Ca\textsuperscript{2+} and Phospholipid-Dependent Protein Kinase (Protein Kinase C) Activity Is Not Necessarily Required for Secretion by Human Neutrophils

By Kenneth J. Balazovich, James E. Smolen, and Laurence A. Boxer

Human neutrophils are known to respond to both chemotactic and chemical stimuli by migration to the site of inflammation, releasing intracellular granule contents and generating superoxide anions (O\textsubscript{2\textsuperscript{-}}).\textsuperscript{1-12} In addition, there appears to be a marked increase in the phosphorylation of a limited number of cellular proteins when neutrophils are treated with these agents.\textsuperscript{11,12} For example, it has been demonstrated that the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine, the tumor promoters phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu), and the diterpene ester mezerein all stimulate these responses.\textsuperscript{4,11,13-18} Phorbol esters are particularly useful as activators since they have been shown to bind directly to protein kinase C (PKC),\textsuperscript{19-21} an enzyme that requires both Ca\textsuperscript{2+} and phospholipid, but not calmodulin, as co-factors.\textsuperscript{22,23} PKC also binds to and is activated by 1,2-diacylglycerols, which are products of arachidonic acid metabolism in the neutrophil. Mezerein and both PMA and PDBu possess many structural and physiological similarities as tumor promoters,\textsuperscript{24} but more relevant to the present study is the finding that although it is not a phorbol diester, mezerein has been shown to displace PDBu from its binding site specifically, in a manner consistent with competitive inhibition of PKC.\textsuperscript{25-29}

Although there is strong circumstantial evidence for phorbol derivatives inducing critical cellular responses through PKC, the exact mechanisms for phorbol-induced O\textsubscript{2\textsuperscript{-}} production, degranulation, and phosphorylation are not known. There is evidence that the phosphorylation of a specific set of cellular proteins is causally related to PMA-induced reactions. For example, the release of serotonin may be related to the phosphorylation of a 47,000 dalton protein in platelets.\textsuperscript{30} In addition, the treatment of mast cells with PMA has been shown to induce the release of histamine and to stimulate the phosphorylation of specific cellular proteins.\textsuperscript{31} More important, rabbit neutrophils have been shown to secrete lysozyme and to phosphorylate endogenous cellular proteins when treated with PMA.\textsuperscript{32} In untreated neutrophils, most of the cellular PKC activity is found in soluble form; the remaining activity is associated with cellular membranes and the cytoskeleton.\textsuperscript{33,34} After neutrophils are treated with phorbol esters, PKC activity is primarily associated with insoluble cellular material (e.g., membranes), a phenomenon that has been termed "translocation" to describe a potential physical shift in PKC activity.\textsuperscript{35-37} In the absence of unambiguous data, however, it is not known if PKC translocation actually represents a change in the physical location of the enzyme (from soluble to insoluble cellular components); an alteration in PKC activities in those locations is also possible, especially in light of evidence that suggests two classes of binding sites on PKC for (\textsuperscript{3}H)PDBu.\textsuperscript{38} Although the mechanism of cellular activation involving PKC is poorly understood, it is currently hypothesized that PMA induces certain neutrophil functions, such as degranulation and O\textsubscript{2\textsuperscript{-}} production, by directly activating PKC.\textsuperscript{10}

This report concerns the potential role of PKC in mediating neutrophil activation. In brief, we confirmed previous results indicating that PMA and PDBu induce PKC activation, PKC translocation, degranulation and O\textsubscript{2\textsuperscript{-}} generation.\textsuperscript{2-7,20,33} Mezerein did not stimulate PKC activity and no PKC translocation was detected, but it did block PMA-stimulated PKC activation and translocation. Mezerein also induced degranulation and O\textsubscript{2\textsuperscript{-}} production to levels comparable with those caused by the phorbol esters. Most important, mezerein stimulated the phosphorylation of a slightly different set of endogenous cellular proteins that either PMA or PDBu In
light of these data, there may be at least two mechanisms for stimulating degranulation and \( \text{O}_2^- \) production, one of which may involve PKC.

