Platelet-Activating Factor Induces Protein Kinase Activity in the Particulate Fraction of Human Neutrophils

By James C. Gay and Ella S. Stitt

Platelet-activating factor (PAF) is a proinflammatory lipid that has both platelet- and phagocyte-stimulating properties. Because several known activators of calcium-, phospholipid-dependent protein kinase (protein kinase c, PKC) also stimulate neutrophil responses and because neutrophil stimuli such as phorbol diesters and the chemotactic peptide f-Met-Leu-Phe are reported to increase protein kinase activity in neutrophil (PMN) particulate fractions, we investigated the effect of PAF on neutrophil protein kinase activities. In neutrophils exposed to 10^{-8} mol/L PAF, cytosolic PKC activity was 521 ± 38 pmol ^32P/10^7 PMN/min (mean ± SEM), which was not significantly lower than cystolic activity in buffer-treated controls (558 ± 32 pmol ^32P/10^7 PMN/min, n = 14). PAF-exposed cells exhibited a concomitant rise in protein kinase activity associated with the particulate fraction with 53 ± 4 pmol ^32P/10^7 PMN/min compared with 32 ± 2 pmol in control cells (n = 14). Particulate protein kinase activity was independent of the presence of calcium and phospholipid in the assay medium. The specific PKC inhibitor H-7 inhibited particulate protein kinase activity, however, which suggested that the enzyme activity assayed in this fraction may be PKC in a constitutively activated form. The increase in particulate protein kinase activity induced by PAF required the presence of cytochalasin B, which was detectable within 5 seconds of exposure to PAF, and was not reversed by washing the cells free of extracellular PAF after initial exposure. Although PAF did not have a direct effect on PKC activity from cytosolic fractions from resting cells, the increase in particulate protein kinase activity induced by PAF was inhibited when the cells were first depleted of calcium by incubation with Quin 2. These results suggest that PAF induces an increase in particulate protein kinase activity in neutrophils by a calcium-dependent mechanism and that the induction of membrane-associated protein kinase activity may be involved in neutrophil-stimulating actions such as superoxide production, which occur at higher concentrations of PAF.

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Submitted June 15, 1987; accepted September 2, 1987.

Supported by grant No. AI 22382-01 from the National Institutes of Health and the Biomedical Research Support Grant Program (Grant No. RR-95424).


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0006-4971/88/7101-0028$3.00/0
Chemical Co, St Louis. Hanks' balanced salt solution (HBSS) was obtained from Gibco, Grand Island, NY. HEPES buffer was purchased from Flow Laboratories, MacLean, VA. γ-32P-ATP was obtained from New England Nuclear, Boston.

All lipids were stored at -4°C in chloroform. Immediately before use, an aliquot from the stock solution was evaporated under N2, redissolved in 2% BSA in saline, and subsequently added to the final incubation media.

Neutrophil suspensions. Human neutrophils were isolated from heparinized venous blood from healthy adult volunteers by Ficoll-Hypaque density gradient centrifugation as previously described. Cells were suspended in HBSS with 10 mmol/L HEPES and NaHCO3 at pH 7.4.

