Effect of Phorbol 12-Myristate 13-Acetate and Its Analogue 4α-Phorbol 12,13-Didecanoate on Protein Phosphorylation and Lysosomal Enzyme Release in Rabbit Neutrophils*

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The co-carcinogenic compound phorbol 12-myristate 13-acetate but not its inactive analogue 4α-phorbol 12,13-didecanoate causes the phosphorylation of several rabbit neutrophil polypeptides whose molecular weights and isoelectric points (pl) are as follows: M, = 40,000, pl = 6.4; M, = 50,000, pl = 4.9; M, = 55,000, pl = 6.3; M, = 64,000, pl = 6.0; M, = 70,000, pl = 5.6; M, = 90,000, pl = 6.0. Most of these phosphorylated proteins are located exclusively in the cytosol; the 64,000 molecular weight protein is found both in the cytosol and the cytoskeleton, and the 40,000 molecular weight protein is found in the nuclear pellet. The 50,000 molecular weight protein is also phosphorylated in whole cells by the chemotactic peptide fMet-Leu-Ph and in cell-free systems by protein kinase C. Using limited proteolysis, one phosphopeptide fragment was phosphorylated by the three stimuli. In addition, phorbol 12-myristate 13-acetate but not 4α-phorbol 12,13-didecanoate causes cell aggregation and the exocytotic release of the specific granules of rabbit neutrophils. In contrast, both compounds increase the amount of actin associated with the cytoskeleton. The divalent cation ionophore A23187 at low concentration and the compound phorbol 12-myristate 13-acetate act synergistically in causing neutrophil degranulation. Lysosomal enzyme release and the phosphorylation of the 50,000 molecular weight polypeptide produced by phorbol 12-myristate 13-acetate are inhibited by trifluoperazine, and these two responses seem to be causally related. These results are discussed in terms of the role of 1,2-diacylglycerol and activation of protein kinase C in specific granule release from rabbit neutrophils.

The present studies were undertaken in order to characterize in detail the effects of PMA and its inactive analogue 4α-PDD on the degree of protein phosphorylation in rabbit neutrophils and to examine the relationship between any possible protein phosphorylation and the various neutrophil responses (lysosomal enzyme release, cell aggregation, and actin association with the cytoskeleton) elicited by PMA.

MATERIALS AND METHODS
Rabbit peritoneal neutrophils (4- to 12-h exudates) were obtained, washed, and handled as previously described, and the cells were resuspended in protein and magnesium-free modified Hanks' balanced salt solution (17). A 5- to 10-min preincubation at 37°C preceded all experimental manipulations, and the experiments were all carried out at 37°C.

Lysosomal Enzyme Release—Lysosomal enzyme release was carried out as previously described (18). Briefly, 0.5-1 × 10⁶ cells/ml were incubated with or without the stimulus (PMA, 4α-PDD, and/or A23187) for a specific period of time, and then the tubes were transferred to ice. The tubes were spun down in a Sorvall RC3 centrifuge (2000 × g for 5 min at 4°C), and the supernatant from each of them was removed. The amount of lysozyme in the supernatant was determined by measuring the change in optical density of a suspension of Micrococcus lysodeikticus (0.16 mg/ml) in phosphate buffer (67 mM Na₂HPO₄, 67 mM NaH₂PO₄, pH 6.27). The total lysozyme was obtained by lysing a cell sample in Triton X-100 (0.5%), and enzyme release was expressed as percent of total lysozyme found...
in the pellet. In all the experiments reported, and unless specified otherwise, the release of lactate dehydrogenase, a measure of cell death, was less than 5% of the total cell content.

**Cell Aggregation—**Neutrophil aggregation was measured by monitoring the change in optical transmission at 600 nm of a cell suspension (2.5 × 10^7 cells/ml in a Zeiss PMQ111 spectrophotometer). Magnesium (0.74 mM, as MgCl₂) was added 1–3 min before the addition of either PMA or 4a-PDD, and the change in optical density was monitored continuously for 15 min.