**MATERIALS AND METHODS**

**Cells.** Whole human blood was obtained by venipuncture from normal, healthy donors 20 to 55 years of age. Neutrophils were isolated from this blood by Ficoll-Hypaque centrifugation and dextran sedimentation, followed by hypotonic lysis to remove residual erythrocytes. Washed cells were twice with Dulbecco's phosphate-buffered saline (PBS) or Hank's buffer (pH 7.5) before use; cells were >95% neutrophils as assayed by differential histologic staining, and were consistently >97% viable as assayed by trypan blue exclusion.

Reagents. Mezerein was obtained from LC Services (Woburn, Mass). Electrophoretic reagents were obtained from Bio-Rad (Rockville Center, NY). All other reagents were obtained from Sigma (St Louis), unless otherwise specified. Phorbol esters and mezerein were dissolved in dimethylsulfoxide and diluted with water to obtain final working stock solutions.

**Protein kinase C: cell stimulation and fractionation.** Cells were washed once in PBS and then incubated at 10^6 cells/mL in Hank's buffer containing the appropriate concentration of stimulus for 5 minutes at 37°C. The cells were then washed once in Hank's buffer to remove unbound stimulus. The final cell pellet was lysed by resuspension at 5 x 10^6 cells/mL in extraction buffer (50 mmol/L Tris-HCl, pH 7.5, 50 mmol/L of 2-mercaptoethanol, 1 mmol/L of phenylmethylsulfonylfluoride, 2 mmol/L of EGTA, and 0.05% Triton X-100) and sonicated on ice twice for 30 seconds each. Control cells were treated in an identical manner, but were subjected to electrophoresis on 10% polyacrylamide gels (160 x 180 mm, 5% acrylamide) and autoradiography. Some assays were conducted in the presence of phospholipid, calcium, and inhibitors, or agents such as 40 mmol/L of NaF or 1 mmol/L of EDTA, 0.25 mmol/L of Aprotinin and 0.1 mmol/L of trypsin inhibitor [Boehringer-Mannheim]). Total cellular protein was precipitated with 25% TCA and extracted with acetone. Final pellets were solubilized in 50 μl of sample buffer [62.5 mmol/L of Tris, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS plus bromphenol blue] and boiled for 3 minutes before freezing. 35 μg of solubilized protein was subjected to electrophoresis on 10% polyacrylamide gels (160 x 180 mm, according to the method of Laemmli). Autoradiography was performed at -70°C for 72 hours using Kodak X-Omat AR film.

**Enzyme release and \( \text{O}_2^\cdot^- \) assays.** Neutrophils were incubated at 10^6 cells/mL in Hank's buffer or PBS (pH 7.5) for 5 minutes at 37°C with or without phorbol, mezerein, or inhibitors. At the end of the incubation period, cells were centrifuged at 1,000 g for 10 minutes at 4°C, and the supernatant was removed. Supernatant material was then assayed for content of vitamin B\(_2\) binding protein or lactate dehydrogenase (LDH). The medium was then replaced with buffer containing a stimulus for 5 minutes, after which the reaction was stopped by the addition of ice-cold buffer, followed by a brief rinse with 10% TCA. Neutrophils were scraped from the dish in 3 mL of ice-cold Collection Buffer (0.25 mol/L of sucrose, 0.1 mol/L of NaF, 10 mmol/L of EDTA, 0.1 mmol/L of PMSF, 0.1 mmol/L of Aprotinin and 0.1 mmol/L of trypsin inhibitor [Boehringer-Mannheim]). Total cellular protein was precipitated with 25% TCA and extracted with acetone. Final pellets were solubilized in 50 μl of sample buffer [62.5 mmol/L of Tris, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS plus bromphenol blue] and boiled for 3 minutes before freezing. 35 μg of solubilized protein was subjected to electrophoresis on 10% polyacrylamide gels (160 x 180 mm, according to the method of Laemmli). Autoradiography was performed at -70°C for 72 hours using Kodak X-Omat AR film.

**Quin 2 assay for intracellular calcium.** Quin 2 loading and fluorescence measurements were performed according to the method of Korchak and co-workers, with the exception that PBS plus 1 mmol/L of Mg\(^{2+}\) and 0.6 mmol/L of Ca\(^{2+}\) was used as the medium.