Neutrophil stimulation. Neutrophils (3 x 10⁷) were pre-warmed at 37°C for ten minutes in the presence of 10⁻⁵ mol/L cytochalasin B. Sodium azide (1 mmol/L) was then added and the cells incubated for an additional three minutes. Cells were then exposed to either PAF or an equal volume of 2% BSA in saline for 5 minutes. The reaction was stopped by the addition of 4 vol ice-cold HBSS and the tubes placed in a melting ice bath. After low-speed centrifugation, the supernatant was discarded and the cell pellet resuspended in extraction buffer consisting of Tris buffer, pH 7.5 (50 mmol/L), 2-mercaptoethanol (50 mmol/L), PMSF (1 mmol/L), EDTA (2 mM), and EGTA (5 mmol/L). Cell breakage was achieved with sonication for 2.5 minutes by using a Fisher Sonic Dismembrator Model 300. After sonication, the cell homogenate was centrifuged at low speed to remove nuclei and unbroken cells. The homogenate was then centrifuged at 100,000 g for 60 minutes at 5°C and the resultant supernatant removed and used as the cytosolic fraction. The 100,000-g pellet (particulate fraction) was resuspended in the original volume of extraction buffer to which had been added Triton X-100 detergent (0.1% final concentration). The reaction was stopped by the addition of 4 vol ice-cold HBSS and the tubes placed in a melting ice bath. After low-speed centrifugation, the supernatant was discarded and the cell pellet resuspended in extraction buffer consisting of Tris buffer, pH 7.5 (50 mmol/L), 2-mercaptoethanol (50 mmol/L), PMSF (1 mmol/L), EDTA (2 mM), and EGTA (5 mmol/L). Cell breakage was achieved with sonication for 2.5 minutes by using a Fisher Sonic Dismembrator Model 300. After sonication, the cell homogenate was centrifuged at low speed to remove nuclei and unbroken cells. The homogenate was then centrifuged at 100,000 g for 60 minutes at 5°C and the resultant supernatant removed and used as the cytosolic fraction. The 100,000-g pellet (particulate fraction) was resuspended in the original volume of extraction buffer to which had been added Triton X-100 detergent (0.1% final concentration). The pellet was then gently stirred at 4°C, usually overnight, before the assay for protein kinase activity. In some experiments the pellet was further fractionated by an additional 100,000-g centrifugation for 60 minutes. The resulting supernatant was used as the Triton X-100 extractable fraction and the remaining pellet, the residual particulate fraction.

Determination of PKC activity. Cytosolic and particulate fractions were assayed by modifications of the methods of Kikkawa et al and Wolfson et al. The standard assay mixture (250 μl) consisted of Tris buffer, pH 7.5 (35 mmol/L), MgCl₂ (10 mmol/L), CaCl₂ (2 mmol/L), PS (5 μg), diolein (0.5 μg), histone (40 μg), ATP (50 mmol/L), EDTA (2 mM), and EGTA (5 mmol/L). Cell fraction sample as an enzyme source. After addition of the enzyme source, the reaction mixture was incubated for ten minutes for cytosolic fractions and 30 minutes for particulate fractions. Protein kinase activity was linear throughout the time periods studied. The reaction was stopped by addition of 1 mL ice-cold 25% trichloroacetic acid (TCA), following which 0.5 mg BSA was added as a carrier. Precipitated proteins were collected on 0.45-μm nitrocellulose filters (GN-6, Gelman Sciences, Inc, Ann Arbor, MI), dissolved in scintillation fluid (Aquasol, New England Nuclear) and counted on a liquid scintillation counter. Results were expressed as pmol 32P incorporated per 10⁴ cell equivalents per minute or pmol 32P per mg protein per minute. In cytosolic and detergent-extractable particulate fractions, PKC activity was determined by subtracting the activity detected in the absence of calcium and lipids. Protein kinase activity in whole and residual particulate fractions was unaffected by added calcium and lipids, as noted by others. Thus, Ca/PL-independent protein kinase activity is reported in these fractions.

Protein determinations were performed by the method of Bradford.

Cell viability. Trypan blue dye exclusion was used to determine viability of neutrophil suspensions. Viability was always >95% and remained so despite exposure to PAF concentrations up to 10⁻⁴ mol/L or calcium chelators.

Statistical methods. Variance is expressed as the mean ± SEM and analysis of variance determined by Student’s t test.

RESULTS

As noted by other investigators, PKC activity in unstimulated neutrophils sonicated in the presence of calcium chelators was detected only in the cytosolic fraction. When neutrophils were exposed to 10⁻⁴ mol/L PAF for ten minutes, no significant change in cytosolic PKC activity was observed (558 ± 32 pmol 32P/10⁶ neutrophils/min in unstimulated cells vs 521 ± 38 pmol 32P/10⁶ neutrophils/min in PAF-treated cells, n = 14, P > .4). At the same time, there was an increase in protein kinase activity associated with the particulate fraction (32 ± 2 pmol 32P for controls vs 53 ± 4 pmol 32P with PAF, n = 14, P < .01). When calculated on the basis of activity per milligram protein, there was also no significant change in cytosolic PKC activity (2,450 ± 153 pmol 32P/mg/min in controls vs 2,348 ± 216 with PAF, n = 12, P = .7), but an increase in particulate protein kinase activity was observed (109 ± 11 pmol 32P for controls vs 223 ± 23 pmol 32P with PAF, n = 12, P < .001). The increase in particulate protein kinase activity was detected at PAF concentrations as low as 10⁻⁵ mol/L (Fig 1).

