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PHORBOL 12-MYRISTATE 13-ACETATE ACTIVATES RABBIT NEUTROPHILS WITHOUT AN APPARENT RISE IN THE LEVEL OF INTRACELLULAR FREE CALCIUM

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SUMMARY: The addition of low concentrations of phorbol 12-myristate 13-acetate to rabbit neutrophils induces cell aggregation, degranulation, increased oxygen consumption and an increase in the amount of actin associated with the cytoskeleton without a rise in the level of intracellular free calcium as measured using the fluorescent probe quin-2. The ability of phorbol 12-myristate 13-acetate to initiate neutrophil responses similar to those produced by the chemotactic factor without causing a rise in the level of intracellular free calcium suggests two possibilities; that there is a second messenger in addition to calcium or that it activates the cells at a point distal to calcium mobilization. The possible role of diacylglycerol in neutrophil activation is discussed.

It is commonly hypothesized that neutrophil activation by chemotactic stimuli is mediated by an increase in the level of intracellular free calcium (1-8). This conclusion is based on the following experimental observations: (a) cell responsiveness is modulated by the concentration of calcium in the suspending medium, (b) introduction of calcium into the cytosol by means other than the first messenger activates the cells, (c) inhibitors which antagonize the activity of the intracellular calcium receptor (calmodulin) inhibit cell activation, and (d) occupancy of the plasma receptors by the first messenger (chemotactic stimuli) causes an increase in the level of intracellular free calcium. Although this evidence suggests very strongly that cell activation can be elicited by a rise in the level of intracellular free calcium, it does not rule out the possibility that cell activation can be initiated by means other than a change in calcium homeostasis.

ABBREVIATIONS

N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid: HEPES
Formyl-Methionyl-Leucyl-Phenylalanine: f-Met-Leu-Phe
Phorbol 12-myristate 13-acetate: PMA
(Ethylenedinitrilo) tetracetic acid: EDTA
Guanosine 3',5'-monophosphate: cyclic GMP

Nanometer: nm
Nanomolar: nM
Nanogram: ng
Microgram: µg

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The co-carcinogenic compound phorbol 12-myristate 13-acetate (PMA) added exogenously causes neutrophil aggregation, enzyme secretion from specific granules, and an increase in the respiratory burst (9-12).

In this paper we wish to report experiments showing that relatively low concentrations of phorbol myristate induce neutrophil aggregation, lysosomal enzyme release, enhanced oxygen consumption and increased cytoskeletal actin without increasing the level of intracellular free calcium. The relationship of these results to cell activation and the possible role of diacylglycerol as an additional second messenger are discussed.

MATERIALS AND METHODS

Rabbit peritoneal neutrophils (4-12 h exudates) were obtained and handled as previously described (13,14). The cells were washed and resuspended (1×10^7 cells/ml) in magnesium and protein free modified Hanks' Balanced Salt Solution buffered with 10 mM Hepes (14,15). The suspensions were preincubated in a temperature controlled rotary bath at 37°C for 10 minutes before they were used.

Neutrophil aggregation was measured as described previously (16) by monitoring the change in optical transmission at 600 nm of a cell suspension ($2-5 \times 10^6$ cell/ml) in a Zeiss PMQ111 spectrophotometer. Magnesium (as $MgSO_4$)was added 1-3 minutes before the addition of the stimulus. Lysosomal enzyme release was carried out as previously described (16). Oxygen consumption was measured as described previously (16) using a Clark-type O_2 electrode (Model 53, Yellow Springs Instrument Co., Yellow Springs, OH). Fluorescence signal of the calcium sensitive fluorescent probe Quin-2 (17) was monitored using an SLM (Model 8000) fluorescence spectrophotometer with temperature controlled cuvette and magnetically driven stirrer (18). Cytoskeletal structures were isolated as proteins insoluble in 1% Triton X-100, and the proteins were electrophoresed through a 5-15% gradient polyacrylamide slab gel (19,20,21).