**Isolation of Cytoskeletal Proteins—**The determination of actin association with the cytoskeleton was carried out as previously described (20–23), and the cytoskeletal structures were isolated as proteins insoluble in 1% Triton X-100 as described by Phillips et al. (19). A 500-μl aliquot from a cell suspension containing 10^7 cells/ml was distributed into various sets of Eppendorf microcentrifuge tubes (1.5 ml capacity). One set was always used as control and the remaining as experimental to which the desired additions were made. When PMA and 4a-PDD were used, 5% of a concentrated stock solution were added to give the desired concentration. The cells were allowed to react for the preset time before the reaction was stopped by the addition of cold Trition stock solution (500 μl). The Triton stock solution contained 2% Triton X-100, 160 mM KC1, 40 mM imidazole HCl, 20 mM EGTA, 2 mM EDTA, and 8 mM sodium azide, pH 7.0. The tubes were placed on ice for 10 min and then centrifuged for 5 min (8000 × g) in an Eppendorf microfuge. The supernatant was then decanted and the pellet dissolved in 50 μl of a solution containing 10% SDS, 5% mercaptoethanol, 20% glycerol, and 6 mM of HCl (pH 6.7) by vortexing in a boiling water bath with vigorous vortexing until dissolved; this usually took 10–15 min. Actin was determined by molecular weight and two-dimensional gel electrophoresis.

**Protein Phosphorylation—**To determine the ability of PMA and 4a-PDD to induce phosphorylation of specific polypeptides, cells, usually 5 ml of 1–3 × 10^7 cells/ml, were incubated with 1 mCi of [32P]orthophosphate for 30 min in a rotary shaker incubator (37 °C). The buffer contained: NaCl, 136 mM; KCl, 4.9 mM; glucose, 5.56 mM; Hepes, 10 mM; CaCl₂, 0.33 mM; pH 7.2. At the end of the incubation period the cells were washed twice with 25 ml of the above buffer and resuspended in buffer to give a final concentration of 3 × 10^6 cells/ml. PMA or 4a-PDD (50 μl) was added from a stock solution to give the desired final concentration (the final concentration of dimethyl sulfoxide did not exceed 0.1%). The reaction was stopped by removing an aliquot of the cell suspension (300 μl) and adding it to 200 μl of stopping solution at 100 °C, and the mixture was boiled for 5 min. The stopping solution consisted of 9% SDS, 13 mM β-mercaptoethanol, 20% glycerol, 86 mM Tris-HCl, 6 mM EDTA, 0.1% bromphenol blue, pH 6.7.

**Polyacrylamide Gel Electrophoresis—**The proteins were electrophoresed as described previously (20, 21) through a 10% straight gel (with 5% polyacrylamide in the stacking gel) according to the method of Laemmli (22). The gel was then stained, dried, and exposed as described in the previous section.

**Preparation of Phosphorylated Subcellular Fractions—**Neutrophils were washed once in phosphate-free Hank's buffer (see phosphorylation by PMA and 4a-PDD for composition) and resuspended in 5% SDS, 4.5 × 10^6 cells/ml. 2 mCi of [32P]orthophosphoric acid were added and the cell placed in a rotary incubator for 30 min. The cells were then centrifuged (2000 × g for 2 min), washed 3 times (25 ml per wash), and then divided into two tubes each containing 10 ml of buffered solution. One tube was used as control while the other received 0.1 μg/ml of PMA. Both control and experimental were incubated for 15 min in a rotary shaker incubator before being placed on ice.

Subcellular fractions were prepared as described previously (25–27) by z modification of the method of Woodin and Wiencke (28). The two sets of cells were kept at 4 °C throughout the preparation. Cells were centrifuged and resuspended in ice-cold sucrose buffer (11.5% sucrose containing 10 mM Hepes, 1 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, pH 7.2) and washed three times with the above buffered solution. They were resuspended in the same buffer (2 ml) and transferred to a hand-held Wheaton Dounce tissue grinder where each sample received 100 strokes. The homogenate was centrifuged at 1200 × g for 5 min and the postnuclear supernatant layered onto a discontinuous sucrose gradient consisting of 4.32 ml of 30% sucrose, 3.36 ml of 40% sucrose, and 4.32 ml of 50% sucrose in a nitrocellulose tube. All the gradient sucrose solutions contained 10 mM Hepes and 1 mM EGTA at pH 7.2. The nuclear fraction from the first spin was resuspended in 2 ml of washing buffer and rehomogenized for a second time with 50 strokes. Again the sample was centrifuged (1200 × g for 5 min) and the postnuclear supernatant applied to the gradient. The pellet of this tube consisted of the nuclear fraction. After centrifuging in a Beckman SW 27 rotor at 120,000 × g for 2 h, various subcellular fractions were obtained: cytosol (the supernatant fraction above 30% sucrose), membrane fraction 1 (at the interface of supernatant and 30% sucrose), membrane fraction 2 (at the interface of 30–40% sucrose), membrane fraction 3 (at the interface of 40–50% sucrose), and the granular fraction (pellet). The membrane fractions were washed with 16.5 mM Tris, pH 7.2 (15:1) and resuspended in 4% SDS, 15 mM β-mercaptoethanol, 86 mM Tris-HCl, bromphenol blue, 0.01%, pH 6.7, boiled for 5 min, and applied to a 10% polyacrylamide gel for analysis (see previous section for detail).