**RESULTS**

**Stimulation of PKC activity by phorbol esters.** To examine the effect of phorbol esters and mezerein on PKC activity in human neutrophils, cell homogenates were separated into soluble and insoluble fractions, each of which was assayed using histone protein as a PKC substrate. PKC activity was defined as the specific incorporation of \( ^{32}\text{Pi} \) into histone (present in excess in the assay) which was dependent on the presence of phospholipid and calcium. Under the conditions of our assay, PKC was dependent on phospholipid and calcium, with optimal activity at 0.5 mmol/L of added calcium and 18 μg/mL of phosphatidylserine. Background activity (ie, in the absence of phospholipid, calcium, and activators) was <30 pmol \(^{32}\text{P}/10\) min/10^6 cell equivalents. Basal PKC activity (in the absence of phorbol) was low, ~33 ± 25 pmol \(^{32}\text{P}/10\) min/10^6 cell equivalents (Table 1). When incubated with 100 mmol/L of PMA or PDBu, soluble

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an analogue and competitive inhibitor of PMA and for protein kinase C (PKC). Aliquots of each fraction were then assayed rate. Activity material contained >75% of the total PKC activity (Table 1). Pretreatment with 10^{-9} mol/L of mezerein only partially blocked PDBu-induced PKC activity (Fig 2). Similar results were found with PMA (data not shown). These data suggest that mezerein blocked previously active PKC, perhaps by competing directly with the binding of PMA and PDBu, a possibility that is consistent with previously published results.25-29

PKC translocation and functional activation of neutrophils by phorbol derivatives. Because mezerein blocked the stimulation of PKC activity by PMA and PDBu, we looked at the effect of these agents on various neutrophil functions. To test the ability of phorbol esters and mezerein to induce PKC translocation, cells were pretreated with various concentrations of PMA, PDBu, or mezerein, lysed, and then separated into soluble and insoluble fractions. Under these conditions, translocation of PKC activity from the soluble to the insoluble fraction was observed at PDBu concentrations from 10^{-7} to 10^{-3} mol/L (Fig 3A), with a maximum at 10^{-4} mol/L. Higher drug concentrations resulted in cell lysis, as indicated by an increase in lactate dehydrogenase release from the cytoplasm (Fig 3, inset). Similar results were obtained in experiments with PMA (data not shown). PKC translocation was not detected in

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<th>Table 1. Human Neutrophil Protein Kinase C Activity</th>
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PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate.

isolated human neutrophils were sonicated and centrifuged at high speed to obtain soluble (supernatant) and insoluble (pellet) fractions. Aliquots of each fraction were then assayed for protein kinase C (PKC) activity by monitoring radioactive phosphate incorporated into histone, and using phorbol esters, mezerein, or 1,2-diolein as stimuli. Results were obtained from at least ten separate experiments, and each experiment was performed in triplicate; the data are means ± 1 SD for these experiments. Numbers in parentheses indicate the percentage of total PKC activity in that fraction.

material contained >75% of the total PKC activity (Table 1). Neither whole neutrophils nor neutrophil sonicates (either with or without nuclei) had any significant PKC activity.

PKC activity stimulated by PMA or PDBu in the soluble fraction from untreated cells was dependent on the concentration of the phorbol esters (Fig 1). In contrast, mezerein, an analogue and competitive inhibitor of PMA and PDBu, did not activate soluble PKC over the same concentration range (Fig 1). PMA and PDBu also activated PKC in the insoluble fraction (Table 1). Mezerein did not activate insoluble PKC above basal levels at any concentration tested (results not shown).