EDTA [(ethylenedinitrilo) tetra acetic acid] was purchased from Eastman. Hepes (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), f-Met-Leu-Phe (formyl-methionyl-leucyl-phenylalanine), (phorbol 12-myristate 13-acetate) (PMA) were all purchased from Sigma Chem. Co. (St. Louis, MO). Quin-2/AM was purchased from Lancaster Synthesis Ltd., England. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

The mechanism by which the the tumor promoter PMA causes various neutrophil responses and the relationship of these responses to cellular calcium homeostasis are not currently understood. In order to investigate whether the PMA-induced changes are mediated through a rise in the intracellular level of free calcium or by means other than calcium mobilization, a series of experiments were performed in which several cell responses and the intracellular levels of Ca^{2+} were measured. Dose-response curves of the effects of PMA on lysosomal

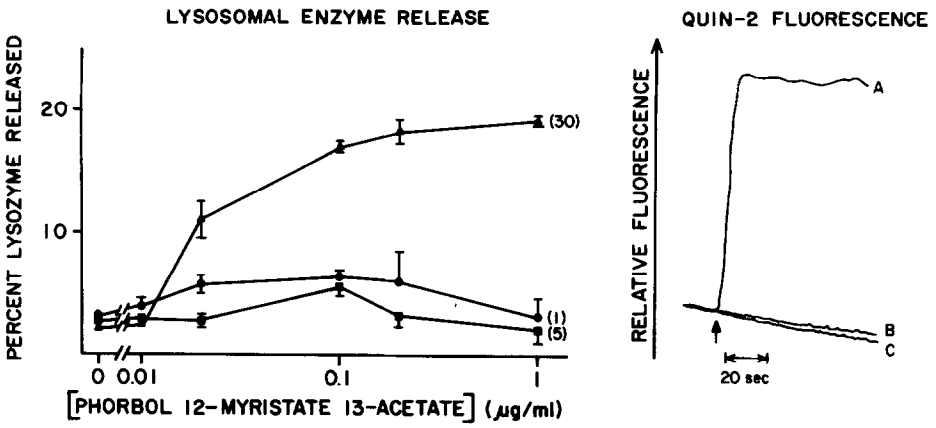

Figure 1. The effects of various concentrations of PMA on lysosomal enzyme release and cell-associated quin-2 fluorescence. Left panel: the number in the parenthesis refers to the time in minutes which the cells were allowed to react with PMA before the lysozyme release was measured. The error is standard error of the mean. Right panel: the arrow indicates the time of PMA addition. A, 1 μg/ml; B, no addition; C, 0.1 μg/ml. These data are taken from a single experiment representative of at least four separate experiments.

enzyme release, oxygen consumption and cell associated quin-2 fluorescence (an indicator of the level of cytoplasmic free Ca^{2+}) are summarized in Figures 1 and 2. It is quite evident that at low concentrations of PMA (< 0.1 μg/ml) significant amounts of both lysosomal enzyme release and oxygen consumption occurred without a concomitant rise in the level of intracellular free calcium as monitored by quin-2 fluorescence. While f-Met-Leu-Phe induced degranulation is rapid and requires the presence of cytochalasin B, PMA induced degranulation is very slow, does not require the presence of cytochalasin B, and is quantitatively much smaller. When monitored over 30 minutes, low concentrations of PMA not only do not cause a rise in the level of intracellular free calcium, but they induce a net loss of cellular calcium as evidenced by the continuous decrease in the cell-associated quin-2 fluorescence. This loss of signal suggests that PMA activates the Ca^{2+}-efflux pump (9,12) leading to a net removal of calcium from the cells and inducing a continuously slow displacement of bound calcium from internal stores. In view of the high affinity of quin-2 (17) for calcium and of the high

