulating agent (Fig. 6A, inset). The rate of O$_2^-$ release upon restimulation (C and C' in Fig. 6A) was comparable to the rate of O$_2^-$ release normally observed with cells not treated with albumin, 1 min after the stimulus was applied.

Albumin inhibited O$_2^-$ release from neutrophils stimulated with 41 µM arachidonate in a stoichiometric fashion (Fig. 6B). Superoxide release from cells stimulated with 82 µM arachidonate was also inhibited by albumin, but concentrations greater than those calculated on the basis of a 1:6 stoichiometry were required (Fig. 6B). At this higher concentration (82 µM) arachidonate may partition into regions of the cell whose components compete effectively with the external albumin.

Greater than 90% of the linoleate bound to neutrophils was removed by exposure of the cells to albumin (50 µM), whereas only about 50% of the palmitate was removed by similar treatment (Fig. 7). This difference may reflect dissimilarities in the interactions between different types of fatty acids and albumin.

**Fig. 8. Ultrastructural alterations induced in neutrophils by arachidonate and reversal with albumin.** A, typical control cell fixed with glutaraldehyde after incubation for 1 min as described under “Methods.” Note that the cell surface is relatively smooth with occasional short projections (arrows). Also evident are portions of the multilobed nucleus (N) and membrane-bound cytoplasmic granules (arrowheads) which are characteristic of this cell type. X 7,400. B, typical cell treated with 82 µM arachidonate for 1 min prior to fixation. The cell has numerous large bleb-like structures (arrowheads) projecting from the cell body. X 9,100. C, typical cell treated with 82 µM arachidonate for 1 min followed by addition of 50 µM delipidated albumin for 1 min prior to fixation. All preparations were stained with uranyl acetate and lead citrate as described under “Methods.” X 8,500.
in the cellular disposition of these fatty acids at the concentrations utilized, since albumin was added at a concentration greater than 3-fold than necessary to bind all of the fatty acid. Albumin binds linoleate and palmitate with approximately the same affinity (32).

Incubation of neutrophils with trypsin (200 units/mol) for 45 min did not affect the subsequent binding of linoleate (Fig. 7). Less drastic conditions (i.e. 147 units/ml of trypsin for 30 min) substantially inhibited the binding of oleate to plasma-lemma from rat liver (34).

**Fluorescence Polarization Studies with Parinaric Acid**—cis- and trans-PnA are highly fluorescent in membranes and very weakly fluorescent in buffer (28, 29); thus the fluorescent emission of these probes in a suspension of cells is due almost entirely to the fraction which is bound. **trans-PnA** has steric similarities to saturated fatty acids (e.g. linear structure) and partitions preferentially into the gel-like lipid phases of membranes, whereas **cis-PnA** has steric similarities to unsaturated fatty acids (e.g. bent configuration) and partitions with a slight preference for liquid crystalline domains (28, 29, 35). These features allow detection of relatively small changes in the fraction of membrane which is gel or liquid crystalline.

Addition of cis- or trans-PnA (1.6 μM) to neutrophils resulted in a rapid increase in fluorescence to stable values. cis-PnA had the more rapid fluorescence enhancement, attaining 80–90% of its steady state intensity in less than 6 s and reached a steady state value in approximately 30 s. trans-PnA appeared to have first a rapid enhancement of fluorescence, attaining half-maximal values in 6–8 s, but rose more slowly to a steady state value in 2–3 min (data not shown).

Addition of fatty acids to suspensions of neutrophils previously incubated with the probes cis- or trans-PnA (1.6 μM) resulted in changes in the fluorescence polarization ratio ($\Delta P$), as shown in Table I. These changes occurred within 30 to 60 s and were stable over 10 min. Myristate, which did not stimulate O$_2$ release, lead to an increase in the fluorescence polarization ratio, whereas arachidonate and linoleate, which did stimulate the cells, led to decreases in the fluorescence polarization ratio. cis-9-Tetradecenoate and cis-9-hexadecenoate also decreased the polarization ratio (data not shown). In model systems, increases or decreases in the PnA fluorescence polarization ratio are associated with increases or decreases, respectively, in the fraction of a membrane which is in a gel state (28, 29). By analogy, it would appear that fatty acids which stimulate the cells to release O$_2$ increase the fraction of the membrane which is liquid crystalline. Since cis- and trans-PnA are structurally similar to the fatty acids being studied here (35), it seems plausible that their locations in the cell are similar to those of the other fatty acids actually being studied.

