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Neutrophil Aggregation: Evidence for a Different Mechanism of Action by Phorbol Myristate Acetate (40962)

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Abstract. Chemotactic factors, the ionophore A23187, and arachidonic acid aggregate polymorphonuclear neutrophils. They appear to act in part by causing neutrophils to take up extracellular calcium and to metabolize arachidonic acid. Hence, the aggregation response to these stimuli requires extracellular calcium, is enhanced by cytochalasin B (which enhances calcium uptake in stimulated cells), and is inhibited by arachidonate antimetabolites. Here, phorbol myristate acetate was also found to aggregate neutrophils. However, this response was relatively insensitive to extracellular calcium, cytochalasin B, and arachidonate antimetabolites. The mechanism of action of the phorbol ester, therefore, may not require calcium influx nor arachidonate metabolism. This suggests that neutrophil aggregation can occur through at least two different mechanisms. Other neutrophil responses (e.g., release of specific granule enzymes and oxidative metabolism) may also proceed through these same mechanisms. Phorbol myristate acetate's aggregating action was also found to be inhibited by deoxyglucose and low ambient temperatures. Thus, its action, similar to the action of other neutrophil stimulants, requires a response by metabolically active cells.

Arachidonic acid aggregates human (1) and degranulates rabbit (2) polymorphonuclear neutrophils (PMNs). It is also implicated in PMN responses to other stimuli. For instance, blockers of arachidonic acid metabolism inhibit not only the arachidonate-induced responses (1, 2) but also the responses of aggregation, degranulation, and generation of reactive oxygen-containing species evoked by chemotactic factors (2–5) and A23187 (2, 6). In addition to arachidonate metabolism, calcium influx appears involved in the PMN response to these agents. Each agent causes neutrophils to take up extracellular calcium (7–11). Moreover, inhibition (4, 9, 11–22) or enhancement (e.g., by cytochalasin B (8, 11, 16)) (1, 2, 4, 8, 11–13, 15–20, 23–26) of this uptake similarly inhibits or enhances the ensuing response. Since calcium uptake triggers arachidonate metabolism (27–31) and, conversely, arachidonate metabolism triggers calcium uptake (2, 11, 32) these two events appear linked in a sequence which transduces cell stimulation into cell response.

Phorbol myristate acetate (PMA) triggers the release of specific granule enzymes (14, 33–39) and the oxidative metabolic burst (15, 40–43) in neutrophils. It has also been noted to clump PMNs (34, 37). Here, the PMN aggregating action of this drug was further studied using a previously described assay system (44). It was found to be a potent aggregating agent. The action of the drug, however, was relatively insensitive to extracellular calcium, cytochalasin B, and arachidonate antimetabolites, and, therefore, contrasts strikingly with the action of other aggregating agents.

Materials and methods. Reagents and buffers. PMA, indomethacin, nordihydroquaiaretic acid (NDGA), cytochalasin B, and deoxyglucose were purchased from Sigma Chemical Company (St. Louis, Mo.). The arachidonate analog, 5,8,11,14-eicosatetraynoic acid (ETYA), was a generous gift of Dr. W. E. Scott (Hoffman–La Roche, Inc., Nutley, N.J.). PMA, indomethacin, cytochalasin B, and ETYA were dissolved in dimethylsulfoxide which, in the final concentrations used here (0.01%, or less), did not influence PMN function nor viability. ETYA was stored at −70°C under a nitrogen atmosphere. The buffer used throughout these studies was a modified Hanks' balanced salt solution free of bivalent cations (44). Where indicated, the appropriate concentrations of calcium and magnesium were added as chloride salts.