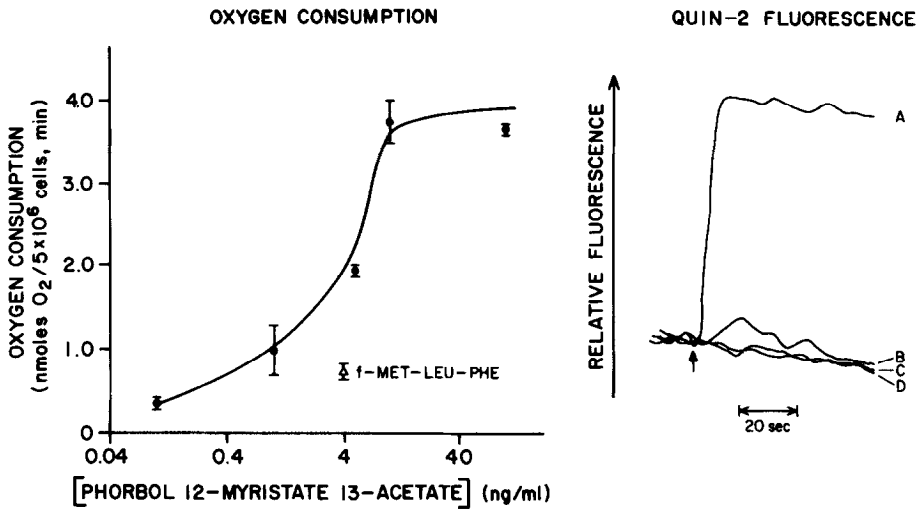

Figure 2. A dose-response curve of the effects of PMA on oxygen-consumption and cell-associated quin-2 fluorescence. Left panel: after the initial delay period which varied between 1-3 minutes depending on PMA concentration, oxygen consumption was linear for at least five minutes. The errors are standard errors of the mean. Right panel: the arrow indicates the time of PMA addition. A, 1 μg/ml; B, 0.1 μg;ml; C, 0.05 μg/ml; D, 0.01 μg/ml. These data are taken from a single experiment representative of at least two separate experiments.

intracellular concentration of quin-2 achieved during loading (17), the inability to detect an increase in fluorescence following stimulation may indeed reflect a lack of calcium mobilization by low concentrations of PMA. When monitored over 30 minutes, PMA at low concentrations not only did not cause a rise in the level of intracellular free calcium, but it induces a net loss of cellular calcium as evidenced by the continuous decrease in the cell-associated quin-2 fluorescence. F-Met-Leu-Phe (10^{-9}M) added to neutrophils 15 seconds after the addition of PMA (0.1 μg/ml) evoked a normal increase in quin-2 signal (Figure 3). Yet the addition of f-Met-Leu-Phe (10^{-9}M) to neutrophils after the cells have been incubated with PMA (0.1 μg/ml) for 3 minutes evoked a significantly reduced quin-2 signal (data not shown). This suggests that the Ca^{2+}-pool which is normally released by f-Met-Leu-Phe can also be released slowly by PMA and, as pointed out earlier, this is accomplished by activating the Ca^{2+}-efflux pump. The activation

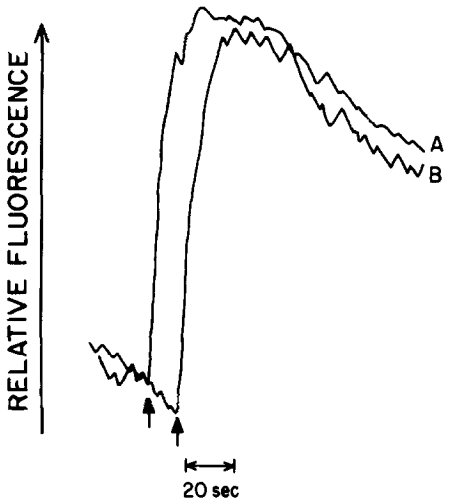

Figure 3. The effects of the chemotactic stimulus f-Met-Leu-Phe (10^{-9}M) on quin-2 fluorescence. A, control cells; B, cells which have been treated with 0.1 μg/ml PMA for 15 seconds. The arrows indicate the time of f-Met-Leu-Phe addition. This is a single experiment representative of at least two experiments.

of the Ca^{2+}-efflux pump reduces the concentration of intracellular free calcium leading to a redistribution of intracellular calcium and thus a release of calcium from internal stores. At a very low concentration PMA also causes neutrophil aggregation, and like f-Met-Leu-Phe, the PMA-induced aggregation requires the presence of Mg^{2+} in the suspending medium (Figure 4). The effects of PMA on both oxygen consumption and aggregation are quantitatively much greater than those induced by f-Met-Leu-Phe, and there is a lag time (1-3 minutes depending on the concentration of PMA used). The lag time needed for PMA to induce lysosomal enzyme release is much longer than that needed for either aggregation or increase of oxygen consumption.