If neutrophils were treated with 10^{-6} mol/L of mezerein before lysis and the isolated soluble material was incubated with various concentrations of PDBu, nearly total inhibition of PDBu-dependent PKC activity was observed (Fig 2). Pretreatment with 10^{-9} mol/L of mezerein only partially blocked PDBu-induced PKC activity (Fig 2). Similar results were found with PMA (data not shown). These data suggest that mezerein blocked previously active PKC, perhaps by competing directly with the binding of PMA and PDBu, a possibility that is consistent with previously published results.25-29

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Fig 1. Phorbol ester- and mezerein-stimulated protein kinase C activity in human neutrophils. Cells were sonicated and centrifuged at 100,000 g to obtain soluble (supernatant) and insoluble (pellet) fractions. Soluble material was assayed for protein kinase C (PKC) activity in a cell-free assay for 10 minutes at 30 °C as described in the Materials and Methods section. Each point represents the mean ± 1 SD of at least ten experiments. The results show dose-response curves for phorbol 12-myristate 13-acetate (PMA) (●), phorbol 12,13-dibutyrate (PDBu) (▲), and for mezerein (◆).

Fig 2. Inhibition by mezerein of phorbol 12,13-dibutyrate (PDBu)-stimulated protein kinase C activity. Human neutrophils were incubated with buffer (●), 10^{-6} mol/L of mezerein (▲), or 10^{-9} mol/L of mezerein (●) for 5 minutes at 37 °C, washed to remove residual drug, and then sonicated. Sonicates were centrifuged at 100,000 g to obtain soluble (supernatant) and insoluble (pellet) fractions. The soluble fractions were then assayed for protein kinase C (PKC) activity using increasing doses of PDBu as a PKC activator in a cell-free assay. Each point represents the mean of two experiments, with each experiment performed in triplicate; SD from the mean were consistently <15%. The dotted line represents basal activity of PKC in the absence of phorbol.
Mezerein-treated cells, and mezerein appeared to inhibit PKC activity (Fig 3B).

Phorbol esters and mezerein were tested for their ability to stimulate the release of vitamin B\(_{12}\) binding protein, a constituent of specific granules, from intact cells. PDBu stimulated the release of intracellular granule contents into the extracellular medium (Fig 4), with maximal degranulation of 63% obtained at 10\(^{-8}\) mol/L. Experiments using identical concentrations of PMA produced similar results (data not shown). Mezerein also induced up to 60% degranulation, but a maximal response was obtained at drug concentrations 3 to 4 orders of magnitude higher. These results were obtained without significant concomitant lactate dehydrogenase release <10\(^{-4}\) mol/L (Fig 3, inset).

Human neutrophils were tested for their ability to generate \(\mathrm{O}_2^-\) in response to phorbol esters and mezerein. Although mezerein proved to be a better stimulus for generating \(\mathrm{O}_2^-\), both mezerein and PDBu induced the production of \(\mathrm{O}_2^-\) in a dose-dependent manner (Fig 5). Maximal production of \(\mathrm{O}_2^-\) was 10 nmol/5 min/10\(^6\) cells using 10\(^{-4}\) mol/L of PDBu or 10\(^{-7}\) mol/L of mezerein.

Our data suggested that mezerein stimulated neutrophils to degranulate and produce \(\mathrm{O}_2^-\) through a mechanism that was independent of PKC, since no PKC translocation and little PKC activity was observed, and that was different from that stimulated by PMA and PDBu. Because PKC is activated by calcium and because phorbol esters decrease the \(K_m\) for calcium, we wanted to determine if mezerein produced its effects by changing intracellular calcium concentrations. Concentrations of 1\(\mu\)mol/L of this compound did not cause a rise in intracellular calcium (data not shown). N-formylmethionyl-leucyl-phenylalanine (FMLP), however, at a 0.1\(\mu\)mol/L of concentration, caused the expected rise in intracellular calcium. These data suggest that mezerein produces its cellular effect without causing a rise in intracellular calcium, much as has been reported for PMA and PDBu.