Also noted by other investigators, protein kinase activity in the particulate fraction was largely independent of exogenous calcium and phospholipid in the assay medium. Figure 1 shows that the PAF dose-response curve for particulate kinase activity obtained in the presence of calcium and PS was essentially superimposable with that obtained in their absence. In contrast, cytosolic protein kinase activity was predominantly (~80%) Ca/PL dependent (PKC by definition) and in the absence of activators was unaffected by PAF concentration. The increase in particulate protein kinase activity was dependent on the presence of cytochalasin B during stimulation (Table 1). Only at the highest PAF concentration examined (10⁻³ mol/L) was any increase in particulate protein kinase activity detected (120% ± 12% of controls, n = 3) in the absence of the microfilament inhibitor.

Because PAF-induced oxidative metabolism begins within seconds of cell exposure, we next examined the time course of the increase in particulate protein kinase activity to determine whether there was a correlation with the time course for PAF-mediated superoxide production. Figure 2 shows that by 5 seconds after exposure to PAF an increase in particulate protein kinase activity was evident. The activity increased to a maximum approximately 30 seconds after exposure and remained elevated over control levels for at least 45 minutes. Measured protein kinase activity tended to decrease with incubation times greater than ten minutes, however, which may reflect the activity of neutrophil phosphatases that remove 32P from sites on the histone substrate molecule.
Fig 1. Dose-response effects of PAF on neutrophil protein kinase activity in cytosolic (A) and particulate (B) fractions. A quantity of $3 \times 10^7$ neutrophils was exposed to either BSA or PAF at the indicated concentration for ten minutes before fractionation. (A) Cytosolic PKC activity and Ca/PL-independent activity was unaffected by PAF. (B) Protein kinase activity in the particulate fraction was independent of the presence of added calcium or phospholipid in the assay mix. (a), with added calcium and phosphatidylycerine; (o), without calcium and phosphatidylycerine. Data points represent means ± SEM from at least six separate experiments.

Table 1. Effect of Incubation Conditions on Particulate Protein Kinase Activity

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Activity (pmol $32P/10^7$ PMN/min)</th>
</tr>
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<tbody>
<tr>
<td>With CB*</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>36 ± 1†</td>
</tr>
<tr>
<td>PAF</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>Without CB*</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>PAF</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>Washed before‡</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>PAF</td>
<td>54 ± 1</td>
</tr>
<tr>
<td>Washed after§</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>PAF</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>Further Particulate Fractionation</td>
<td></td>
</tr>
<tr>
<td>Extractable</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>PAF</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>Residual</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>PAF</td>
<td>36 ± 1</td>
</tr>
</tbody>
</table>

Abbreviations: PMN, polymorphonuclear leukocytes; CB, cytochalasin B.

*Neutrophils were exposed to either 2% BSA (buffer) or $10^{-8}$ mol/L PAF in the presence or absence of $10^{-5}$ mol/L cytochalasin B before fractionation and determination of kinase activity in the whole particulate fraction.

†Mean ± SEM of triplicate determinations from two to three separate experiments.

‡Neutrophils were washed twice in HBSS then exposed to buffer or $10^{-8}$ mol/L PAF. Incubation was continued for five minutes, after which the cells were subjected to fractionation.

§Neutrophils were exposed to buffer or PAF for five minutes and subsequently washed twice in HBSS to remove extracellular PAF. After resuspension in HBSS, incubation was continued for five minutes before cell fractionation.

Particulate fractions of buffer- and PAF-treated neutrophils were further fractionated by a second 100,000-g centrifugation for 60 minutes. The supernatant was used as the extractable pellet sample and the pellet as the residual sample. Activity in the extractable pellet was Ca/PL dependent, whereas that in the residual pellet was Ca/PL independent.