It is very clear from these results that PMA at low concentrations can initiate various neutrophil responses without a rise in the intracellular level of free calcium. This suggests either the presence of an additional second messenger or that PMA activates the cells at a point distal to calcium mobilization possibly by increasing the intracellular level of cyclic GMP or by substituting for diacylglycerol to stimulate the native protein kinase C activity (22,23). Furthermore, phorbol esters

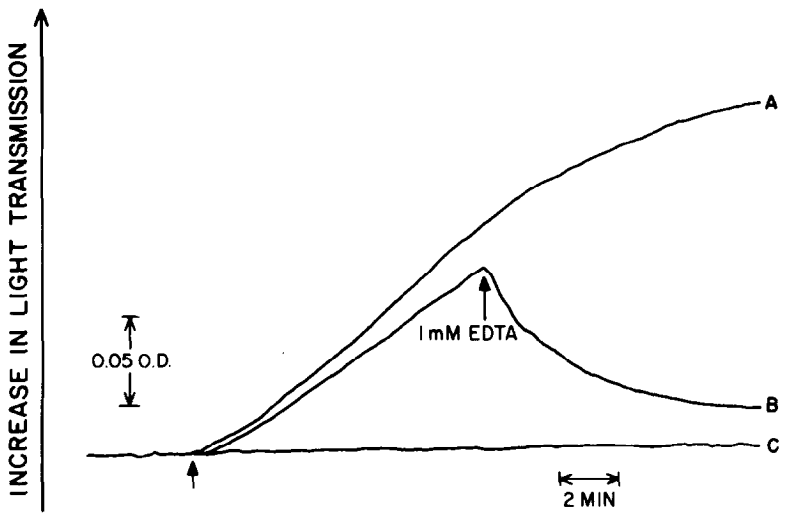

Figure 4. Effect of PMA on neutrophil aggregation in the presence and absence of 0.74 mM Mg^{2+}. A, 1 ng/ml in the presence of Mg^{2+}, B, 0.3 ng/ml; C, 1 ng/ml PMA in the absence of Mg^{2+}. The first arrow indicates the time when PMA was added, and the second arrow indicates the time when 1 mM EDTA was added. This is a single experiment representative of at least two experiments.

stimulate platelets to release serotonin without activating the phosphatidylinositol cycle to produce diglyceride (22). In view of the present findings it is reasonable to suggest that diacylglycerol in addition to calcium may serve the role of a second messenger in neutrophil activation by chemotactic stimuli. This view is also supported by our findings that occupancy of the receptors by the chemotactic factor f-Met-Leu-Phe leads to a rapid increase in the level of 2,3 diacylglycerol and that PMA causes the specific phosphorylation of a few proteins (unpublished data).

Like f-Met-Leu-Phe, PMA increases the amount of actin associated with the cytoskeleton (Figure 5). It is interesting to point out that PMA increases cytoskeletal actin and at the same time induces lysosomal enzyme release in the absence of cytochalasin B. In addition, at low concentrations (0.01 μg/ml) PMA increases cytoskeletal actin without causing significant amounts of lysosomal enzyme release. It is generally found that chemotactic factors do not cause rabbit neutrophils in suspension to degranulate unless cytochalasin B is present.

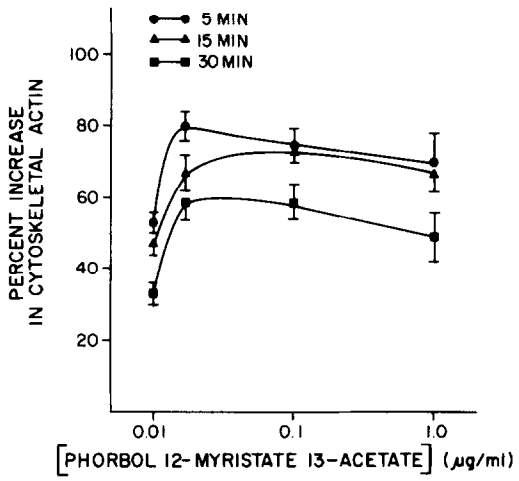

Figure 5. Dose-response curve of the effect of PMA on the amount of actin associated with the cytoskeleton. The number in parenthesis refers to the time in minutes in which the cells were allowed to react with PMA before the cytoskeletons were isolated. The unit on the ordinate axis refers to the percent increase of cytoskeletal actin as compared to its respective control. The error is standard error of the mean.

It is commonly hypothesized that cytochalasin B is needed for this process because it interferes with microfilament function and thus decreases actin association with the cytoskeleton. In light of this result, this hypothesis may have to be reevaluated. But it must be kept in mind that PMA releases little lysozyme (20%) and that this release is much slower and does not require a rise in the level of intracellular free calcium. It is quite possible that secretory mechanisms other than those utilized by chemotactic factors exist and are activated, albeit, somewhat inefficiently, by PMA.

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