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Research Article

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Receptor-mediated Regulation of Superoxide Production in Human Neutrophils Stimulated by Phorbol Myristate Acetate

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ABSTRACT Human neutrophils contain receptors for phorbol myristate acetate (PMA), a complex lipid that induces them to generate superoxide (O_2^-). Binding of PMA to these receptors displays specificity, reversibility, and high affinity. The receptor's apparent K_D was ~0.29 nM and multiple copies (~$2.1\pm0.6 \times 10^5$) were present per neutrophil. We found that the timing and magnitude of the neutrophil's respiratory burst were set independently. The onset of O_2^- production occurred after a lag that was inversely proportional to the initial concentration of added PMA. The extent (rate) of O_2^- production was directly proportional to the fractional occupancy of the receptor by PMA. Dual regulatory controls, such as those we noted when neutrophils were stimulated by PMA, could afford metabolic stability in the face of transient or low intensity stimuli without compromising quick and powerful responses to larger disturbances.

INTRODUCTION

Phorbol myristate acetate (PMA)[1] has diverse effects on cellular growth, differentiation, and metabolism. An extraordinarily potent tumor promoter (1), PMA is also a strong inflammatory agent (2) that induces superoxide (O_2^-) production in neutrophils (3, 4) by activating their NADPH oxidase (5). Recently, Driedger and Blumberg (6) reported that specific, high affinity receptors for phorbol diesters, such as PMA, exist in chick embryo fibroblasts. Shoyab and Todaro (7) extended these observations to include a variety of normal and transformed avian and mammalian cells. In an independent

pursuit of the interactions between phorbol diesters and human leukocytes, we have developed alternative methods to study PMA binding to cells. We describe these methods herein, report that human neutrophils contain specific receptors for PMA, and demonstrate that the rate and extent of occupancy of these receptors governs the activation of the neutrophil's respiratory burst after exposure to PMA.

METHODS

Neutrophils. Neutrophils were prepared from heparinized venous blood by described standard techniques (5). After hypotonic lysis to remove any erythrocytes, granulocytes (>95% viable by trypan blue, <2% contamination by mononuclear cells) were suspended at 1×10^7 ml^{-1} and kept at 23.5°C in a described (5) millipore-filtered balanced salt solution, (BS-H), that contained 5 μM albumin and was buffered with 10 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4.

O_2^- production. Superoxide was measured spectrophotometrically at 550 nm with a Gilford model 222A recording spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Reaction mixtures in 10-cm path length semimicro quartz cuvettes contained: 5×10^5 granulocytes in 1 ml of BS-H with 50 μM ferricytochrome C (Sigma Chemical Co., St. Louis, Mo.) $\pm$30 μg/ml superoxide dismutase (Miles Laboratories, Inc., Elkhart, Ind.). PMA (Consolidated Midland Corp., Brewster, N. Y. or Lifesystems, Newton, Mass.), was prepared in dimethylsulfoxide (DMSO) at $\times$100 the final desired concentrations and stored at 23.5°C, in the dark, in 2-ml Wheaton borosilicate glass micro vials (Scientific Products Div., American Hospital Supply Corp., McGaw Park, Ill.). After recording base-line O_2^- production (nil) for 5 min, 10 μl of PMA solution or DMSO (for controls) was admixed and O_2^- generation was recorded for 15–95 min. Absolute rates of maximal O_2^- production were calculated from the linear portion of the response, using an extinction coefficient of 18.5 mM^{-1} cm^{-1} (8).

We calculated a relative rate of O_2^- production by dividing the observed rate (Δ) by the rate manifested by neutrophils exposed to 1.2 ng/ml of PMA (Δ_a) or 100 ng/ml of PMA (Δ_{max}). Under our standard conditions, 100 ng/ml triggered a maximal response, and $\Delta_a = 0.74 \Delta_{max}$. The expression Δ/Δ_{max} expresses the rate of O_2^- production relative to the maximal

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[1] Abbreviations used in this paper: DMSO, dimethylsulfoxide; BS-H, a balanced salt solution; K_D, dissociation constant; PMA, phorbol myristate acetate; PMN, neutrophil; R_t, receptor sites/cell; R_o, occupied receptor sites/cell; Δ, rate of O_2^- production; Δ_{max}, maximal rate of O_2^- production.

response given by that individual's cells under given conditions. Lag time and activation time were calculated by the methods of Cohen and Chovaniec (9, Fig. 2).

