Low doses of aliphatic alcohols produce divergent effects on the function of chemoattractant receptors on human polymorphonuclear leukocytes (PMNs) since they enhance chemotaxis but inhibit stimulation of superoxide production by chemoattractants. As such, alcohols can provide useful pharmacologic tools to probe the mechanisms of stimulus-response coupling in leukocytes. A role for protein kinase C has been implicated in the activation of the respiratory burst in PMNs. Although the vast majority of this enzyme activity is located in the cytosolic fraction of unactivated PMNs, protein kinase C activity appears in the particulate fraction of the cells when they are stimulated to produce superoxide by either chemoattractants or by phorbol myristate acetate (PMA). Doses of the alcohols that selectively inhibited stimulation of superoxide production by chemoattractants also inhibited the appearance of protein kinase C activity as well as an undefined protein kinase activity in the particulate fraction of the cells. In contrast, the alcohols did not affect either the ability of PMA to stimulate the production of superoxide in PMNs nor the appearance of protein kinase activity in the cells' particulate fraction. PMA is known to bind and activate protein kinase C directly, thus bypassing receptor-mediated events. These data suggest that alcohols inhibit the stimulation of the respiratory burst by chemoattractants in PMNs by blocking the ability of receptor occupancy to induce the appearance of protein kinase activity in particulate fractions. These results moreover suggest that the appearance of protein kinase activity in the particulate fraction may be required for activation of the respiratory burst in PMNs.

CHEMOTRACTANT receptor occupancy in leukocytes results in a variety of functional responses including morphologic polarization, chemotaxis, secretion of lysosomal enzymes, and activation of the respiratory burst.1,2 Several lines of evidence suggest that motility-related functions are regulated quite differently from secretion and superoxide production.3,4 The concentrations of chemoattractants required to initiate the former are about 20-fold less than those required to stimulate the latter.2,4,5 In addition, low doses of aliphatic alcohols increased the affinity of the oligopeptide chemoattractant receptor, enhanced PMN chemotaxis, yet selectively depressed chemoattractant-mediated superoxide production and lysosomal enzyme secretion.6 The alcohols appeared to alter stimulus-response coupling by the chemoattractant receptor since superoxide release induced by phorbol myristate acetate (PMA) was unaffected.4 Recent evidence suggests a role for protein kinase C (PKC) in superoxide production by PMNs, human monocytes, and mouse macrophage cell lines stimulated by PMA or oleoyl-acetyl glycerol.7-10 PKC activity in unstimulated human PMNs was almost entirely cytosolic when the cells were disrupted in the presence of ethylene glycol tetra-acetic acid (EGTA), but chemoattractants and PMA induced the appearance of PKC activity in the particulate fractions.5,11 The induction of PKC activity in the particulate fraction by chemoattractants was correlated with activation of the respiratory burst rather than with motility-related functions in that it occurred at high doses of chemoattractant and was greatly enhanced by cytochalasin B.11 In the present work, we have used low doses of alcohols as a pharmacologic probe to determine whether superoxide release stimulated by the oligopeptide chemoattractant receptor requires the appearance of PKC activity in the particulate fractions of PMNs.

MATERIALS AND METHODS

PMA, phosphatidylserine (PS), 1,2-diolein, histone (type III), cytochrome c, cytochalasin B, f-Met-Leu-Phe (FMLP) and adenosine triphosphate (ATP) were from Sigma Chemical Co (St Louis). Gamma[32P]ATP was from New England Nuclear (Boston). Tri-fluperoxamine (TFP) and purified calmodulin were generously supplied by Drs Marty Vogel and David Sedwick, Duke University Medical Center.

