Role of Extracellular Calcium in Neutrophil Degranulation Responses to 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine

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The rabbit polymorphonuclear neutrophil degranulation response to 1-O-alkyl-2-O-acetyl-*sn*-glycero-3-phosphocholine depends on extracellular calcium. In the absence of this bivalent cation, neutrophil suspensions pretreated with cytochalasin B responded to the lipid by releasing minimal amounts of lysozyme and β-glucuronidase. Incremental increases in extracellular calcium over a range of 20–200 μM led to increasing amounts of lipid-stimulated enzyme release. In contrast, extracellular magnesium neither supported nor enhanced the degranulation responses. Verapamil (25–200 μg/ml), a calcium channel blocker, inhibited degranulation. Neutrophil suspensions exposed to the phosphocholine stimulus rapidly took up radiolabeled extracellular calcium. The kinetics of this calcium uptake were similar to the kinetics of enzyme release, and the amount of calcium taken up correlated closely with the amount of released lysozyme and β-glucuronidase. Finally, in a dosage which blocked degranulation, verapamil inhibited calcium uptake. Thus, the rapid association of extracellular calcium with the neutrophil may mediate, at least in part, the degranulating actions of the phosphocholine stimulus. (Am J Pathol 1981, 105:107–113)

PLATELET-ACTIVATING FACTOR, a compound recently identified as 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (AAGPC)[1,2] is an endogenously formed mediator of anaphylactic reactions.[3,4] *In vitro,* it activates platelets and polymorphonuclear neutrophils (PMNs) and stimulates contraction of various smooth muscle preparations, including guinea pig lung.[4–9] Each of these *in vitro* actions of AAGPC may be related to the phospholipid's *in vivo* anaphylactic toxicity. Thus, by stimulating aggregation and degranulation of platelets and PMNs and by constricting bronchial smooth muscle, AAGPC may clog pulmonary blood vessels with hematogenous cells, injure pulmonary capillary endothelium, cause bronchoconstriction, and disrupt pulmonary function.[3–10] The mechanisms by which cells respond to AAGPC, therefore, are of interest. We reported previously that extracellular calcium was required for the rabbit PMN-stimulating actions of AAGPC and suggested that the phospholipid might act by causing these cells to take up extracellular calcium.[8] Many studies implicate calcium uptake in PMN responses to other stimuli.[11–18] Here we report on studies that support this mechanism of action for AAGPC in the rabbit PMN degranulation response.

Materials and Methods

Reagents and Buffers

$^{45}Ca^{2+}$, ^{3}H-inulin, ^{3}H-sucrose, and Aquasol were purchased from New England Nuclear Corporation (Boston, Mass). Cytochalasin B, ethylenediamine tetraacetic acid (EDTA), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, Mo); n-butylphthalate and formic acid (88%) were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Verapamil hydrochloride was a generous

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gift of Knolls Pharmaceutical Co. (Whippany, NJ). The buffer used for these studies was a modified Hanks' balanced salt solution.[15] Where indicated, calcium and magnesium were added to this buffer as chloride salts.

Preparation, Storage, and Use of AAGPC

AAGPC was prepared and its structure confirmed by mass spectroscopy as described.[5] It stimulated platelet aggregation and secretion of radiolabeled serotonin in subnanomolar concentrations virtually identical to those previously reported.[1-5] The phospholipid was stored in chloroform:methanol (1:1, v:v) at −70 C. Shortly before use, AAGPC was blown dry with nitrogen. The compound was brought up in BSA (2.5 mg/ml) buffer and further diluted in this same BSA buffer. For all studies, the final concentration of BSA was either 125 μg/ml (in degranulation assays) or 62.5 μg/ml (for uptake of radiolabeled substances).

Cell Preparations

PMNs were obtained from the rabbit peritoneum, washed, and freed of contaminating erythrocytes as described.[8] The final leukocyte preparation consisted of more than 95% PMNs and fewer than 20 platelets/100 PMNs.

Degranulation Assay

PMNs (2.6×10^6 cells/ml) were suspended in buffer (which contained an indicated concentration of Ca^{2+} and Mg^{2+}) at 37 C for 20 minutes, exposed simultaneously to cytochalasin B (5 μg/ml) and AAGPC, and at specified times thereafter placed on ice. The cell suspensions were centrifuged at 4 C at 200*g* for 3 minutes in order to obtain supernatant fluid, which was assayed for lysozyme, β-glucuronidase, and lactic acid dehydrogenase, as described.[8] Where indicated, verapamil was added to the cell suspension 5–10 minutes before a stimulus.