Isotopic studies. 20-³H(N)-PMA (17.2 Ci/mmol in toluene/ethanol, 9:1) was purchased from New England Nuclear, Boston, Mass. The material was dried at 0°C under a stream of N₂, reconstituted at 4 μg/ml in DMSO, and stored at −20°C. When subsequently diluted in DMSO, the isotope was fully active, relative to unlabeled PMA when tested by analysis of activation rate (Fig. 2).

We tested neutrophils for specific binding of [³H]PMA under conditions that paralleled those used in our functional assays. Our method of separating bound from free [³H]PMA was based on the technique described by Feinberg et al. (10). Silicone oil (Versilube F-50, Harwick Chemical, Pico Rivera, Calif.), 0.4 ml, was added to conical polyethylene 1.5 ml microcentrifuge tubes (West Coast Scientific, Berkeley, Calif.) and overlaid with 1 ml of BS-H containing 5×10^5 granulocytes at 23.5°C or 37°C. Binding was initiated by adding 0.1–1.0 ng of [³H]PMA±200 ng of nonradioactive PMA. The unlabeled compound was added just before or with the isotope. After 15 (37°C) or 30 min (23.5°C) 4–12 replicate tubes were centrifuged (8,000 g, 60 s) in an Eppendorf model 3200 centrifuge (Brinkman Instruments, Inc., Westbury, N. Y.). 250-μl aliquots of each supernatant were removed for liquid scintillation counting, and the remaining supernatant and silicone oil were discarded. The pelleted cells were analyzed for radioactivity by placing the bottom of the tube, amputated with a razor blade, in scintillation vials. All samples were dissolved in 10 ml of Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) and counted on a Beckman LS-330 liquid scintillation counter with external standards for quench correction. "Specific" uptake was defined as the difference between cell-associated radioactivity after incubation with [³H]PMA and cell-associated radioactivity after incubation with [³H]PMA + 200 ng/ml of nonradioactive PMA. The latter was considered to represent nonspecific uptake.

In preliminary experiments we noted that: (a) solubilizers, such as NCS (Amersham Corp., Arlington Heights, Ill.), were unnecessary for extracting [³H]PMA from the cell pellet; (b) contamination of the cell pellet by extracellular fluid was negligible (<0.1 μl), based on studies with ¹⁴C-inulin (New England Nuclear); and (c) [³H]PMA adsorbed to the walls of glass or plastic vessels and partitioned into silicone oil. For example, when 5×10^5 PMN in 1 ml of HBSS was incubated for 30 min at 23.5°C with 1.0 ng of [³H]PMA in microcentrifuge tubes containing 0.4 ml of silicone oil, and appropriate samples of supernatant, oil, and the tube itself were counted, the [³H]PMA was distributed as follows: cell-associated, 31%; "free" in HBSS, 35.5%; adherent to the centrifuge tube, 17.5% and in the silicone oil, 9.5% (recovery, 93.2%); (d) binding of [³H]PMA to the walls of our quartz cuvettes was similar in extent to binding by plastic tubes; and (e) silicone oil extracted no radioactivity from pelleted, [³H]PMA-labelled neutrophils for at least 60 min.

Receptor occupancy was calculated from measured values for specifically bound [³H]PMA and estimated values for total receptor sites obtained by Scatchard plots (11) analyzed by the method of least mean squares. Bound/free ratios were calculated in two ways. Differing in certain basic assumptions, these analyses nonetheless yielded very similar estimates of total receptor sites/cell (R_t) and receptor occupancy (R_o/R_t). In Method A, free [³H]PMA was equated to the total concentration measured (by liquid scintillation counting) in the supernatants. In Method B, free [³H]PMA was calculated by subtracting both specifically and nonspecifically bound isotope from added isotope. The latter method assumed that PMA adsorbed to the test tube walls was in equilibrium

with that present in solution, and was equally available for binding to neutrophils.

RESULTS

O_2^- *production.* No O_2^- was released by unstimulated neutrophils or by controls exposed only to DMSO. After exposure to 0.1–1.2 ng/ml of PMA, 5×10^5 neutrophils released O_2^- after a variable but characteristic lag. Fig. 1 demonstrates that the curve relating lag time, in minutes, to these PMA concentrations much resembles a rectangular hyperbola, whose vertical asymptote is displaced somewhat to the right of the y axis.