Cell preparation and isolation of subcellular fractions. Human blood PMNs were isolated as previously described.12 Cells (1 x 109 in 5.0 mL) were preincubated in Hanks' balanced salt solution (HBSS) containing 10 mmol/L HEPES and 4.2 mmol/L NaHCO3 (GIBCO, Grand Island, NY), pH 7.5, at 37 °C for five minutes with 10 mmol/L cytochalasin B with or without alcohols. FMLP (100 nM/mL) was added for one minute or PMA (1 ng/mL to 1 μg/mL) was added for five minutes. Reactions were stopped by addition of a tenfold excess of ice-cold, calcium-free HBSS containing the appropriate concentration of alcohol. Cells were centrifuged, washed with alcohol-free HBSS, and the cell pellets were suspended in extraction buffer consisting of 0.05 mol/L Tris-HCL, 0.05 mol/L 2-mercaptoethanol (2-ME), 2 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), pH 7.5, and sonicated for 30 seconds at a setting of 35% using an Artek Sonic 300 Dismembrator equipped with a microtip (Artek Systems Corp, Farmingdale, NY). Following centrifugation for one hour at 100,000 g, the supernatant (extractable particulate fraction) was removed and the nonextractable pellet resuspended in 0.1% Triton X-100 (Sigma) in extraction buffer. Following centrifugation for one hour at 100,000 g, the supernatant (extractable particulate fraction) was removed and the nonextractable pellet resuspended in 1.0% Triton X-100 in extraction buffer.

Assay of protein kinase C. Reaction mixtures contained 0.03 mol/L Tris-HCL, pH 7.5, 0.01 mol/L MgCl2, 50 μmol/L ATP, 2 x 106 cpm [γ-32P]ATP, 0.4 mmol/L EGTA, 0.01 mol/L 2-ME and 0.2

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mmol/L PMSF. Where indicated, 5 µg of PS, 0.5 mg of olein, and 0.6 mmol/L CaCl₂ were included.²,³ Reactions were started by adding 0.05 mL of enzyme preparation in extraction buffer containing 0.05% Triton X-100 (final reaction volume 0.25 mL) and incubated at 30 °C for five minutes for cytosolic fractions or 60 minutes for particulate fractions. The reactions were terminated with 1 mL of 25% trichloroacetic acid and 50 µg of bovine serum albumin, filtered, and counted.¹⁴ PKC activity in cytosolic and Triton-extractable particulate fractions is expressed as pmol [³²P] incorporated/min/10⁷ cells and is given as the difference between the activity in the presence of PS, olein, and calcium and that in the absence of these agents. A protein kinase activity present in the nonextractable particulate fraction was assessed as stated except that the PS, olein, and calcium were not added since these substances did not affect the activity of this kinase. The concentrations of alcohols used in these studies did not affect any kinase activity when added directly to the enzyme assays (data not shown).

**Assay of superoxide.** Superoxide release by human neutrophils was assayed by monitoring the superoxide dismutase–inhibitable reduction of ferricytochrome c using an extinction coefficient of 29.5 mmol/L/cm as described⁶ except that reaction mixtures contained 2.5 × 10⁶ cells.

**Determination of microviscosity parameter.** Microviscosity was quantified by measuring the fluorescence polarization of diphenylhexatriene with a microviscosimeter MV-1 (Elscent, Haifa, Israel) as described previously.⁴

**RESULTS**

Effects of aliphatic alcohols on superoxide production and appearance of PKC in particulate fractions of PMNs. Since chemoattractants produce an increase in PKC in PMN particulate fractions,¹¹ we sought to determine whether this phenomenon was required for superoxide production by these agents. Cells were treated with buffer or aliphatic alcohols in the presence of cytochalasin B prior to stimulation with the oligopeptide chemoattractant FMLP or with PMA. Superoxide release was quantified in intact cells, and PKC activity was measured in subcellular fractions. Incubation of PMNs with butanol (0.25%), pentanol (0.1%) or isoamyl alcohol (0.15%) at concentrations that produced equivalent decreases in the membrane microviscosity parameter⁴ (data not shown) inhibited superoxide release in response to FMLP (Table 1) by 89%, 80%, and 85%, respectively, in a representative experiment. The average inhibition of superoxide generation in five experiments with those same alcohols was 83%, 73%, and 81%, respectively. There was no significant effect on PMA-induced superoxide release. Additional experiments and previously published data⁴ showed that butanol and pentanol have no effect on superoxide generation by PMA at doses of this agent ranging from 1 ng/mL to 1 µg/mL. As noted previously, FMLP did not alter PKC activity in the cytosol¹⁳ and treatment of cells with aliphatic alcohols did not affect this (Table 2). PMA produced a profound decrease in cytosolic PKC activity, a finding that has previously been noted in PMNs and other cell types.⁴,¹³ Butanol and pentanol enhanced the disappearance of cytosolic PKC activity produced by PMA. FMLP and PMA increased both the PKC activity found in the Triton X-100–extractable pellet and the kinase activity associated with the detergent-nonextractable particulate fraction (Table 2). Incubation of cells with the alcohols inhibited the FMLP-induced increase in PKC activity measured in both the extractable and nonextractable particulate fractions to basal or near-basal levels. In contrast, the alcohol did not significantly affect the increase in particulate fraction kinase activity produced by 1 µg/mL PMA (Table 2). The alcohols similarly did not alter the increase in this activity when submaximal doses of PMA were used (1 ng/mL to 100 ng/mL, data not shown). Table 3 shows summary data from five experiments in which the fractional stimulation of particulate fraction protein kinase activities was measured in cells incubated with buffer or the various alcohols in the presence of FMLP or PMA. Although all the alcohols diminished the increase of kinase activity induced by FMLP, in both the extractable and nonextractable fractions, they did not significantly alter PMA-induced stimulation of protein kinase activity in either particulate cell fraction.