Phorbol ester-induced phosphorylation of endogenous cellular proteins. We performed an SDS-PAGE analysis of endogenous cellular phosphorylation patterns generated by treating cells with phorbol esters or mezerein in an attempt to understand how such different responses could be elicited by similar agents. Cells were metabolically labeled with radioactive phosphorus (ie, \(^{32}\)Pi) and then incubated with 10\(^{-8}\) mol/L of PMA, PDBu, or mezerein for 5 minutes. Cellular protein was then collected and phosphorylation patterns were analyzed by separating 35 \(\mu\)g of protein by SDS-PAGE and visualizing phosphoproteins by autoradiography. The results of these experiments are presented in Fig 6. Control cells, which were incubated in the absence of phorbol or mezerein (Fig 6, D'), had at least 11 phosphoproteins visible by these methods. The high degree of phosphorylation observed in cells incubated without a stimulus was probably due to partial activation of control cells secondary to their adherence to Petri plates during phosphate loading. In all cases, treated cells had an increase in both the number of phosphoproteins (as compared with the control) and in the...
Fig 5. Effect of phorbol esters and mezerein on human neutrophil superoxide anion production. Neutrophils were incubated with various concentrations of either phorbol 12,13-dibutyrate (PDBu, \( \bullet \)) or mezerein (\( \bullet \)) for 5 minutes at 37 °C. The incubation medium was then assayed for superoxide dismutase-inhibitable superoxide anion \( (\mathrm{O}_2^-) \) production, monitored by the reduction of cytochrome c in the assay medium. Each point represents the mean ± SD of at least three experiments.

density of labeling of bands. Phosphoprotein patterns for cells treated with PMA (Fig 6, A') and with PDBu (Fig 6, B'), both used at 100 ng/mL, were nearly identical. Cells treated with 10^{-8} mol/L of mezerein (Fig 6, D'), however, had a higher degree of phosphorylation of proteins at 45, 38, 25, 24, and 15 kd as compared with phorbol-treated cells. More important, additional labeling was observed at 32 and 14 kd, and a doublet was observed at 20 kd, (arrows, Fig 6), results that were not observed in phorbol-treated cells. Although none of the phosphoproteins generated by phorbol or mezerein treatment were identified in this study, these preliminary results suggest that mezerein-treated intact neutrophils phosphorylate a slightly different subset of cellular proteins than do phorbol-treated cells.

DISCUSSION

Tumor-promoting phorbol esters elicit a series of responses when used as stimuli for intact neutrophils.\(^{1-17}\) For example, rabbit neutrophils release lysozyme from intracellular specific granules and also phosphorylate a limited number of cellular proteins when stimulated with PMA.\(^{32}\) In addition, Andrews and Babior\(^{12,42}\) and White and colleagues\(^{32}\) showed that phorbol derivatives stimulate the phosphorylation of a limited number of endogenous cellular proteins in human and in rabbit neutrophils. Correlations between the presence of phorbol esters and protein phosphorylation have been demonstrated in fibroblasts,\(^{48}\) mast cells,\(^{51}\) platelets,\(^{20}\) and lymphocytes,\(^{35,36}\) as well as in neutrophils.\(^{11,12,28,32,49}\) Although it is known that some phorbol esters bind directly to PKC, details of the activation mechanism after binding that contributes to these effects are not known.

We confirmed previous results demonstrating that PMA, PDBu, and mezerein all induce \( \mathrm{O}_2^- \) production, degranulation and phosphorylation events in the human neutrophil. Mezerein stimulated higher levels of \( \mathrm{O}_2^- \) production, though lesser amounts of degranulation, than did PDBu at similar concentrations. This observation compares favorably with that of Goldstein and co-workers\(^{18}\) who demonstrated that mezerein at 10^{-8} to 1.5 × 10^{-7} was a better inducer of \( \mathrm{O}_2^- \) production than was PMA.