**Chemicals—**EGTA and Hepes were purchased from Sigma; PMA and 4a-PDD were obtained from CMC Cancer Research Chemicals, Brewster, NY. All electrophoresis chemicals were supplied by BioRad. Trifluoperazine was obtained from Dr. H. Greene, Smith Kline and French Labs, Philadelphia, PA.

**RESULTS**

Effects of Phorbol 12-Myristate 13-Acetate and 4a-Phorbol 13,13-Didecanoate on Protein Phosphorylation—The exogenous addition of PMA to suspension of rabbit neutrophils causes the phosphorylation of several polypeptides whose molecular weights (M₅) and isoelectric points (pI) are as follows: M₅ = 40,000, pI = 6.4; M₅ = 50,000, pI = 4.9; M₅ = 55,000, pI = 6.3; M₅ = 64,000, pI = 6.0; M₅ = 70,000, pI = 5.6; M₅ = 90,000, pI = 6.0 (Fig. 1). The time course and the dose-response curves of the PMA-induced protein phosphorylation are shown in Figs. 2 and 3. In addition to these proteins there were also small but consistent increases in the phosphorylation of three other proteins (M₅ = 31,000, M₅ = 27,000, and M₅ = 130,000). The 130,000 molecular weight protein (possibly vinculin) was found to be associated with the cytoskeleton. The time course of PMA-stimulated phosphorylation is slow with the optimum phosphorylation reached within 10 min, and the concentration causing 50% maximal response is about 0.05 μg/ml for most of these proteins. The dose-response and the time course curves are very similar to those previously described regarding PMA-induced lysozyme enzyme release in these cells (9). These increases in the PMA-induced phosphorylation are not due to a change in the specific activity of the ATP pool since there is no effect of PMA on the total labeled phosphate incorporated in the trichloroacetic acid-precipitable material (data not shown). Since PMA does not cause a rise in the level of intracellular calcium, the observed protein phosphorylation cannot, therefore, be mediated through an increase in the intracellular level of free calcium.

To investigate the subcellular distribution of these phosphorylated polypeptides, cells were first labeled with 32P and then homogenized and the subcellular fractions isolated on a discontinuous sucrose gradient. The distributions of these phosphorylated polypeptides as determined by subcellular
PMA Induces Neutrophil Protein Phosphorylation

FIG. 1. Effect of PMA on protein phosphorylation in rabbit neutrophils. Cells were prelabeled with \(^{32}P\)O\(_4\) for 30 min, washed three times, and then treated with PMA (0.1 \(\mu\)g/ml) for 10 min. Thereafter the cells were lysed and the whole cell proteins were resolved on two-dimensional gel electrophoresis. A, control; B, PMA-activated cells. The arrows indicate the 90,000 (pp90), 70,000 (pp70), 64,000 (pp64), 55,000 (pp55), 50,000 (pp50), and 40,000 (pp40) molecular weight polypeptides.

Comparison of the Effects of Phorbol 12-Myristate 13-Acetate and 4\(\alpha\)-Phorbol 12,13-Didecanoate on Lysosomal Enzyme Release, Cell Aggregation, and Actin Association with the Cytoskeleton—In order to examine the relationship between the observed protein phosphorylation and other known actions of PMA, we have compared the effects of PMA and 4\(\alpha\)-PDD on lysosomal enzyme release, cell aggregation, and the amount of actin associated with cytoskeleton. We have found that in contrast to the lack of effect of 4\(\alpha\)-PDD on protein phosphorylation both 4\(\alpha\)-PDD and PMA cause a significant \((P < 0.001)\) increase in the amounts of actin associated with the cytoskeleton (Triton X-100 insoluble), and the time course of the stimulus-dependent change in the amount of actin associated with the cytoskeleton is rapid reaching a maximum increase within 1 min after the addition of either of the compounds (data not shown). On the other hand 4\(\alpha\)-PDD, unlike PMA, does not cause lysosomal enzyme release in rabbit neutrophils in suspension (Fig. 4). The time course of
PMA-induced specific granule release is slow. The kinetics of phosphorylation is similar to those of enzyme release.