The addition of 0.1 ng/ml of PMA induced no O_2^- production, even after 95 min of incubation. Moreover, whereas 0.2 ng/ml of PMA invariably stimulated normal neutrophils to produce O_2^-, only about half of the populations tested showed a response to 0.1 ng/ml in the time allotted (50–95 min). Both observations suggest that the presence of a minimal, threshold concentration of PMA is needed to activate or sustain the response. When neutrophils were incubated with very large (e.g., 10 μg/ml) concentrations of PMA, a lag time of ~40–50 s persisted at 37°C.

Although lag time was a convenient and reproducible index of the activation of O_2^- generation, others have favored using activation time for this purpose. In our
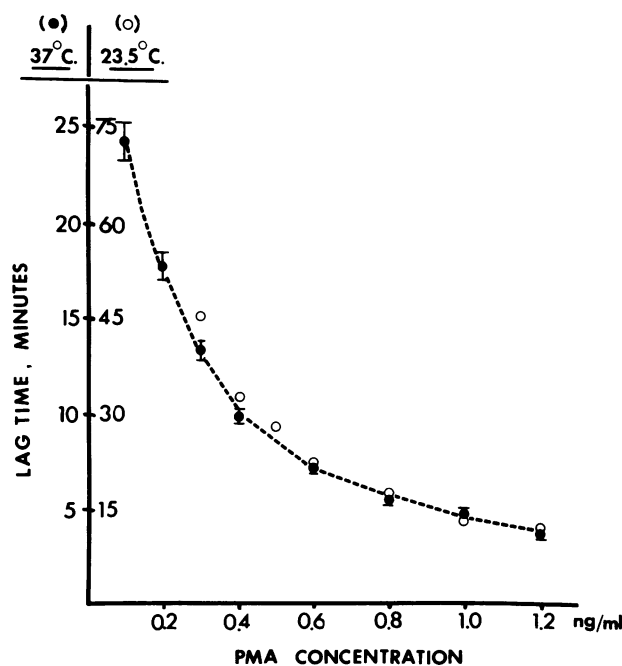

FIGURE 1 Lag time and PMA concentration. Neutrophils (5×10^5/ml) were exposed to PMA at 37°C (●, mean±SEM, $n = 15$) or 23.5°C (○, mean, $n = 2$) and the lag was measured as described. Note the different ordinate scales for 37°C and 23.5°C.

system, the lag time also closely approximates the time necessary for a half-maximal rate of cytochrome C reduction to be attained. As defined by Cohen and Chovaniec (9), activation time is the time elapsed between the addition of a stimulus and the development of a linear rate of cytochrome C reduction. The two indices of activation are closely related. For PMA concentrations between 0.2 and 1.2 ng/ml, we found that activation time equalled 1.35 ± 0.036 times the lag time. For this reason, plots of the activation time against PMA concentration also resembled rectangular hyperbolas, virtually identical in shape to that shown in Fig. 1, but shifted slightly upward on the horizontal axis (data not shown).

Fig. 2 shows that the reciprocals of the lag times are linearly related to PMA concentration, verifying the hyperbolic nature of the curves shown in Fig. 1 for the PMA concentrations noted. We found such linear transformations of the lag time data to be reproducible, and used them to good effect in bioassaying [³H]PMA and related compounds (data not shown).

The rate of O_2^- production also depended on PMA concentration, as shown in Fig. 3. This figure shows both absolute and relative rates of O_2^- production for the same set of 15 experiments analyzed in Figs. 1 and 2. The normalized function Δ/Δ_a showed virtually identical dependency on PMA concentrations at 37°C and 23.5°C (data not shown), although the absolute rate of O_2^- production at any given PMA concentration was 2.6-fold higher at 37°C.

FIGURE 3 Rates of O_2^- production. The left hand ordinate shows the absolute rate of O_2^- production ($\bullet$, mean$\pm$SEM, $n = 15$) and the right hand ordinate shows the normalized function, Δ/Δ_a, defined in the text.