**TABLE I**

| Fatty acid | trans-PnA | cis-PnA |
|------------|-----------|---------|
| μM         | $\Delta P$| $\Delta P$|
| Myristate  | 0.67      | $-0.022 \pm 0.06$ | $+0.114 \pm 0.021$ |
| 3.3        | $+0.064 \pm 0.019$ |          |
| 10.0       | $+0.155 \pm 0.016$ |          |
| Arachidonate | 2.7      | $-0.023 \pm 0.019$ |          |
| 5.4        | $-0.050 \pm 0.026$ | $-0.044 \pm 0.030$ |
| 15.0       | $-0.086 \pm 0.048$ | $-0.083 \pm 0.035$ |
| Linoleate  | 2.0       | $-0.011 \pm 0.013$ |          |
| 4.5        | $-0.041 \pm 0.029$ | $-0.047 \pm 0.023$ |
| 8.0        | $-0.081 \pm 0.020$ |          |

*Expressed as the mean ± S.D. for several determinations.
Ultrastructural Observations—Treatment of neutrophils with cis-unsaturated fatty acids which stimulated O₂ release, effected alterations in cell shape (i.e., linoleate (89 µM), arachidionate (82 µM), cis-11-octadecenoate (176 µM), cis-9-hexadecenoate (147 µM), cis-9-tetradecenoate (330 µM), 8,11,14-all-cis-eicosatrienioate (82 µM)). Similar treatment of neutrophils with fatty acids which did not stimulate O₂ release had no effect on the morphology (i.e., palmitate (97 µM), cis-11-eicosanoate (81 µM), 9-trans-12-trans-octadecadienoate (89 µM), 178 µM)). The most dramatic changes occurred with arachidonate where large bleb-like structures projecting from the bodies of the cells were observed. These blebs contained cytoplasmic constituents including granules, glycogen particles, and ground substance and often appeared to be pinching off from the cell body (Fig. 8B). Essentially all of the cells treated with arachidonate exhibited these alterations (8 different preparations of cell were employed). These morphological changes were reversed (at least to the extent that blebs were no longer present) by treatment with delipidated albumin (Fig. 8C). Neutrophils treated with other “active” fatty acids also exhibited bleb-like structures (e.g., linoleate (Fig. 9A) with the exception of petroselinate. Petroselinate (390 µM) induced the formation of slender spiny projections from the cell surface (Fig. 9B). Thus, all of the fatty acids tested which stimulated O₂ release induced the formation of evaginations from cells. That these changes were not the result of production of O₂ was indicated by the fact that inclusion of ferricytochrome c (0.875 mM) or superoxide dismutase (20 µg/ml) in the medium did not inhibit the phenomenon. Further, cells from patients with chronic granulomatous disease, which do not produce O₂ or H₂O₂ (for review, see Refs. 2 and 3) also gave positive results, though the morphological changes were not as dramatic. These morphological alterations are unlike those noted with most other stimuli, where intracellular changes occur such as phagosome formation with particulate stimuli and vesicle formation with soluble stimuli (e.g. phorbol 12-myristate 13-acetate (5), chemotactic peptides (36)).