With respect to neutrophil aggregation, while PMA is a potent aggregatory agent producing a maximum response in rabbit neutrophils at a concentration as low as 2.5 ng/ml, 4a-PDD on the other hand at concentrations as high as 500 ng/ml has little if any effect (data not shown). Neutrophil aggregation is fairly rapid, and the doses required to elicit a response by PMA are much lower than those required for lysosomal enzyme release or phosphorylation.

Effects of Trifluoperazine on Protein Phosphorylation, Actin Association with the Cytoskeleton, and Lysosomal Enzyme Release Produced by Phorbol 12-Myristate 13-Acetate—In order to investigate the relationship among the various neutrophil responses (protein phosphorylation, lysosomal enzyme release, and actin association with the cytoskeleton) elicited by PMA we have studied the effects of TFP on these changes. It was found that TFP at concentrations as high as 20 μM had no effect on the stimulus-dependent increases in cytoskeletal actin (data not shown). In contrast, TFP inhibited in a dose-dependent manner lysozyme release and the phosphorylation of the 50,000 molecular weight protein induced by PMA in rabbit neutrophils (Fig. 5). Trifluoperazine up to 20 μM did not inhibit the phosphorylation of the remaining proteins (data not shown). Higher concentrations were not tested because they were lytic to rabbit neutrophils.

Comparison between the Lysosomal Enzyme Release and the Degree of Phosphorylation in the 50,000 Molecular Weight Protein Produced by Phorbol 12-Myristate 13-Acetate—It appears from the results obtained so far that there is a great deal of similarity in the characteristics of two of the responses (the lysosomal enzyme release and the phosphorylation of the 50,000 molecular weight polypeptide) produced in rabbit neutrophils by the PMA. In order to establish this correlation further we have investigated the relationship between these two responses under different experimental conditions. First, the two responses were determined at different times following stimulation using a fixed concentration (0.1 μg/ml) of PMA. Second, they were determined at a fixed time after stimulation using different concentrations of PMA. Third, they were determined at different concentrations of TFP using a fixed time after stimulation and a single concentration of PMA. The results of these studies are summarized in Fig. 6. It is clear that a linear relationship exists between the level of phosphorylation of the 50,000 molecular weight protein and the extent of the lysozyme release induced by PMA. A linear regression analysis of the results yielded a correlation coefficient of 0.96 and a slope of 0.9.
Phosphorylation of the 50,000 Molecular Weight Protein by PMA, fMet-Leu-Phe, and the Protein Kinase C System—In order to investigate further the role of the phosphorylation of this 50,000 molecular weight protein in exocytosis and to examine the role of the protein kinase C system in this phosphorylation, three sets of experiments were carried out. In the first set of experiments, we have examined the effect of the chemotactic peptide fMet-Leu-Phe on the phosphorylation of the 50,000 molecular weight protein. This is necessary for the observed PMA-induced phosphorylation to be of any physiological significance and to be involved in exocytosis. In these experiments, the cells were treated as described under “Materials and Methods” except that fMet-Leu-Phe (10⁻⁹ M) was used as a stimulus. The results summarized in Table I clearly show that fMet-Leu-Phe in whole cell preparation is able to induce the phosphorylation of this protein.

In the second set of experiments, we have examined in a cell-free system (the cytosol) the effect of the addition of calcium and phosphatidylserine on the degree of phosphorylation of the 50,000 molecular weight. This is needed to test for the role of the protein kinase C system in this phosphorylation. The standard reaction mixture in these experiments contained 50 mM Tris (pH 7.4), 10 mM MgCl₂, 0.4 mM EGTA, 40 μg of cytosolic proteins, and 20 μM [γ⁻³²P]ATP (1–3 × 10⁵ cpm/pmol) in the presence or absence of calcium or phosphatidylserine. The final volume was 100 μl, and the phosphorylation reaction was initiated by the addition of [γ⁻³²P]ATP and terminated by the addition of 50 μl of “SDS-stop” solution. It was found that the addition of calcium (0.4 mM) and phosphatidylserine (10 μg/100 μl) together to a cell-free system (the cytosol) produced more than a 5-fold increase in the degree of phosphorylation of the 50,000 molecular weight protein (see Fig. 7).