Under our usual conditions, we maintained a constant cell concentration (5×10^5 PMN/ml) and varied the amount of PMA added. If we instead added a constant amount of PMA to varying numbers of PMN, we obtained results as illustrated in Fig. 4. Although lag time was independent of cell concentration over
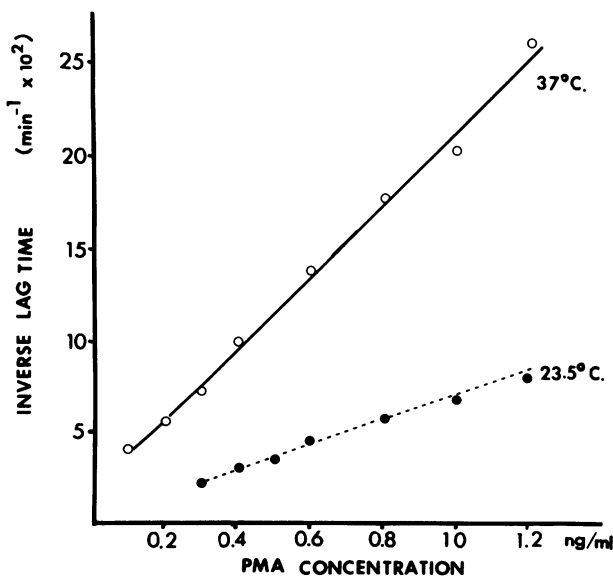
FIGURE 2 Inverse lag time and PMA concentration. The reciprocals of the mean lag times shown in Fig. 1 are plotted against PMA concentration. Note that the resultant function is linear and that the slope of the line at 37°C is three times that at 23.5°C.
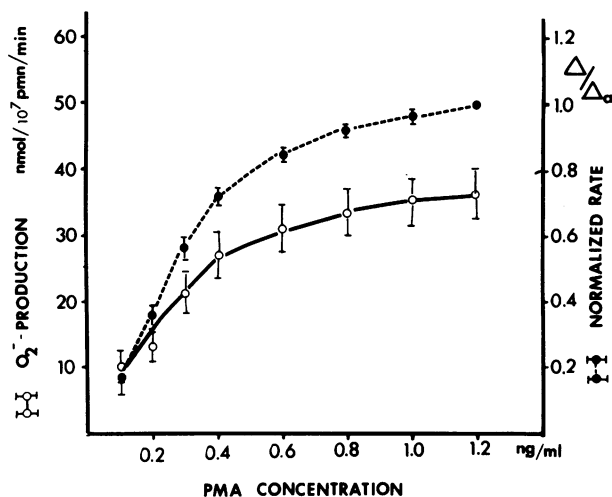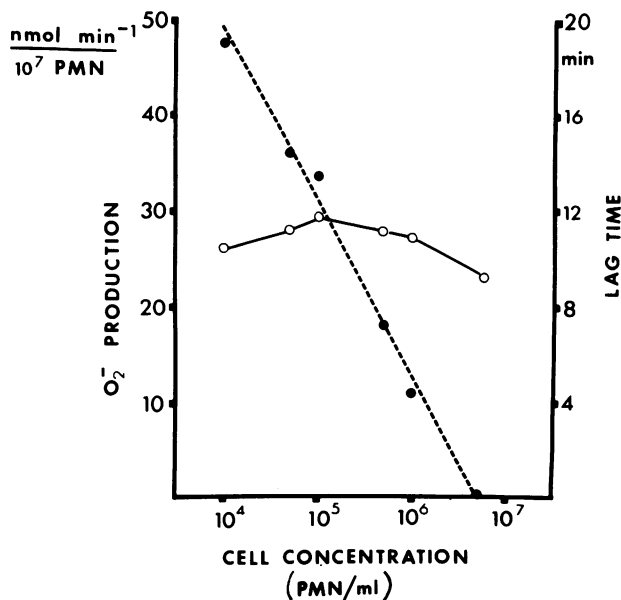
FIGURE 4 Dissociation of lag time and rate of O_2^- production. Varying numbers of neutrophils were exposed to a constant concentration of PMA (ca 0.5 ng/ml). Note that whereas the lag is relatively independent of cell concentration, the rate of O_2^- production is not under conditions when PMA concentration is limiting. ($\bullet$) signifies the rate of O_2^- production and (○) shows the lag time.

a 500-fold range, the rate of O_2^- production/cell was profoundly affected. In control experiments performed with saturating concentrations (1 μg/ml) of PMA, O_2^- production/cell was essentially independent of cell concentrations over this range.