**Correlation of inhibition of FMLP-stimulated PKC redistribution with superoxide production.** PMNs were incubated with buffer or with various concentrations of pentanol or butanol in the presence of cytochalasin B, and superoxide release and PKC activity in particulate fractions were measured following stimulation of the cells with FMLP or PMA. Butanol and pentanol inhibited FMLP-induced superoxide release in a dose-dependent fashion (Fig 1A). The doses of butanol and pentanol that produced 50% inhibition (ID₅₀) of superoxide were 0.16% and 0.074% respectively. In contrast, the alcohols did not alter superoxide release in response to PMA (Fig 1A). Both alcohols also produced a dose-dependent inhibition of the appearance of PKC activity in the detergent-extractable particulate fraction of PMNs in response to FMLP with ID₅₀'s of 0.11% and 0.056%, respectively, for butanol and pentanol (Fig 1B). The appearance of kinase activity in the detergent-insoluble particulate fraction of cells treated with FMLP also showed dose-dependent

### Table 1. Effect of Aliphatic Alcohols on Superoxide Production

<table>
<thead>
<tr>
<th>Cells Incubated With</th>
<th>Superoxide Release by Cells Stimulated With</th>
<th>No Stimulant</th>
<th>FMLP</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butanol, 0.25%</td>
<td>0 ± 0</td>
<td>1.3 ± 0.5</td>
<td>11.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Pentanol, 0.1%</td>
<td>0 ± 0</td>
<td>2.5 ± 0.6</td>
<td>10.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Isoamyl alcohol, 0.15%</td>
<td>0 ± 0</td>
<td>1.8 ± 0.2</td>
<td>10.6 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

*PMNs (2.5 x 10⁵) were preincubated with the indicated percentage (vol/vol) of alcohol and 10 µmol/L cytochalasin B for five minutes at 37 °C prior to the addition of stimulants.

†Cells were incubated with no stimulant, 100 nmol/mL FMLP, or 1 µg/mL PMA, and superoxide release was measured for ten minutes. Superoxide release was calculated as nmol ferricytochrome c reduced/10 min/10⁶ cells. Values represent the mean ± SD of duplicate determinations obtained in a representative experiment. Values in parentheses indicate percentage of inhibition. In a total of five experiments, the average percentage of inhibition by the same alcohols for FMLP was 83 ± 12, 73 ± 11, and 81 ± 6 for butanol, pentanol, and isoamyl alcohol, respectively. The alcohols produced no significant inhibition of PMA-induced superoxide release (−1 ± 1, 4 ± 4, 0.1 ± 0.3, respectively).
inhibition in the presence of increasing concentrations of the
alcohols with an ID_{50} of 0.078% for butanol and 0.054% for
pentanol (Fig 1C). In contrast, the PMA-stimulated appear-
ance of PKC activity in the detergent-soluble particulate
fraction (Fig 1B) and that of protein kinase activity in the
nonextractable subcellular fraction (Fig 1C) were not signif-
ically affected by any of the doses of alcohols tested.