In the third set, we have examined the sites of phosphorylation of this protein by the various stimuli. In these experiments, gel pieces containing the 50,000 phosphoprotein were obtained from the two-dimensional gels and then subjected to limited proteolysis in SDS-polyacrylamide gel according to the method initially described by Huttner et al. Autoradiographic patterns of phosphorylation under various conditions are summarized in Table I. The finding that there is no synergistic effect after 15 min following the addition of the two stimuli is due to the fact that A23187 can induce degranulation from the specific granules (i.e. part of the PMA-induced release has been released by A23187). Also the rate of phosphorylation of the 50,000 molecular weight protein is enhanced when A23187 and PMA are added together.

Enzyme Release and Phosphorylation of the 50,000 Molecular Weight Protein—It is generally agreed that intracellular calcium ions play an important role in neutrophil degranulation and that PMA induces degranulation in the absence of a rise in the level of intracellular free calcium. In order to investigate the relationship between calcium ions and PMA activation of neutrophils we have studied the effect of PMA and A23187 added together on lysosomal enzyme release and phosphorylation of the 50,000 molecular weight protein. In these experiments, low concentrations of the divalent cation ionophore A23187 were used to avoid significant ionophore-induced degranulation. The results of these studies are summarized in Fig. 8. Note that in the first 5 min of incubation, during which the ionophore produced little or no effect on its own, the Ca²⁺ ionophore-induced calcium mobilization (this concentration of ionophore induces Ca²⁺ mobilization as measured by increased quin-2 signal) and PMA-induced protein kinase C activation act synergistically in causing neutrophil degranulation. The finding that there is no synergistic effect after 15 min following the addition of the two stimuli is due to the fact that A23187 can induce degranulation from the specific as well as the other granules whereas PMA can induce degranulation only from the specific granules (i.e. part of the PMA-induced release has been released by A23187). Also the rate of phosphorylation of the 50,000 molecular weight protein is enhanced when A23187 and PMA are added together.

**Discussion**

The addition of low concentrations of the tumor promoter phorbol 12-myristate 13-acetate to rabbit neutrophils in sus...
the 64,000 molecular weight polypeptide is found in both the nuclear pellet and the plasma membrane, respectively. It is tempting to speculate similarities to vinculin as described by wick and Stossel (31). This protein binds to actin and is involved in the regulation of actin polymerization in neutrophils (31, 32). Our finding that it is found in both the cytosol and the cytoskeleton; the 40,000 and 31,000 molecular weight polypeptides are found in the nuclear pellet and the plasma membrane, respectively. It is tempting to speculate that the protein with an apparent molecular weight of 64,000 may be the same as the leukocyte protein isolated by Southwick and Stossel (31). This protein binds to actin and is involved in the regulation of actin polymerization in neutrophils (31, 32). Our finding that it is found in both the cytosol and the cytoskeleton is consistent with this view. The protein with 130,000 molecular weight which is found to be associated with the cytoskeleton is probably vinculin. The patterns of PMA-induced protein phosphorylation are similar to those observed by Andrews and Babior (11) but significantly different from those reported by Schneider et al. (10). In spite of some differences among these three studies a protein in the region between 47,000 and 50,000 molecular weight was found to be phosphorylated in all these studies.

In contrast, the addition of 4α-phorbol 12,13-didecanoate, an inactive analogue of PMA, does not stimulate protein phosphorylation, lysosomal enzyme release, or cell aggregation. On the other hand, both PMA and 4α-PDD increase the amount of actin associated with the cytoskeleton. The ability of 4α-PDD to stimulate actin association but not cell aggregation or lysozyme release suggests strongly that either actin association with the cytoskeleton or the phosphorylation of the 50,000 molecular weight polypeptide is involved in specific granule release or neutrophil aggregation, or if it is, that these functions require something in addition which is supplied by stimulation with PMA but not 4α-PDD. Trifluoperazine at concentrations which are similar to those that are known to inhibit protein kinase C activation (33) abolishes completely both neutrophil degranulation and the phosphorylation of the 50,000 molecular weight polypeptide induced by PMA. On the other hand, it has no effect on the increases in cytoskeletal actin produced by either PMA or 4α-PDD.