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Aggregation assay. The aggregation assay has been previously described (44). Cells were prepared by centrifuging blood from normal donors (who abstained from anti-inflammatory agents for at least 10 days) over Ficoll–Hypaque discontinuous gradients to obtain a PMN-enriched layer which, after being twice exposed to hypotonic medium to remove erythrocytes, contained greater than 97% PMNs (44) and virtually no platelets (1). Reagents were made 37°C and pH 7.4 before use and all experiments (unless noted otherwise) were performed at 37°C. PMN suspension (1 ml, 4600 cells/μl) was placed in a plastic vial and stirred continuously with a magnetic bar. After 4 min, calcium and/or magnesium (concentrations specified) was added to the suspension; 1 min thereafter, PMA was added. Just before and at 1/4, 1/2, 1, 2, 4, 8, 11, and 15 min after adding PMA, 20-μl samples of the suspension were taken, diluted in 10 ml of Isoton (Coulter Electronics, Hialeah, Fla.), and immediately analyzed for total and large particle concentrations with a Model ZBI Coulter counter (Coulter Electronics, Hialeah, Fla.). Large particles were particles with (volume) sizes greater than 1.8 times that of unaggregated PMNs. Results are reported as the large-particle percentage (LPP) or the maximal change in the LPP (MLPP). The LPP is 100 times the large-particle concentration divided by the total particle concentration; the MLPP is the highest LPP found at 1/4, 1/2, 1, 2, 4, 8, 11, or 15 min after adding PMA minus the LPP found just before the addition.

Cell toxicity. In order to determine if the reagents studied here were toxic, the supernatant fluid of PMN suspensions was assayed for the cytosolic enzyme lactic acid dehydrogenase (18). The agents used here did not increase the activity of this enzyme in the supernatant fluid after incubation with the suspensions for 20 min.

Results. Aggregation response. PMNs suspended in physiological medium containing 1.4 m*M* calcium and 0.7 m*M* magnesium aggregated when exposed to PMA. This effect was evidenced by rises in the LPP of the suspensions following PMA treatment (Fig. 1). Such rises reflect the formation of aggregated cells which are larger than 1.8 times the size of unaggregated PMNs and, therefore, are recorded as large particles (44). The action of the phorbol ester was dose dependent: 6×10^{-6}–6×10^{-8} *M* induced maximal responses; 6×10^{-9}, 2×10^{-9}, and 6×10^{-10} *M* induced successively lower magnitudes of response; and 6×10^{-11} *M* was inactive (Fig. 1). Within 11 min, the four highest concentrations of PMA caused the cells to clump macroscopically and settle out of the continuously stirred suspension; after this clumping, the suspension contained less than 45% of the original cells and showed a

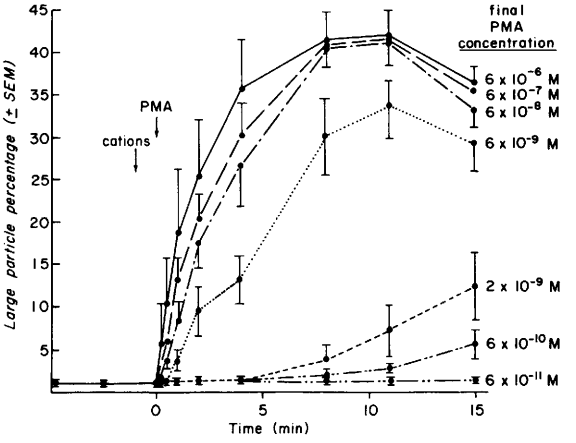

FIG. 1. Large-particle percentage after adding various concentration of phorbol myristate acetate (PMA) to neutrophil suspensions. Each curve gives mean values for six experiments.

slight decline in the LPP as recorded with the Coulter counter. At lower PMA concentrations, these events did not occur; rather, the agent induced progressively more slowly developing responses without inducing macroscopic clumping: 6×10^{-9}, 2×10^{-9}, and 6×10^{-10} *M* required, respectively, 1, 4, and 8 min to initiate aggregation (Fig. 1). Thus, the magnitude and rapidity of the PMA-induced response are dose dependent.

Effect of glucose, deoxyglucose, and ambient temperature. In the absence of extracellular glucose, PMA induced a normal response (Table I). However, 10 m*M* deoxyglucose in the absence (but not in the presence) of glucose strongly inhibited this response (Table I). Decreasing the ambient temperature to 3°C was also inhibitory. At this temperature, 6×10^{-8} *M* PMA had no apparent effect on the cells (Table I). The phorbol ester, therefore, does not appear to act like a polyvalent ligand which nonspecifically agglutinates the cells. On the contrary, its bioactivity appears to require a response by metabolically active neutrophils.