Unlike the detergent-extractable kinase, which displayed
the phospholipid and calcium requirements characteristic of
PKC, the detergent-insoluble protein kinase activity iso-
lated from PMNs was not dependent upon the presence of
exogenously added calcium and lipid. This may be a conse-
quence of these components remaining in the residual mem-
brane present in these fractions. To further characterize this
detergent-insoluble kinase activity, aliquots of the detergent-
nonextractable material from PMA-treated cells were incu-
bated with EGTA to establish calcium dependence or with
TFP, a phenothiazine that has been shown to inhibit both
PKC and the calmodulin-dependent protein kinase. EGTA
(1 mmol/L) inhibited the kinase activity by 62%, whereas
TFP, at concentrations of 50 and 100 μmol/L, inhibited the
activity by 39% and 77%, respectively (data not shown).

DISCUSSION

In several cell types including human PMNs, phorbol ester
tumor promoters translocate cytosolic PKC activity to a
membrane-containing fraction. This response may be
involved in tumor promotion as well as in the activation of
the respiratory burst by the active phorbol esters. It has
been shown that the active phorbol esters directly bind to
PKC in intact cells, and this enzyme is therefore thought to
be the receptor for these tumor promoters. Chemoattrac-

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Table 2. Effect of Alcohols on the Subcellular Distribution of Protein Kinase Activity in PMNs Treated With Stimulants

<table>
<thead>
<tr>
<th>Cells Stimulated With</th>
<th>Protein Kinase Activity (pmol/min/10^6 Cells) Isolated From Cells Treated With†</th>
<th>Buffer</th>
<th>Butanol§</th>
<th>Pentanol</th>
<th>Isoamyl Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td></td>
<td>394 ± 3</td>
<td>418 ± 34</td>
<td>385 ± 36</td>
<td>424 ± 42</td>
</tr>
<tr>
<td>FM LP</td>
<td></td>
<td>438 ± 47</td>
<td>418 ± 19</td>
<td>512 ± 3</td>
<td>441 ± 32</td>
</tr>
<tr>
<td>PMA</td>
<td></td>
<td>63 ± 15</td>
<td>0</td>
<td>0</td>
<td>79 ± 100</td>
</tr>
<tr>
<td>Particulate fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extractable</td>
<td></td>
<td>34 ± 5</td>
<td>27 ± 9</td>
<td>22 ± 2</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>FM LP</td>
<td></td>
<td>63 ± 4</td>
<td>37 ± 1</td>
<td>32 ± 6</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>PMA</td>
<td></td>
<td>121 ± 9</td>
<td>117 ± 9</td>
<td>102 ± 6</td>
<td>117 ± 17</td>
</tr>
<tr>
<td>Nonextractable</td>
<td></td>
<td>35 ± 1</td>
<td>20 ± 3</td>
<td>23 ± 4</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>FM LP</td>
<td></td>
<td>52 ± 10</td>
<td>28 ± 8</td>
<td>22 ± 2</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>PMA</td>
<td></td>
<td>146 ± 22</td>
<td>137 ± 7</td>
<td>150 ± 31</td>
<td>115 ± 1</td>
</tr>
</tbody>
</table>

†Where indicated, cells were incubated with 100 nmol/L FMLP for 60 seconds or with 1 μmg/mL PMA for five minutes. All samples contained 10
μmol/L cytochalasin B.

§Concentrations of alcohols: butanol, 0.25%; pentanol, 0.1%; isoamyl alcohol, 0.15%.

<table>
<thead>
<tr>
<th>Particulate Cell Fraction*</th>
<th>Fractional Stimulation of Protein Kinase Activity Isolated From Cells Incubated With†</th>
<th>Buffer</th>
<th>Butanol§</th>
<th>Pentanol</th>
<th>Isoamyl Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extractable</td>
<td></td>
<td>2.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.3 ± 0.6</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>FM LP</td>
<td></td>
<td>5.3 ± 0.6</td>
<td>4.7 ± 0.4</td>
<td>5.1 ± 0.7</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>PMA</td>
<td></td>
<td>2.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Nonextractable</td>
<td></td>
<td>6.9 ± 0.4</td>
<td>6.5 ± 0.7</td>
<td>8.0 ± 2.0</td>
<td>6.8 ± 1.3</td>
</tr>
</tbody>
</table>

*Following removal of cytosol, the particulate cell fractions were extracted with 1% Triton X-100 as indicated in Materials and Methods. Extractable and
nonextractable portions were assayed for PKC activity (extractable) and protein kinase (nonextractable) activities.