Trifluoperazine is known to inhibit with different ID₅₀ both protein kinase C system and calmodulin-dependent protein kinase. The lack of inhibitory effect of TFP (up to 20 μM) of the remaining phosphorylated proteins suggests at least two possibilities. First, the phosphorylation of these proteins by PMA is mediated by yet unknown types of protein kinases. Second, the phosphorylation of these proteins requires less activity of protein kinase C than the 50,000 protein and thus higher concentrations of TFP are needed to inhibit their phosphorylations. Unfortunately, this point cannot be tested since concentrations of TFP higher than 20 μM produce significant lyses in rabbit neutrophils. At any rate, the inhibition of the phosphorylation of 50,000 molecular weight protein and exocytosis by TFP correlate well with each other.

The phosphorylation by PMA of the above polypeptides does not seem to be causally related to the PMA-induced increase in actin association with the cytoskeleton. This conclusion is based on the experimental finding that although the compound 4α-phorbol 12,13-didecanoate produces significant increases in cytoskeletal actin, it fails to increase the phosphorylation of any of these polypeptides. Furthermore, PMA-induced increases in cytoskeletal actin are rapid while its effect on phosphorylation is significantly slower. Likewise, the observed PMA-stimulated phosphorylation is not likely to be involved in neutrophil aggregation. This conclusion is based on two main experimental observations. First, concentrations of PMA which produce maximum cell aggregatory response have little or no effect on polypeptide phosphorylation. Second, PMA effects on neutrophil aggregation and polypeptide phosphorylation can be dissociated in time; the first response occurs at a much faster rate than the second.

The 50,000 molecular weight protein is also phosphorylated in whole cells by the chemo tactic factor fMet-Leu-Phe. This result is in agreement with those reported by Andrews and Babior (11) and Schneider et al. (10). In addition, this protein is phosphorylated in cell-free systems by the addition of calcium and phosphatidylserine. Using limited proteolysis, one phosphopeptide fragment is phosphorylated by the three stimuli. Based on these findings it is reasonable to conclude that the phosphorylation of this protein by PMA and fMet-Leu-Phe in intact cells is mediated by the protein kinase C system.

Based on the results reported above, it appears that the phosphorylation of the 50,000 molecular weight polypeptide and the release of specific granules produced by PMA are causally related. PMA has been shown to activate the protein kinase C system directly (12, 15, 16). It is reasonable to hypothesize that the activation of this enzyme which leads among other things to the phosphorylation of the 50,000 molecular weight protein is intimately involved in specific granule release. This hypothesis is supported by several lines of experimental evidence. First, 4α-PDD which does not activate protein kinase C at the concentrations used in the present experiments does not cause lysosomal enzyme release or the phosphorylation of the 50,000 molecular weight polypeptide. Second, the time course and the dose-response curves of the two responses (degranulation and 50,000 molecular weight phosphorylation) are very similar. Third, both responses are inhibited by the same concentrations of trifluoperazine. The concentrations which inhibit both neutrophil responses are similar to those that are known to inhibit protein kinase C activity (33). Fourth, the rates of both responses can be enhanced when the ionophore A23187 and PMA are added together.

Phorbol 12-myristate 13-acetate which is not produced by neutrophils when stimulated by chemotactic factors can sub-
stitute for the diacylglycerides in their ability to stimulate native protein kinase C and greatly increase the affinity of the enzyme for calcium ions (12, 15, 16). The present results imply that lysosomal enzyme release induced by the chemotactic factors such as fMet-Leu-Phe is partially mediated through the activation of protein kinase C. This view is supported by the findings that receptor occupancy by the synthetic chemotactic factor fMet-Leu-Phe leads to rapid and transient rises in the level of 1,2-diacylglycerol and in the production of phosphatidic acid in rabbit neutrophils (34–36).

At present the exact roles of intracellular calcium ions and protein kinase C in exocytosis of neutrophils are not fully understood. The most likely hypothesis is that under physiological stimulation both systems (Ca\(^{2+}\) and protein kinase C) are needed for optimal response. Calcium ions through the activation of protein kinase C. This view is supported by the findings that receptor occupancy by the chemotactic factor met-Leu-Phe leads to rapid and transient rises in the level of 1,2-diacylglycerol and in the production of phosphatidic acid in rabbit neutrophils (34–36).

According to this hypothesis increasing above basal level the activity of either of these two systems (protein kinase C without an increase in Ca\(^{2+}\) such as in the case of PMA or a small rise in Ca\(^{2+}\) without an increase in protein kinase C such as low concentrations of A23187) alone without the other causes some exocytosis since both systems have some activities under unstimulated conditions.

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