We next examined the reversibility of the PAF effect with vigorous cell washing (Table 1). Cells were exposed to PAF for five minutes, and extracellular PAF was removed by two centrifugations with resuspension of cells in buffer without PAF. Particulate activity remained elevated. Thus the PAF effect on particulate protein kinase activity did not require the continued presence of PAF in the extracellular environment, although cellular uptake or persistent receptor binding of PAF may perpetuate the response.

Upon further separation of the pellets into detergent-extractable and -nonextractable fractions, most of the increase in protein kinase activity was localized to the extractable pellet (Table 1). Extractable protein kinase activity again became Ca/PL dependent although the residual particulate protein kinase activity remained independent of the presence of activators. The total activity in these fractions together was greater than that recovered in the

Fig 2. Time course of PAF-mediated increase in particulate protein kinase activity. Neutrophils were exposed to $10^{-8}$ mol/L PAF for the indicated times before cell fractionation and assay of protein kinase activity. Data points represent means ± SEM of triplicate determinations from three experiments. Results for time points of 5 seconds and longer are statistically different from time 0 at a P value of <.05.
unfractionated pellet, which suggested the liberation of activity in the fractionation process or the loss of an inhibitor of protein kinase activity.

To determine the specificity of PAF in increasing particulate protein kinase activity, we studied the activity in neutrophils exposed to lipids structurally similar to PAF. Neither lyso-PAF, GPC, nor monopalmitin increased particulate protein kinase activity at concentrations up to 10^{-5} mol/L (Fig 3), in accord with reports that these compounds are either inactive or require much higher concentrations to elicit responses compared with PAF.\textsuperscript{12,13} In contrast, OAG not only markedly increased particulate protein kinase activity but also elicited a decrease in cytosolic PKC activity in manner similar to that reported for PMA.\textsuperscript{8} Therefore, despite the fact that PAF and OAG share a glycerol backbone and 2-acetyl substitutions, the mechanisms of increasing particulate protein kinase activity appear to be different, with OAG possibly causing translocation of PKC activity from the cytosolic to the particulate fraction and PAF possibly causing an increase in the activity of the protein kinase that is present in the particulate fraction of unstimulated cells.

To investigate the mechanism whereby PAF augmented particulate protein kinase activity, we sought evidence for PAF as a direct activator of PKC. When the cytosolic fraction of unstimulated neutrophils was used as the PKC source, no direct effect of PAF could be demonstrated (Table 2). PAF could not substitute for phospholipid or diolein in promoting enzyme activity. PAF also had no direct effect on the activity of Ca/PL-independent particulate protein kinase from resting cells (data not shown).

To further study the mechanism of the PAF effect, the role of calcium in promoting particulate protein kinase activity was investigated. First, neutrophils were incubated in HBSS without calcium and with Quin 2 (30 \textmu mol/L) as an intracellular chelator of calcium before exposure to PAF. This approach has been shown to severely reduce intracellular calcium levels in human neutrophils.\textsuperscript{14} In cells so treated the increase in particulate protein kinase activity associated with exposure to PAF was markedly inhibited (Fig 4), although cytosolic PKC activity was unaltered (data not shown). Next, neutrophils were incubated with HBSS without calcium and with 1 \textmu mol/L EGTA before stimulation. This procedure also inhibited the increase in particulate protein kinase activity in PAF-treated cells (Fig 4). These data suggest that PAF-mediated modulation of intracellular calcium directly augments particulate protein kinase activity.

Because the protein kinase activity in the particulate fraction was independent of added calcium and phospholipid, it cannot be stated with certainty that this represents PKC. To further characterize the enzymatic activity, histone phosphorylation by the particulate fraction was measured in the presence of H-7 (100 \textmu mol/L), an inhibitor of PKC.\textsuperscript{15} Although it is reported that the Ki value of H-7 for PKC is 6 \textmu mol/L,\textsuperscript{11} in preliminary studies we found little inhibition of cytosolic PKC or particulate Ca/PL-independent protein kinase activity at this concentration. At 100 \textmu mol/L H-7, however, both cytosolic PKC activity and particulate Ca/PL-independent activity were inhibited to a similar degree (inhibition by 100 \textmu mol/L H-7, 68% ± 6% for cytosolic PKC, 51% ± 4% for particulate protein kinase activity from control cells, and 61% ± 4% for particulate protein kinase activity from PAF-treated cells; data from six to nine separate experiments). Thus, it would appear that the H-7–inhibited protein kinase activity in particulate fractions was PKC. We speculate that higher concentrations of H-7 are required to inhibit PKC in neutrophil fractions than are required to inhibit the activity of more purified enzyme.