We found that mezerein at 10^{-8} to 10^{-7} mol/L, unlike PMA and PDBu, induced no neutrophil PKC activity, nor did it lead to PKC translocation (Figs 2 and 3). Our results do not rule out the possibility that PKC had translocated to the insoluble fraction when treated with mezerein but was not active or was not detected under our assay conditions. For example, mezerein-activated PKC may not phosphorylate histone in our assay. Recent experiments have suggested this is not the case, since PMA and PDBu stimulated the phosphorylation of myosin light chain in our assay, but no such activity was detected at any concentration of mezerein.
tested (unpublished observations). We showed that mezerein blocked PDBu-stimulated PKC activity (Fig 3). In addition, whereas $10^{-8}$ mol/L of mezerein completely blocked both PMA- and PDBu-stimulated PKC activity both before and after cell disruption, $10^{-9}$ mol/L of mezerein only partially inhibited this event. These results are in direct contradiction to certain previously published reports involving mezerein. For example, both crude and partially purified PKC from mouse brain cytosol gave comparable activities with either PMA ($10^{-8}$ to $3 \times 10^{-4}$ mol/L) or mezerein. In contrast, our results were obtained with crude soluble and insoluble fractions of neutrophils (Table 1 and Figs 1–3), and we consistently obtained inhibition of PKC activity using a wide range of concentrations. The contrary results were reported only for narrow mezerein concentration ranges, although we demonstrated clear dose–response effects for concentrations from $10^{-8}$ to $10^{-4}$ mol/L. Although we have not directly shown that mezerein is a competitive inhibitor of PMA in our assay, our results demonstrate that pretreatment of whole cells with mezerein inhibits subsequent stimulation by PDBu (Fig 2) and are consistent with the hypothesis that mezerein and certain phorbol esters (ie, PMA and PDBu) may be competing for similar binding sites on the PKC molecule. It is possible that mezerein has inhibited or activated cellular pathways other than those stimulated by PMA and PDBu, and that PKC inhibition is simply peripheral to the specific effect of this agent.

The important implication from our results is that PKC activity, as measured by commonly used techniques, can be dissociated from well-known and very important neutrophil functions, namely degranulation and O$_2^-$ production, in the presence of mezerein. Significant differences were discovered in the phosphorylation patterns of endogenous neutrophil proteins from cells treated with mezerein, a compound that may stimulate cellular functions through a different mechanism than that used by PMA and PDBu. It is also noteworthy that cellular activation by mezerein does not cause a rise in intracellular calcium as monitored by Quin-2 fluorescence. Our work provides additional evidence that it is possible to dissociate PKC activity, as monitored by histone phosphorylation, from certain other neutrophil functions. Intact neutrophils treated with either PMA or PDBu (in contrast to mezerein) exhibited both an activation of PKC and translocation of enzymatic activity from the soluble to the insoluble fraction. These results are in good agreement with those of Kraft and co-workers and other researchers who showed a similar shift in PKC activity. Although mezerein appeared to inhibit PKC translocation, further experimentation is clearly required to prove this point. In certain cases, translocation of PKC may be necessary to elicit subsequent events in the activation pathway, such as endocytosis, degranulation, and O$_2^-$ production. For example, activation of PKC and its translocation to such cytoskeletal elements as microfilaments may trigger cell motility or surface protein redistribution, since the myosin light chain is phosphorylated by PKC. Phosphorylation of the myosin light chain has been shown in at least one system to lead to some of these events. PDBu has been shown to induce the phosphorylation of surface receptors for insulin and for epidermal growth factor and to induce the redistribution and capping of concanavalin A and Lyt-3 surface receptors in human lymphocytes.

Our preliminary studies using mezerein suggest that PKC activity can be inhibited under conditions in which neutrophils are stimulated to degranulate and generate O$_2^-$. In addition, in this study, mezerein induced the phosphorylation of a slightly different subset of cellular proteins than either PMA or PDBu (Fig 6), a regulatory event which may or may not mediate the same secretory responses as the phorbols. Previous models have linked PKC-generated phosphorylation to later events in the stimulus–response pathway in neutrophils. In light of the present data, it is now necessary to reconsider the existing model to accommodate the possibility that a PKC-independent mechanism may be operating in activated neutrophils. Any proposed alternate mechanisms must be independent of increases in intracellular calcium, since mezerein did not cause a rise in Quin-2 fluorescence. It must also take into consideration the activity of kinases other than PKC, since there were clearly differences in the phosphoproteins generated by mezerein or phorbol. As a minimum, it must be noted that PKC activity, as monitored by the cell-free and endogenous protein phosphorylation assays, is not an absolute prerequisite for neutrophil stimulation.

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Ca2+ and phospholipid-dependent protein kinase (protein kinase C) activity is not necessarily required for secretion by human neutrophils

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