Overall, these data suggested that the rate of O_2^- production by neutrophils was affected by the relative amount of PMA/neutrophil, whereas the lag time (and presumably the activation process) was determined by the initial concentration of the exciting agent, PMA.

Binding of ^{3}H-PMA. After considering the shape of the curve shown in Fig. 3, we used [^{3}H]PMA to test neutrophils for the presence of a saturable, PMA-responsive receptor. Our studies showed that [^{3}H]PMA uptake was rapid, and had both specific and nonspecific components (Fig. 5). Nonspecific uptake, a linear function of [^{3}H]PMA concentration, accounted for ~20% of the added isotope under our experimental conditions. In contrast, specific uptake was saturable. Specific and nonspecific uptake reached equilibrium by 30 min at 23.5°C or 15 min at 37°C. We selected 23.5°C (room temperature) for routine use because this afforded convenient and precise temperature control, maintenance of a muted, but easily measurable meta-

FIGURE 5 Uptake of [^{3}H]PMA. The main figure shows uptake of [^{3}H]PMA by 5 × 10^5 PMN exposed for 30 min at 23.5°C to varying concentrations of [^{3}H]PMA ±200 ng/ml nonradioactive PMA. Each data point represents the mean of six replicate samples. The inset shows kinetics of [^{3}H]PMA uptake by 5 × 10^5 PMN exposed to 2 ng [^{3}H]PMA ±200 ng nonradioactive PMA at 23.5°C. Each data point represents the mean of three replicate samples. Total, specific and nonspecific uptake are defined in the text. In these experiments we measured approximately 21,000 cpm/ng [^{3}H]PMA.

bolic response, and less pronounced nonspecific binding of [^{3}H]PMA to microcentrifuge tubes (data not shown).

Technical considerations. Although the consistent differences between total and nonspecific uptake of [^{3}H]PMA illustrated in Fig. 5 strongly suggested specific uptake of the ligand, it might be argued that they were artifacts resulting from the different metabolic or physiologic behavior of neutrophils exposed to high (nonspecific uptake conditions) and low (total uptake conditions) concentrations of PMA. This possibility was excluded in two ways.

First, we compared binding of [^{3}H]PMA (0.5 ng/ml) to neutrophils from three subjects at 23.5°C and 0°C. The former conditions permitted a metabolic response, whereas the latter conditions did not. Preliminary studies revealed that specific and nonspecific uptake required >2 h to approach equilibrium at 0°C. For this reason, we allowed 2.5 h for binding at 0°C, while maintaining our usual 30-min period at 23.5°C. The following uptakes (mean±SEM, n = 3, cpm/5 × 10^5 neutrophils) were observed: at 23.5°C specific 1081±73; nonspecific 3213±49; at 0°C, specific 683±89; nonspecific 1514±88. Thus, specific uptake was demonstrated to be independent of neutrophil activation.

In addition, we tested PMA uptake by neutrophils that had been inactivated by mild heating (50°C, 15 min). Such neutrophils failed to produce any superoxide even after exposure to high concentrations of PMA, nor did they develop the vacuolization attending such exposure in normal neutrophils. Curiously, they retained the ability to exclude trypan blue (a conventional indicator of cell viability). These heat-inactivated cells maintained their ability to bind PMA specifically at 23.5°C. This is apparent from the data displayed in Fig. 6, which also demonstrates the specific binding of [^{3}H]PMA to be fully reversible. Ready reversibility of specific [^{3}H]PMA binding was also observed when native unheated neutrophils were studied in the same fashion (data not shown). Cells, "lightly" fixed by exposure to 2% glutaraldehyde in 75 mM phosphate buffer, pH 7.4, for 90 min at 0°C showed no specific binding of [^{3}H]PMA on subsequent testing.