**Table 2. Lack of a Direct Effect of PAF on PKC Activity**

<table>
<thead>
<tr>
<th>Activators</th>
<th>Activity (pmol 32P/10^7 PMN/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>244 ± 26*</td>
</tr>
<tr>
<td>Ca</td>
<td>220 ± 27</td>
</tr>
<tr>
<td>Ca + PS</td>
<td>592 ± 41</td>
</tr>
<tr>
<td>Ca + diolein</td>
<td>249 ± 22</td>
</tr>
<tr>
<td>Ca + PS + diolein</td>
<td>589 ± 43</td>
</tr>
</tbody>
</table>

By using the cytosolic fraction from unstimulated neutrophils as the enzyme source, PKC activity was determined under assay conditions using the addition of various mediators: Ca, 2.0 mmol/L; PS, 5 \textmu g; diolein, 0.5 \textmu g; PAF, 1.1 \mu g.

*Mean ± SEM of triplicate determinations from four separate experiments. No statistical difference was observed between results with v without PAF.

![Fig 3. Ability of lipids with structural similarity to PAF to influence neutrophil protein kinase activity.](image-url)

Neither GPC nor lyso-PAF had an effect on cytosolic PKC or particulate protein kinase activity at concentrations up to 10^{-5} mol/L. Monopalmitin was likewise without effect (data not shown). OAG induced both a decrease in cytosolic PKC activity (A) and an increase in particulate protein kinase activity (B). Data points represent means of three to four separate experiments.
cells exposed to PAF, and little increase in particulate activity (Table 3).

Preparations.17 This hypothesis is consistent with the experience of other investigators who have used higher H-7 concentrations to inhibit PMA-stimulated neutrophil responses.18,19 Under the influence of 100 μmol/L H-7, protein kinase activity was reduced in both control pellets and pellets from cells exposed to PAF, and little increase in particulate activity was detectable from PAF-treated cells in the presence of either inhibitor (Table 3).

DISCUSSION

The direct neutrophil-stimulating actions of PAF are well described. As with other chemotactants,20 induction of O2\textsuperscript{-} production requires agonist concentrations several orders of magnitude higher than are required to initiate chemotaxis.1 In the present report, we demonstrate that exposure of neutrophils to PAF concentrations of 10\textsuperscript{-7} mol/L and higher, concentrations at which PAF stimulates oxidative metabolism,1,5 was associated with an increase in protein kinase activity in the particulate fraction. Cytosolic PKC activity did not change significantly. The hypothesis that particulate protein kinase activity is involved in the generation of O2\textsuperscript{-} induced by PAF was further supported by the finding that cytochalasin B was required for optimal induction of O2\textsuperscript{-} production and particulate protein kinase activity and that both O2\textsuperscript{-} production and increased particulate protein kinase activity occurred within seconds of exposure to PAF.