Effect of extracellular calcium and magnesium. To study the effects of these bivalent cations, neutrophils were suspended in medium containing 1.4 m*M* calcium, 0.7 m*M* magnesium, or neither cation; exposed to PMA; and assayed for their maximal aggregation response (MLPP). Figure 2 gives the MLPP found after adding various concentrations of PMA to cells suspended with both cations (upper curve), magnesium alone (center curve), or calcium alone (lower curve). Although not shown, similar studies were performed with cells suspended with neither cation; these results were identical to those found for cells suspended with only calcium: over the entire range of concentrations studied, PMA did not aggregate PMNs suspended in magnesium-free medium. Contrastingly, PMNs suspended without calcium but with 0.7 m*M* magnesium exhibited a prominent response which was about 40% of the magnitude, and retained the sigmoidal dose–response curve shape, of the response of cells suspended with both cations (compare the two upper curves of Fig. 2). Although

TABLE I. INFLUENCE OF AMBIENT TEMPERATURE AND DEOXYGLUCOSE ON THE NEUTROPHIL AGGREGATION RESPONSE TO PHORBOL MYRISTATE ACETATE (PMA)[a]

Simulator PMA (n*M*)	Condition	Time (min) after adding PMA					
		1	2	4	8	11	15
6	Glucose, 37°C	3.6 ± 1.2[b]	9.4 ± 2.8	12.9 ± 3.4	30.1 ± 4.6	33.6 ± 4.1	29.1 ± 3.1
6	No glucose, 37°C	1.7 ± 0.4	5.7 ± 2.2	10.3 ± 3.4	25.5 ± 4.7	34.1 ± 4.2	31.1 ± 2.4
6	Deoxyglucose, 37°C	1.0 ± 0.3	1.6 ± 0.7*	2.7 ± 1.2*	5.1 ± 1.7*	6.9 ± 2.5*	8.0 ± 2.8*
6	Glucose plus deoxyglucose, 37°C	4.0 ± 1.5	8.9 ± 2.4	17.6 ± 3.2	32.0 ± 5.1	40.8 ± 4.4	39.9 ± 3.7
60	Glucose, 37°C	10.5 ± 2.9	17.4 ± 3.1	26.7 ± 4.7	42.3 ± 2.3	45.5 ± 2.7	33.7 ± 2.2
60	Glucose, 3°C	0.8 ± 0.1**	0.8 ± 0.2**	0.7 ± 0.1**	0.9 ± 0.3**	0.6 ± 0.1**	0.7 ± 0.1**

[a] Neutrophil suspensions were preincubated with glucose (10 m*M*), deoxyglucose (10 m*M*), or neither agent for 5 min and 1.4 m*M* calcium and 0.7 m*M* magnesium for 1 min before exposure to PMA. The experiments were performed at the indicated ambient temperature.

[b] Mean large-particle percentage, ±SEM, found at the indicated time after adding PMA. Each value is the mean for at least four experiments.

* $P < 0.05$, compared to cells preincubated without deoxyglucose.

** $P < 0.05$, compared to cells studied at 37°C.

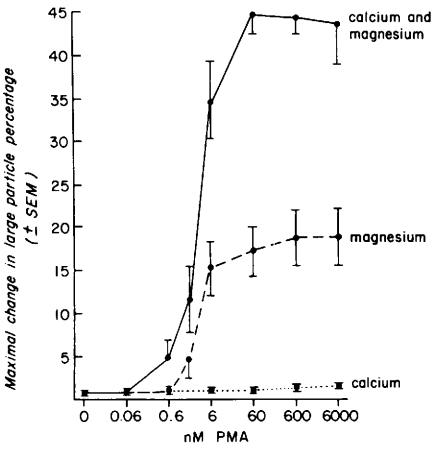

FIG. 2. Effect of extracellular calcium and magnesium on the maximal change in the large-particle percentage of neutrophil suspensions exposed to varying concentrations of PMA. Cells were preincubated with 1.4 m*M* calcium and/or 0.7 m*M* magnesium for 1 min before exposure to PMA. Each point is the mean of at least six experiments.