Because PMA is soluble in silicone oil, it was also necessary to consider the possible "stripping" of [^{3}H]PMA bound to neutrophils during the few seconds of centrifugation wherein they traversed the silicone oil barrier. We obtained neutrophils from five normal subjects, suspended them at 5 × 10^5 neutrophils/ml and exposed them to 0.5 ng/ml [^{3}H]PMA ±200 ng/ml nonradioactive PMA for 30 min at 23.5°C. Replicate sets, six each, were centrifuged in tubes with or without the silicone oil, and the specific binding was calculated (mean±SEM cpm/5 × 10^5 neutrophils). Specific uptake was 1204±216 in the absence of silicone oil and
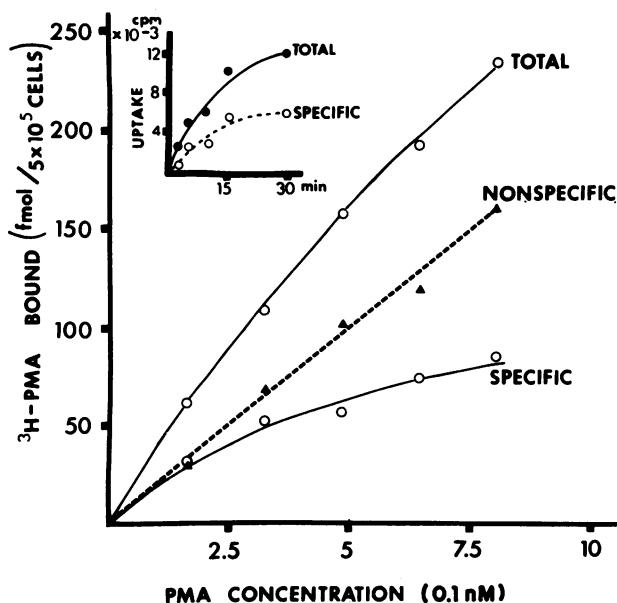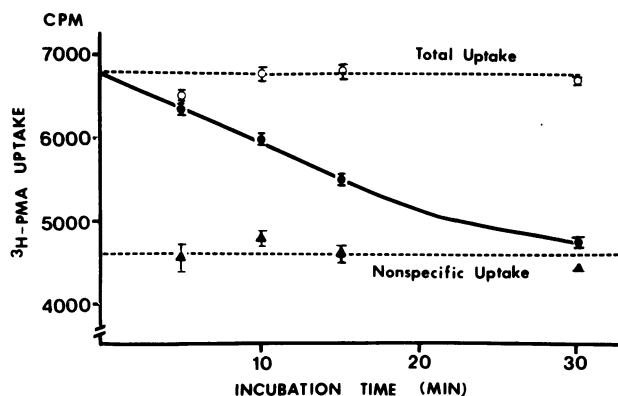
CPM

FIGURE 6 Reversibility of [³H]PMA binding. Neutrophils, heat inactivated at 50°C for 15 min, were exposed to 0.5 ng/ml [³H]PMA with (▲) or without (○, ●) 200 ng of non-radioactive PMA for 90 min. After adding 200 ng of PMA (●) or an equivalent volume of DMSO (○) to the indicated cells, samples were removed for measurements of binding at the incubation times shown. Note that specific binding was fully reversible.

FIGURE 7 Scatchard plot of [³H]PMA binding. Specific binding of [³H]PMA to 5×10^5 PMN was determined as described in the text. Each point shown represents the mean difference between twelve replicate samples exposed to [³H]PMA and twelve replicate samples exposed to the same concentration of [³H]PMA + 200 ng/ml of nonradioactive PMA. This is experiment 1 of Table I. Note that both methods of calculating binding described the text afforded the same value for R_t, whereas Method A (top curve) yielded a lower value for K_D (0.16 nM) than did Method B (0.42 nM).

1165±156 in its presence. We concluded that silicone oil, as used in our system, did not perturb the measurements of PMA uptake.

PMA receptors. The characteristics of the PMA receptor of human neutrophils are illustrated in Fig. 7 and summarized in Table I. At saturation, $\sim 2.1 \pm 0.6 \times 10^5$ PMA molecules were specifically bound per neutrophil (R_t). The apparent K_D of the neutrophil's PMA-receptor was ~ 0.29 nM (0.18 ng ml⁻¹).

Our experimental data permitted calculations of fractional receptor occupancy (R_o/R_t) at the various PMA concentrations tested. When R_o/R_t was compared to the fractional rate of O_2^- production (Δ/Δ_{max}), the functions proved to be indistinguishable (Fig. 7). Thus, Δ/Δ_{max} was directly proportional to R_o/R_t.