Uptake of Radiolabeled Substances

$^{45}Ca^{2+}$ uptake was performed by a slight modification of the procedure reported by Naccahe et al.[13,14] PMN suspensions (5×10^6 cells/ml) were incubated with 3 μCi/ml $^{45}Ca^{2+}$ plus 0.5 mM unlabeled calcium for 40 minutes at 37 C at a final suspension volume of 15 ml in 50-ml polycarbonate test tubes. As reported by Naccache et al,[13] the amount of $^{45}Ca^{2+}$ in cells did not change after this time, i.e., equilibrium with radiolabel was reached. (At this time pelleted cells from 1 ml of suspension contained between 4000–6000 cpm). The cells were then challenged with cytochalasin B plus a stimulus. Just before and at indicated times after this challenge, 1-ml samples of the suspension were taken in duplicate, placed over 0.5 ml of n-butyl phthalate (in a 1.5-ml Eppendorf plastic tube), and centrifuged at 8000*g* for 30–45 seconds. The pelleted cells were isolated by cutting off the bottom of the Eppendorf tubes and placing these bottoms in 4-ml scintillation vials containing 0.5 ml of formic acid. The vials were incubated in a shaking water bath at 37 C for 30 minutes. Thereafter, 3 ml of Aquasol were added to the scintillation vials and the contents were thoroughly mixed before counting the duplicate samples with a Beckman Scintillation Counter, LS 100C (Beckman Instrument Co., Irvine, Calif). For some studies, PMNs were incubated with either ^{3}H-inulin or ^{3}H-sucrose instead of $^{45}Ca^{2+}$; the incubation, stimulation, and cell isolation procedures were those described above. The two saccharides served as markers of extracellular fluid. PMNs preincubated with them for up to 60 minutes were freed of 99.96% of the extracellular fluid after isolation. Furthermore, PMNs exhibited a 6.1 ± 3.0 (SEM for 7 studies), 6.8 ± 3.5, 8.8 ± 2.9, 0.6 ± 4.4, 7.1 ± 6.0, and 8.9 ± 4.5 percentage increase in tritium label at 0.5, 1, 2, 4, 8, and 15 minutes, respectively, after exposure to 100 nM AAGPC plus 5 μg/ml cytochalasin B. Thus, the isolation procedure effectively freed PMNs of extracellular fluid, and AAGPC did not appreciably increase the amount of extracellular fluid associated with the isolated cells.

Cellular Toxicity

PMNs exposed to the stimuli, inhibitor, and reaction conditions used here released less than 5% of their total cellular content of lactic acid dehydrogenase.

Results

Influence of Calcium and Magnesium on Degranulation

Dose-response curves for rabbit peritoneal PMN degranulation responses to AAGPC are published.[8] At all doses of AAGPC (ie, 0.23–2,300 nM), degranulation occurred only when cells were also treated with cytochalasin B. By itself, this latter compound caused a small but significant amount of enzyme release. For instance, PMNs challenged with 230 nM

AAGPC, 230 nM AAGPC plus 5 μg/ml cytochalasin B, or 5 μg/ml cytochalasin B released 9.2 ± 2.1%, 61.4 ± 10.1%, and 24.2 ± 1.9% (SEM), respectively, of their total content of lysozyme. These cells were preincubated with 1.4 mM calcium and 0.7 mM magnesium, challenged with AAGPC and/or cytochalasin B simultaneously, and assayed for enzyme release 5 minutes after challenge. Identically treated cells that were challenged only with BSA released 6.7 ± 2.4% lysozyme. In addition, at all doses of AAGPC, the kinetics of enzyme release were rapid; responses became maximal within 1-3 minutes of challenge (data not shown). In view of these findings, the remaining studies were performed on cytochalasin-B-treated PMNs that were challenged for 4-5 minutes (exact time indicated).