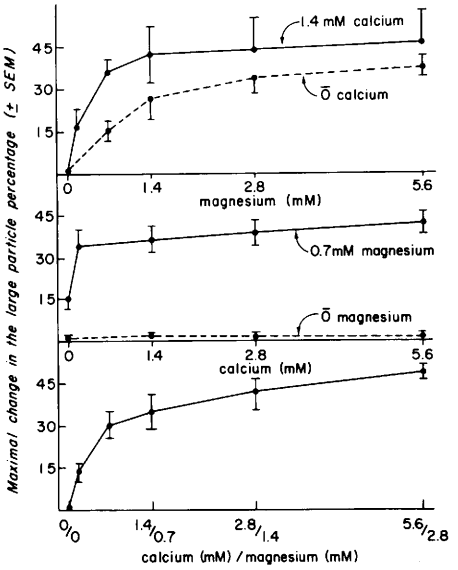

FIG. 3. Effect of various concentration of calcium and magnesium on the maximal change in large-particle percentage of neutrophil suspensions exposed to 6 n*M* phorbol myristate acetate. Cells were preincubated for 1 min with 1.4 m*M* or no calcium and varying magnesium (upper panel), 0.7 m*M* or no magnesium and varying calcium (center panel), or varying concentrations of both bivalent cations (lower panel) before exposure to the drug.

not shown by the data of Fig. 2, cells suspended with only magnesium aggregated more slowly than cells suspended with both cations at all PMA concentrations. Most frequently, their response did not peak during the observation period, i.e., at 15 min, the LPP of these suspensions continued to rise.

In order to further study the influence of bivalent cations upon this response, the concentrations of calcium and magnesium were varied while the concentration of stimulus, PMA, was held constant at 6×10^{-9} *M*. In studies not shown, results similar to those described below were found with higher and lower concentrations of PMA; and for all concentrations of both bivalent cations, appropriate controls were performed to insure that unstimulated cells did not aggregate spontaneously. Figure 3 (upper panel) indicates that cells suspended without calcium aggregated very prominently in the presence of higher concentrations of magnesium; above 1.4 m*M*, magnesium by itself supported responses of magnitude similar to that seen in cells suspended with high concentrations of both cations. In these studies lack of extracellular calcium was still associated with a somewhat more slowly developing response which did not peak during the observation period. However, adding only 0.18 m*M* calcium to cells suspended with 0.7 m*M* magnesium also augmented PMA-induced aggregation, and the magnitude of this response (Fig. 3, center panel, solid line), as well as its rapidity of onset (not shown), matched that found in PMNs suspended with high concentrations of both cations. By itself, calcium in concentrations of 0.18–5.6 m*M* did not allow PMNs to aggregate (Fig. 3, center panel, lower curve). These data indicate that magnesium, but not calcium, can support PMA-induced aggregation. In the presence of lower concentrations of magnesium, only a small amount of calcium is required for a fully normal response; in the presence of higher magnesium concentrations, calcium is not required for the cells to aggregate maximally. Calcium increases the speed with which the cells react to PMA. And, in its presence macroscopic clumping more frequently occurs.

TABLE II. INFLUENCE OF CYTOCHALASIN B (CB) ON THE AGGREGATION RESPONSE TO PHORBOL MYRISTATE ACETATE (PMA) OF NEUTROPHIL SUSPENSIONS PREINCUBATED WITH CALCIUM AND MAGNESIUM OR MAGNESIUM[a] ALONE

Aggregator, PMA (n*M*)	Calcium	Magnesium	CB	Time (min) after adding PMA			
				2	8	11	15
6	1.4	0.7	0.5	16.3 ± 4.4[b]	34.7 ± 4.2	32.4 ± 3.3	26.3 ± 3.0
6	1.4	0.7	0	9.4 ± 2.8	30.1 ± 4.6	33.6 ± 4.1	29.1 ± 3.1
2	1.4	0.7	0.5	0.9 ± 0.1	9.0 ± 4.0	13.0 ± 5.0	17.0 ± 4.6
2	1.4	0.7	0	0.7 ± 0.1	3.6 ± 1.6	7.0 ± 3.0	12.1 ± 4.0
6	0	0.7	0.5	2.3 ± 0.3	7.5 ± 1.4	8.9 ± 1.5	11.0 ± 2.0
6	0	0.7	0	1.4 ± 0.2	6.0 ± 1.2	9.3 ± 2.0	16.0 ± 3.7

[a] Neutrophil suspensions were preincubated with cytochalasin B (μg/ml) for 5 min and calcium/magnesium (m*M*) for 1 min before exposure to PMA.

[b] Mean large-particle percentage, ±SEM, found at the indicated time after adding PMA. Each value is the mean of at least eight experiments.