DISCUSSION

Application of the law of mass action to dose-response relationships predicts that the magnitude of a response will be directly proportional to the fractional occupancy of receptors by drug molecules. A maximal response is expected when all receptors are occupied, and 50% of the maximal response when half of the receptors are occupied (12). More quantitatively, this relationship can be expressed as follows: $\Delta/\Delta_{max} = (X)/K_D + (X)$, wherein Δ is the observed response at a given concentration of free drug, Δ_{max} is the maximal response to that drug, K_D is the dissociation constant of the receptor, and (X) is the concentration of free drug. If the amount of added drug actually bound is relatively small, then the concentration of drug added can be substituted for (X) without appreciably affecting accuracy (12).
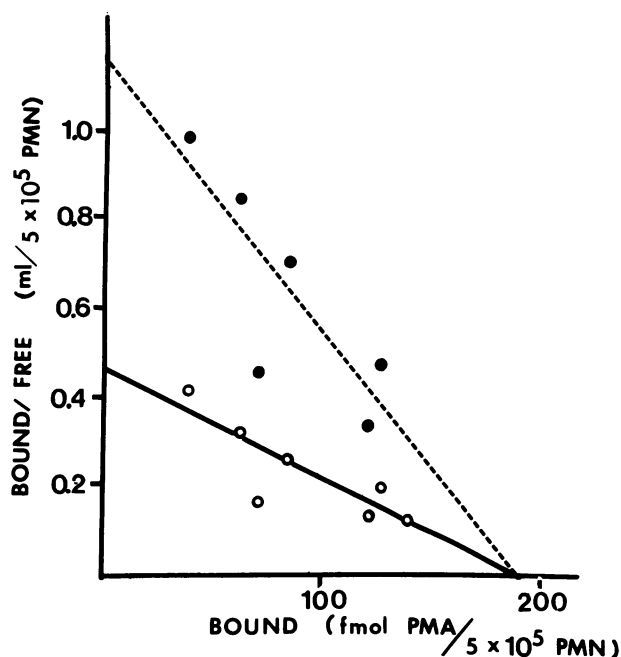
As PMA showed substantial binding, we calculated receptor occupancy by determining experimentally the concentrations of free and bound [³H]-PMA and deriving maximal binding from Scatchard plots. When fractional receptor occupancy was compared with the relative

TABLE I
Properties of the PMA Receptor of Human Neutrophils

| Experiment | Temp | R_t* | | K_D* | |
		A‡	B§	A‡	B§
		$\times 10^5$/cell		$\times 10^{10}$ M	
1	37	2.3	2.3	1.6	4.2
2	23.5	3.0	3.1	4.2	8.9
3	23.5	NT	1.0	NT	3.6

* R_t signifies receptor sites/cell and K_D represents the apparent dissociation constant of the receptor.
‡ Calculation A was done by using measured [³H]PMA concentrations in supernatants to determine "free" ligand.
§ Calculation B includes [³H]PMA adsorbed to the test tube wall in the "free" [³H]PMA compartment.

metabolic response (Δ/Δ_{max}) at equivalent PMA concentrations, the functions coincided almost perfectly (Fig. 8). We interpret this to indicate that receptor occupancy governs the relative rate of O_2^- production.

In contrast, steady-state receptor occupancy did not regulate the length of the lag before initiation of O_2^- production. Instead, this lag was inversely proportional to the initial PMA concentration, suggesting that it might be established by the initial rate of PMA-receptor combination. We will consider this aspect of the PMA-neutrophil interaction more fully in a future communication.

Newburger et al. (13) have also activated human neutrophils with PMA, and concluded that the activation process governing lag time and the reaction responsible for O_2^- production are separable. Our data support this conclusion as well.

Why do human neutrophils possess high affinity receptors for a structurally complex lipid from Euphorbiaceous plants? Moreover, why is a single biological response, O_2^- generation, regulated in a dual manner by the single stimulus, PMA? Although neither question can presently be answered definitively, both warrant consideration.