A number of neutrophil stimuli have been shown to increase particulate protein kinase activity in neutrophils. The list of active agonists includes such diverse agents as phorbol esters, synthetic diacylglycerols, chemotactic peptides, C5a, calcium ionophores, and more recently, PAF and leukotriene B\textsubscript{4}.8,10,21,22 These observations provide evidence linking particulate protein kinase activity to activation of various neutrophil functions. The present paper and a recent report by O’Flaherty and Nishihira23 offer evidence suggesting that at least some of the neutrophil-stimulating actions of PAF may be mediated by PKC. In an earlier paper Nishihira et al were unable to detect increased particulate phorbol binding in neutrophils stimulated with PAF.21 In the subsequent article, using more rapid fractionation techniques these investigators reported that PAF evoked a reversible decrease in cytosolic phorbol binding as well as a modest and reversible increase in particulate binding.22 Histone phosphorylation assays of PKC activity are said to parallel phorbol binding studies although phosphorylation data are not presented. Our results differ in several regards. First, we were unable to detect a significant decrease in cytosolic PKC activity in response to PAF. Our observation is similar to the reported effect of chemotactic peptides on neutrophil particulate protein kinase activity.10 Although measurements of PKC by phorbol binding and histone phosphorylation generally correlate, it is not known whether this holds true in all situations and with all stimuli. Differences in fractionation techniques, calcium chelator concentrations, and other assay conditions may account for differences in reported results. One important difference between our fractionation techniques and those of O’Flaherty and Nishihira is that their method allows very rapid (minutes) isolation of the cytosolic fraction whereas our fractionation procedure required approximately two hours from cell stimulation to completion. It is possible, therefore, that transient decreases in cytosolic PKC activity would be missed by our technique. A second difference is that we observed increases in particulate protein kinase activity in response to PAF that persisted to at least 45 minutes, whereas O’Flaherty and Nishihira found the particulate response to be reversible in 15 to 30 minutes. The reasons for this discrepancy are unclear, but the nature of such differences may greatly enhance our knowledge of the biochemistry and functions of neutrophil protein kinases.

Because particulate protein kinase activity induced by PAF did not require the presence of added calcium and phospholipid during assay, one cannot definitively identify this activity as PKC at the present time. Several lines of evidence, however, suggest that PKC may indeed be the responsible phosphorylating enzyme in the particulate fraction. First, several reports demonstrate that PKC that is bound to membranes may exist in a constitutively activated form that is independent of the presence of added activators.11,12 Second, when whole pellets from control and PAF-treated neutrophils were subjected to additional frac-

**Table 3. Effect of H-7 on Particulate Protein Kinase Activity**

<table>
<thead>
<tr>
<th>Activity</th>
<th>No Inhibitor</th>
<th>H-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibitor</td>
<td>29 ± 2*</td>
<td>16 ± 4 (45)†</td>
</tr>
<tr>
<td>PAF</td>
<td>59 ± 3</td>
<td>21 ± 5 (64)</td>
</tr>
</tbody>
</table>

Particulate fractions from cells exposed to BSA (control) or 10\textsuperscript{-8} mmol/L PAF were assayed for protein kinase activity in the presence or absence of 100 μmol/L H-7.

*Mean ± SEM of three separate experiments.
†Percent inhibition of results obtained in the absence of any inhibitor.
tion into Triton X-extractable and -nonextractable fractions, the extractable portion again became Ca/PL dependent. This suggests that at least a portion of particulate protein kinase activity is altered by the fractionation procedure to an activity that meets the definition of PKC. Third, particulate activity was inhibited by the PKC inhibitor H-7. Finally, it is possible that adequate quantities of calcium and phospholipid exist in the neutrophil plasma membrane to satisfy activation requirements for PKC. Thus, PKC remains a strong candidate for the particulate protein kinase activity detected by our assay conditions.