Influence of cytochalasin B. Cytochalasin B dramatically enhances the speed and extent of calcium uptake in PMNs exposed to chemotactic factors or A23187 (9, 19). Table II shows that it did not significantly enhance PMA-induced aggregation. There was, however, a tendency for the drug to increase the response found in the presence and, contrastingly, to inhibit the response found in the absence, of calcium. At no time did this tendency reach significance ($P < 0.1$, Student's unpaired t test). Any action of cytochalasin B, therefore, appears minimal.

Influence of arachidonate antimetabolites. Cells preincubated with 100 or 200 μ*M* indomethacin, 10 μ*M* ETYA, or 10 μ*M* NDGA for 5 min responded normally to 6×10^{-9} *M* PMA (Table III) as well as to higher and lower concentrations of the stimulator (not shown). Lower concentrations of the antimetabolites were similarly ineffective in blocking the response (not shown). At 33 μ*M*, ETYA inhibited 6×10^{-9} *M* PMA by less than 30% (Table 3) and NDGA inhibited by about 75% (not shown). Higher PMA concentrations overcame this inhibition (not shown).

Discussion. Chemotactic factors, A23187, and arachidonic acid aggregate PMNs but their potency is comparatively modest. On a molar basis, they are active only at one to three orders of magnitude greater concentrations than PMA and, in general, their effects are much less prominent at all concentrations (1, 5, 18, 25, 44, 45). Only A23187 irreversibly aggregates the cells (18); chemotactic factors and arachidonic acid induce responses which reverse within minutes (5, 18). None of these agents aggregate PMNs suspended in calcium-free medium; indeed, their action requires comparatively high concentrations of both calcium and magnesium in order to induce detectable effects (17, 18). Cytochalasin B dramatically enhances [1, 17–19, 23, 25] whereas 100 μ*M* indomethacin (6, 25), 10 μ*M* ETYA (6, 25), and 10 μ*M* NDGA (personal observation) totally abrogate the response to these agents. It is evident, therefore, that aggregation induced by chemotactic factors, A23187, and arachidonic acid is very sensitive to extracellular calcium, cytochalasin B, and arachidonate antimetabolites.

PMA, on the other hand, potently induces PMN aggregation which does not reverse over 15 min (Fig. 1). Its action does not require extracellular calcium (Figs. 2 and 3), is insignificantly affected by cytochalasin B (Table II), and is minimally influenced by inhibitory concentrations of three arachidonate antimetabolites (Table III). Other actions of PMA also appear insensitive to the agents studied here. For instance, the drug aggregates platelets but its effects, unlike those of other platelet-aggregating agents such as adenosine diphosphate, are relatively uninfluenced by calcium chelators and arachidonate antimetabolites (46, 47). Again, PMA-induced release of specific granules and generation

TABLE III. INFLUENCE OF ARACHIDONIC ACID ANTIMETABOLITES ON THE NEUTROPHIL AGGREGATION RESPONSE TO PHORBOL MYRISTATE ACETATE (PMA)[a]

Agent	Concn (μM)	Time (min) after adding PMA						
		½	1	2	4	8	11	15
None		1.0 ± 0.1[b]	3.6 ± 1.2	9.4 ± 2.8	12.9 ± 3.4	30.1 ± 4.6	33.6 ± 4.1	29.1 ± 3.1
Indomethacin	100	1.2 ± 0.1	3.7 ± 1.7	8.8 ± 3.5	17.5 ± 6.4	31.4 ± 6.1	30.9 ± 4.0	25.5 ± 2.8
Indomethacin	200	1.2 ± 0.1	3.3 ± 1.4	9.1 ± 3.7	11.9 ± 2.8	31.5 ± 5.1	32.6 ± 4.9	29.4 ± 4.0
ETYA	10	0.9 ± 0.1	2.3 ± 0.9	7.1 ± 2.1	14.5 ± 3.6	29.7 ± 3.2	30.1 ± 4.0	26.2 ± 3.7
ETYA	33	1.0 ± 0.1	2.0 ± 0.4	5.5 ± 1.6	11.2 ± 1.9	21.2 ± 2.9	22.1 ± 2.9*	20.5 ± 2.4
NDGA	10	1.0 ± 0.1	1.5 ± 1.2	1.8 ± 6.0	6.1 ± 1.5	18.0 ± 4.0	25.1 ± 4.5	31.2 ± 4.4

[a] Neutrophil suspensions were preincubated with the indicated agent for 5 min and bivalent cations (1.4 m*M* calcium and 0.7 m*M* magnesium) for 1 min before exposure to 6 n*M* PMA.