†Cells were incubated with the indicated stimulant as described in the legend to Table 2.

§Concentrations of alcohols: butanol, 0.25%; pentanol, 0.1%; isoamyl alcohol, 0.15%.
dependent and correlated with inhibition of superoxide production. The present study shows that low doses of aliphatic alcohols affect neither PMA-induced superoxide release nor enhancement of PKC activity in PMN particulate fractions. The alcohols have been shown to alter chemoattractant-mediated events at the level of the cell surface receptor, producing an increase in the affinity of the receptor, an increase in PMN chemotaxis, but a corresponding selective inhibition of chemoattractant-mediated superoxide production. The present study shows that low doses of these same alcohols inhibit the FMLP-induced appearance of both PKC and the nonextractable protein kinase activity in particulate cell fractions. This effect of alcohols was dose dependent and correlated with inhibition of superoxide production. In contrast, the alcohols affected neither PMA-induced superoxide release nor enhancement of PKC activity in particulate fractions.

The protein kinase activity is the detergent-insoluble particulate fraction cannot be termed PKC since it is not stimulated by exogenously added calcium and PS. This may be due to the presence of residual lipid and calcium in the detergent-insoluble preparations. This detergent nonextractable protein kinase activity was, however, inhibited by EGTA and TFP, the latter of which is a known inhibitor of both PKC and the calmodulin-dependent, cyclic guanosine monophosphate (cGMP)-stimulated protein kinase associated with cytoskeletal fractions. The insoluble kinase activity also was not stimulated further by cGMP nor by calmodulin and cGMP (data not shown), suggesting that it may be PKC in a form that does not require exogenously added Ca2+ or lipids. However, at this time we cannot specifically identify the kinase activity that appears in the detergent-insoluble particulate fractions of PMNs following FMLP or PMA stimulation.

The present studies suggest that stimulation of the respiratory burst in human PMNs by the chemoattractant FMLP may require the appearance of protein kinase activity in particulate fractions. We base this conclusion on the ability of the alcohols to block both the induction of the respiratory burst and the appearance of particulate-associated kinase activity mediated by chemoattractants. Indeed, NADPH oxidase, the enzyme responsible for the production of superoxide, is located in the particulate fraction of stimulated PMNs, although it is not yet known if this enzyme system is directly regulated by phosphorylation. Chemotaxis is actually enhanced by doses of alcohols that inhibit the appearance of protein kinase activity in the particulate fractions; so this increase in particulate fraction–associated activity is apparently not required for directed motility.

The effects of the alcohols appear to be on a chemoattractant–receptor–coupling mechanism required for activation of the respiratory burst that occurs distal to ligand binding. Since the doses of alcohols used enhance the average affinity of the chemoattractant receptor as well as membrane fluidity, they could act by altering the ability of the receptor to make contact with extramembranous or transmembranous transduction units such as the guanine nucleotide regulatory unit or phospholipase C with which this receptor appears to interact. The relationship between the affinity of the receptor and its ability to initiate the redistribution of protein kinase activity to the particulate fraction is currently unknown. However, the data suggest that the affinity state of the chemoattractant receptor reflects its ability to mediate such a redistribution of kinase activity and that this process may play a role in determining whether PMNs migrate or release cytotoxic products in response to chemoattractants.

The alcohols did not have nonspecific toxic effects since neither superoxide production nor changes in particulate fraction–associated PKC alterations induced by PMA were affected. One would not expect to see an effect of the alcohols on these PMA-induced functions since phorbol esters directly bind to and activate PKC, thus bypassing the more complexly regulated receptor-coupling mechanisms operating in chemoattractant-mediated stimulation. The apparent requirement for the appearance of kinase activity in the particulate fraction for stimulation of superoxide release suggests that subcellular redistribution of protein kinases during cellular activation leads to phosphorylation of membrane-associated proteins. This process may be a necessary...
step in the biochemical transduction of chemoattractant-receptor–mediated signals for activation of the respiratory burst.

ACKNOWLEDGMENT

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Chemoattractant-mediated stimulation of the respiratory burst in human polymorphonuclear leukocytes may require appearance of protein kinase activity in the cells' particulate fraction

MC Pike, L Jakoi, LC McPhail and R Snyderman