That other plant-derived molecules (opiates, colchicine, digitalis, etc.) have proven to "fit" receptors designed for endogenous ligands, suggests that serendipitous molecular mimicry might also be responsible for the existence of PMA receptors in mammalian cells. This implies the existence of endogenous ligands

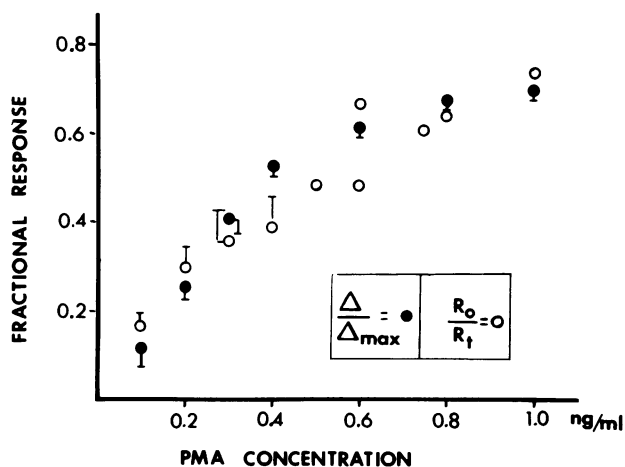FIGURE 8 Correlation between receptor occupancy and O_2^- production. Mean O_2^- production is expressed relative to the maximal response (Δ/Δ_{max}) as described in the text and was derived from the set of fifteen experiments also shown in Fig. 3. Receptor occupancy (R_o/R_t) was determined in three experiments summarized in Table I. The error bar shows ± 1 SEM for both sets of data. Open circles without error bars represent single experimental determinations of R_o/R_t from the experiments shown in Table I.

whose identity and biological functions remain to be discovered.

The other question, which deals with the regulation of the respiratory burst, might best be considered by considering neutrophil function. Neutrophils are patrolling arsenals, equipped with sensors that detect microorganisms or their molecular footprints, and weaponry to secure their destruction. By making its response time inversely proportional to the strength of the initial disturbance, the neutrophil may be permitted to migrate closer to the source of the disturbance before detonating its respiratory burst. By rendering the size of this detonation proportional to steady state receptor occupancy, the neutrophil may also be equipped to screen out "false-alarms", i.e., transient signals that disappear spontaneously or if the neutrophil is swept away from the disturbance by the circulation. Should the PMA receptor prove also to have an affinity for "natural" ligands such as chemotactic peptides or complement components, this speculation would gain considerable experimental support. Alternatively, the PMA receptor may prove to be an integral part of the cellular transduction system that links receptor-mediated surface events to other portions of the cellular machinery.

Although we studied phorbol esters to examine aspects of leukocyte oxidative metabolism, most of the burgeoning literature on these compounds concerns their extraordinary activity as tumor promoters. It is noteworthy, therefore, that two other recent reports describe the presence of specific receptors in mammalian cells. Although Lee and Weinstein (14) had earlier been unable to demonstrate a PMA receptor in HeLa cells, Driedger and Blumberg (16) studied binding of [^{3}H]PDBu (20-^{3}H phorbol-12,13-dibutyrate), a less potent analog of PMA, to particulate preparations of chick embryo fibroblasts. They found $\sim 7 \times 10^4$ specific binding sites/cell, and reported that the K_D for PDBu was 25 nM. Binding was inhibited by PMA ($K_i = .2$ nM) and other phorbol esters. Their experimentally determined K_D and K_i values were reasonably close to experimentally measured 50% effective doses for fibronectin loss.

Shoyab and Todaro (7) reported that [^{3}H]PDBu also binds specifically to normal and transformed cells of mammalian and avian origin. Mink lung cells, for example, were reported to possess 2.0×10^5 receptors with an apparent K_D of 1.3 nM. This number of receptor sites accords with our findings on human neutrophils.

Since our initial submission of this manuscript, we have also used [^{3}H]PDBu to examine the phorbol receptor of human neutrophils. We observed the following values (mean$\pm$SEM, $n = 6$): $K_D = 11 \pm 0.3$ nM; $R_T = 2.98 \pm 0.26 \times 10^5$. The affinity ($K_i$) of PMA for its receptor, calculated from its competition of [^{3}H]PDBu binding, was 2.5×0.25 nM. This indirect measure-

ment is virtually identical to the mean value, 0.29 nM, noted in the present experiments with [³H]PMA. These experiments will be fully described in a subsequent communication.

In view of the present evidence concerning the existence and functional significance of the PMA-receptor in mature human neutrophils, and recent evidence showing profound effects of phorbol esters on the growth and differentiation of leukemic leukocytes (15–19), purification of the PMA-receptor and precise delineation of its biological ligends and activities is an important objective for future research.

ACKNOWLEDGMENTS

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