[b] Mean large-particle percentage, ±SEM, found at the indicated time after adding PMA. Cells preincubated with the indicated agents for up to 20 min did not aggregate unless exposed to PMA. Each value is the mean of at least six experiments.

* $P < 0.05$ compared to the value found at the same time in cells not preincubated with an agent.

of reactive species of oxygen by neutrophils is virtually uninfluenced by extracellular calcium (14, 15, 36) and cytochalasin B (14, 15, 49). Combined with the data of this report, these results imply that certain platelet and neutrophil responses to PMA proceed relatively independently of extracellular calcium and arachidonic acid. Indeed, recent studies indicate that chemotactic factors (48) and A23187 (30) cause PMNs to mobilize their endogenous arachidonate pools but PMA does not (49).

Table IV summarizes the numerous relationships between PMN stimuli, responses, and their relative sensitivity to extracellular calcium, cytochalasin B, and arachidonate antimetabolites. The consistent pattern of sensitivities evidenced in this table as well as earlier mentioned data suggest that PMN aggregation, like specific granule enzyme release (14, 36) and oxidative metabolism (3, 15), can proceed through two distinctly different mechanisms. One mechanism is evoked by stimuli (e.g., certain chemotactic factors, A23187, arachidonic acid) which cause PMNs to take up extracellular calcium and mobilize and/or metabolize arachidonic acid. This mechanism, therefore, is sensitive to extracellular calcium, cytochalasin B, and arachidonate antimetabolites. The second mechanism is relatively insensitive to these agents. Stimuli operating through it (e.g., PMA) may act directly upon the cell membrane (37, 38, 41, 46) and/or cause rearrangements of cellular calcium (36, 50). They do not appear to require influxes of extracellular calcium or arachidonate metabolism.

These two mechanisms of action need not operate independently. For instance, extracellular calcium does enhance the PMN aggregation response to PMA in the presence of lower concentrations of magnesium (Fig. 3); cytochalasin B is required for PMA-induced release of *azurophilic* granule enzymes (35, 39); and chemotactic factors can induce release of specific granule enzymes in the absence of extracellular calcium (13, 20). A stimulant, then, may enlist more than one mechanism to effect PMN responses. Furthermore, studies on these relationships have

TABLE IV. SENSITIVITY TO CALCIUM, CYTOCHALASIN B, AND ARACHIDONATE ANTIMETABOLITES OF NEUTROPHIL RESPONSES EVOKED BY VARIOUS STIMULI[a]

Response	Agent	Stimulus				Reference
		Chemotactins	A23187	Arachidonate	PMA	
Aggregation	Calcium	+	+	+	−	(6,17–19,25)
Aggregation	Cytochalasin B	+ +	+ +	+ +	−	(1,6,17–19,23,25)
Aggregation	Arachidonate antimetabolites	+ +	+ +	+ +	−	(1,5,17)
Degranulation	Calcium	+ +	+ +	+ +	−	(4,10–14,16,20,32,34,36,38)
Degranulation	Cytochalasin B	+ +	+ +	+ +	−	(2,11–14,16,20,32,35,39)
Degranulation	Arachidonate antimetabolites	+ +	+ +	+ +	?	(2,11,32)
Oxidative	Calcium	+ +	+ +	?	−	(15,21,22,24,33,50)
Oxidative	Cytochalasin B	+ +	+ +	?	−	(15,21,50)
Oxidative	Arachidonate antimetabolites	+	?	?	?	(3)

[a] In all studies, neutrophils were preincubated with calcium, cytochalasin B, and/or arachidonate antimetabolite (indomethacin or ETYA) before exposure to chemotactins (C5a or synthetic formylated oligopeptides), A23187, arachidonic acid, or PMA. The ensuing response was compared to the response obtained without the agent. Symbols: +, the response is relatively sensitive (i.e., calcium and cytochalasin B enhance, arachidonate antimetabolites inhibit) to the indicated agent; −, the response is relatively insensitive to the indicated agent; ?, unknown.

employed PMNs obtained from different species (human, rabbit, or guinea pig) or sites (blood or peritoneum); these different cell preparations exhibit different responses to some of the stimuli studied here (1). The data of Table IV serve only to indicate that one mechanism appears to predominate under a specified set of conditions which are outlined in the references given. Further studies on these mechanisms are clearly required.

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