The molecular mechanism whereby PAF increases particulate protein kinase activity remains to be fully elucidated. The characteristics of activation by PAF are similar to those reported for the chemotactic peptide FMLP. Pike et al. observed that FMLP increased particulate protein kinase activity without a change in cytosolic PKC activity and that the increase was dependent on the presence of cytochalasin B. The increase in particulate fraction kinase activity initiated by FMLP and PAF could be due to increased activity of protein kinase that is membrane bound in resting cells. The end result is an increase in total PKC activity over that found in resting cells. In contrast, we share the experience of other investigators who have reported that in neutrophils exposed to PMA the increase in particulate protein kinase activity does not equal the loss in cytosolic PKC activity. Therefore, baseline total protein kinase activity is not recovered in cells stimulated with PMA. Whether this lack of total recovery is related to incomplete extraction of particulate enzyme, intracellular PKC inhibitors, or altered substrate specificity is not clear at the present time. Although it would be helpful to discover whether the remainder of the PKC in PMA-treated cells is indeed active or has been somehow inactivated, the marked increase in particulate protein kinase activity remains a potentially very important component of the mechanism whereby PMA effects neutrophil responses. The biochemical effects of PAF and FMLP on neutrophil protein kinase activity are clearly different from those of PMA and suggest that the actions of stimuli that bind to specific membrane receptors differ from those that directly activate PKC. We were unable to demonstrate any direct effect of PAF on PKC activity in isolated cytosolic fractions, which is in agreement with O'Flaherty and Nishihira. We did find, however, that increases in particulate protein kinase activity were inhibited by chelation of calcium during stimulation. PAF is known to increase intracellular calcium levels in both platelets and neutrophils. It is therefore possible that calcium that is taken up from the extracellular milieu or released from intracellular stores is available to bind to membrane-bound PKC and enhance phosphotransferase activity. A recent report that demonstrates that increasing cytosolic calcium in intact differentiated HL-60 cells augments phorbol ester binding is in keeping with this hypothesis. Alternatively, increased intracellular calcium may increase the activity of calpain, a calcium-requiring proteinase that has been shown to cleave PKC to a form no longer dependent on added calcium and phospholipid. However, Melloni et al. observed that when calpain was active PKC disengaged from the membrane in a soluble form. In our studies, cell fractionation was performed in the presence of calcium chelators, a step designed to prevent proteolytic degradation of PKC by calcium-dependent proteases including calpain. In this situation, intracellular calcium may be increased by PAF during stimulation although calcium chelation may inactivate calpain during fractionation. Indeed, Melloni et al. found that when calcium levels increased but calpain was inhibited PKC remained membrane bound. Therefore, we postulate that our assay conditions detect increased amounts of PKC activity associated with the membrane as a result of increased intracellular calcium induced by PAF. Once membrane association occurs, PKC then may lose its requirement for calcium and phospholipid. PAF has also been shown to stimulate phosphatidylinositol turnover in platelets, and the diacylglycerol liberated in this process could also stimulate PKC activity. If the mechanisms of activation of PKC by PMA and chemotaxtractants are different, then combined exposure to PMA and either PAF or FMLP may enhance particulate protein kinase activity. Preliminary experience from our laboratory indicates that this is indeed the case.

We noted that total protein kinase activity from pellets that had been further fractionated to detergent-extractable and residual portions was greater than that noted in unfractionated pellets, although the relative increase in PAF-treated cells over control cells was similar (Table 1). This suggested to us that the second pellet fractionation liberated protein kinase activity or that this procedure resulted in the loss of protein kinase–inhibitory activity present in the unfractionated pellets. Balazovich et al. have reported a PKC inhibitor in human neutrophils that is associated with specific granules. Thus, there may be more latent protein kinase activity in the unfractionated pellet that may be activated under specific conditions. Furthermore, because our data suggest that particulate protein kinase activity may not represent activity translocated from the cytosol during stimulation, a reversal of inhibitory activity would be an alternative explanation for increased particulate activity not paralleled by decreased cytosolic activity. The activity of such PKC inhibitors is a potential point of regulation by neutrophil stimuli, and PAF-induced reversal of an endogenous particulate PKC inhibitor represents another possible mechanism for the PAF effect.

The present study further supports the hypothesis that the appearance of protein kinase activity in the particulate fraction of human neutrophils and the associated protein phosphorylation that follows are key steps in the induction of \( \text{O}_2^- \) generation induced by some stimuli. Several groups of investigators have described phosphorylation of components of the \( \text{O}_2^- \)-forming NADPH oxidase system of neutrophils by PKC. PKC-mediated phosphorylation may also be important in other neutrophil responses evoked by higher PAF concentrations such as aggregation and adherence but likely plays little or no role in mediating chemotaxis because no increase in particulate protein kinase activity was noted at optimal chemotactic concentrations of PAF. The physiologic relevance of PAF-induced phosphorylation is further supported by the recent observation in our laboratory that the phosphorylation of endogenous particu-
late proteins is also increased by PAF (unpublished observations). The identification of the phosphorylated proteins and further study of the molecular biochemistry of the enzyme will help to elucidate its contribution(s) to the important role of the neutrophil in host defense.

ACKNOWLEDGMENT

The authors wish to thank Dr Linda McPhail for her valuable assistance with the protein kinase assay, Dr Larry Boxer for helpful suggestions, and Lucinda Jackson for expert manuscript preparation.

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Platelet-activating factor induces protein kinase activity in the particulate fraction of human neutrophils

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