DISCUSSION

Fatty acids readily partition into membranes and affect protein-lipid interactions with consequent functional changes in cells (for review see Ref. 12). cis- Unsaturated fatty acids may exert their cellular effects by increasing the fraction of membrane that is liquid crystalline (i.e. Refs. 12–15 and 37), and this may pertain to the effects reported in this paper (see below). The fluorescence polarization data with cis- and trans-PnA are consistent with such an interpretation (Table I). Previous studies on human neutrophils with the fluorescent probe diphenylhexatriene produced similar results (12). The Hill coefficient of 1.7 for the saturable binding of linoleate to neutrophils (Fig. 5B) may, therefore, represent the conversion of membranes or regions of membranes to a state into which further binding of linoleate is facilitated. Since the total P-lipid content of human neutrophils is about 6 x 10⁹ molecules/cell (38, 39) and the saturable binding of linoleate to neutrophils plateaus at 1.6 ± 0.3 x 10¹⁰ molecules/cell (Fig. 5), the ratio of linoleate to P-lipid is very high at maximal binding (i.e. 1:3 to 4:5). In model systems, fatty acids (e.g. cis- and trans-PnA) are known to undergo rapid (i.e. seconds to minutes) transverse diffusion or “flip-flop” through bilayers (28). The very high binding of linoleate to neutrophils may result in significant quantities of externally added linoleate being present in the cytoplasm. If it is assumed that the partition coefficient for linoleate in synthetic P-lipid vesicles and natural membrane approximates that published for cis-PnA (5 x 10¹⁰ to 5 x 10¹°) (29), the cytoplasmic concentration of linoleate at maximum binding can be estimated to be in the micromolar range (i.e., 3 to 30 µM). These concentrations are in the vicinity of those reported to activate soluble enzymes in vitro (e.g. Refs. 17 and 40). This possibility cannot be excluded as causative of the effects observed. However, these calculations also indicated that >99% of the linoleate bound to neutrophils resided in lipid. The high correlation (r = 0.96) between the saturable binding and O₂ release with linoleate (Fig. 4B) thus favors the involvement of membrane phenomena in O₂ release. The fact that such high levels of linoleate are required to stimulate O₂ release makes it unlikely that this compound functions in vivo as a second messenger initiating O₂ production, but is consistent with changes in the physical properties of the membranes.

The reversibility of O₂ release observed with fatty acids and albumin (Fig. 6) confirms previous studies which demonstrated reversibility with other stimulating agents (e.g. Refs. 41 and 42). The ability to reverse morphological changes in neutrophils (Fig. 8) has not been reported previously. These morphological changes induced by fatty acids are not presently understood. They may, however, be due to alterations in cytoskeleton-membrane interactions (20). The reversible nature of these phenomena, along with the ability of albumin to remove >90% of the linoleate from neutrophils (Fig. 7), indicates that covalent incorporation of fatty acids into P-lipids, glycereides, or protein (e.g. see Ref. 19) is not a large factor in the cellular responses reported here.

Although the biochemical mechanism by which cis-unsaturated fatty acids stimulate O₂ release is unknown, some modest speculation may be appropriate. Phosphatidylinositol-specific phospholipase C from liver and brain is activated by cis-unsaturated fatty acids (18, 43) with the same order of magnitude (e.g. arachidonate > 6,9,12-all-cis-octadecatrienoate > linoleate > cis-9-octadecenoate) and over a similar concentration range as that reported here for O₂ release from neutrophils (18) (Fig. 2). This phospholipase was not activated by saturated fatty acids (18). Stimulation of the phospholipase by fatty acids was attributed to alterations induced in the substrate (i.e. membranes (43) or micelles (44)) by these compounds. A similar enzyme activation via the condition of its substrate may occur in intact neutrophils upon insertion of fatty acids into the cell membranes, leading to increased activity of cellular phospholipase C. Rapid turn-over of phosphatidylinositol upon stimulation of neutrophils has been reported (e.g. Refs. 4, 39, and 45). The metabolism of inositol-containing P-lipids is thought to be causally related to increases in intracellular Ca²⁺ (46). In addition, a product of the lipase, diacylglycerol, is the physiological activator of protein kinase C (47). Both Ca²⁺ mobilization and protein kinase C have been implicated in stimulation of neutrophils (e.g. see Refs. 39 and 48–51).

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