The amount of lysozyme (Figure 1) and β-glucuronidase (Figure 2) released by varying doses of

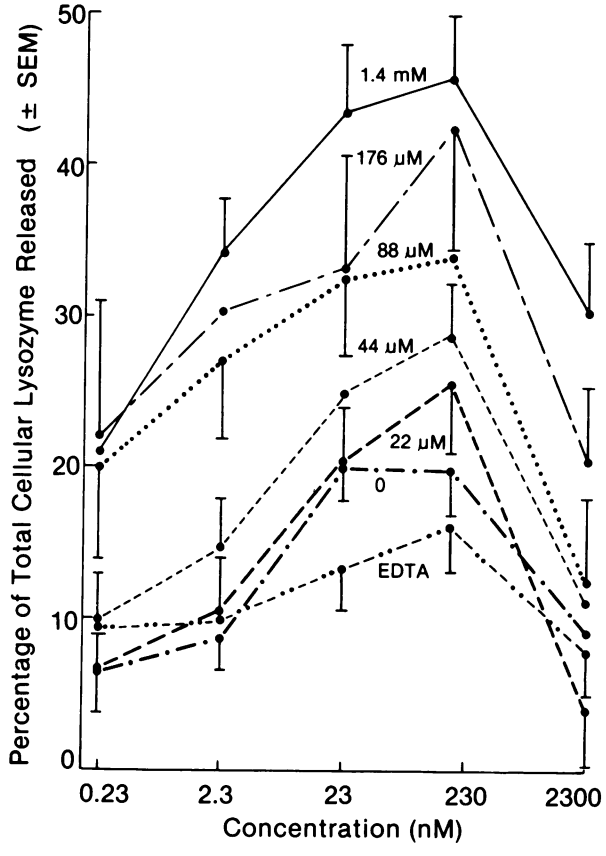

Figure 1—Influence of extracellular calcium concentrations on neutrophil release of lysozyme stimulated by varying doses of AAGPC. Each curve gives the amount of enzyme released at the specified calcium concentration (upper six curves) or in the presence of 2 mM EDTA (lowest curve). Each point gives mean values for at least six experiments.

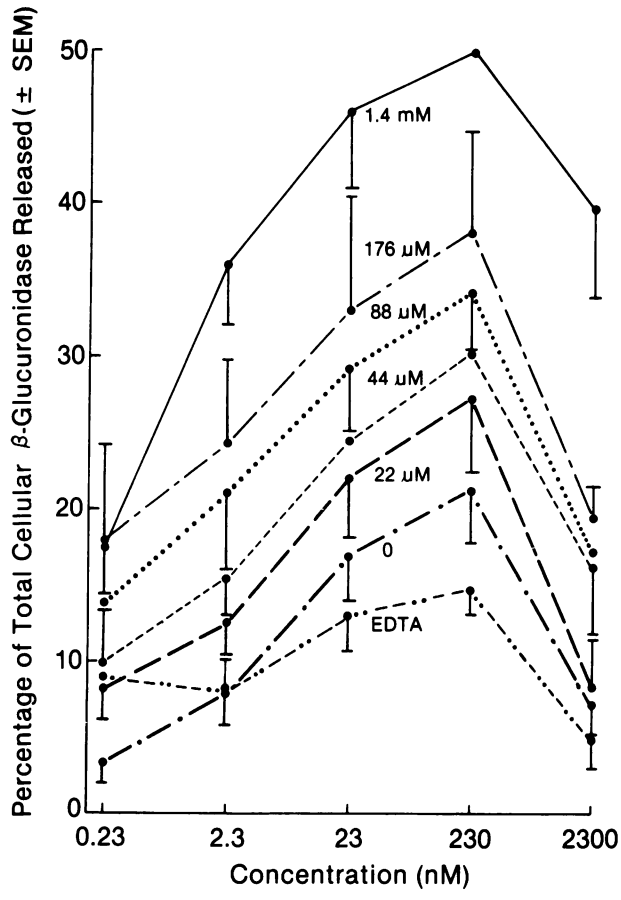

Figure 2—Influence of extracellular calcium concentrations on neutrophil release of β-glucuronidase stimulated by varying doses of AAGPC. Each curve gives the amount of enzyme released at the specified calcium concentration (upper six curves) or in the presence of 2 mM EDTA (lowest curve). Each point gives the mean values for at least six experiments.

AAGPC increased with increases in extracellular calcium. Without added calcium our Hanks' buffer contained less than 20 μM calcium. This trace of the bivalent cation may have contributed to degranulation because EDTA, when added to this buffer, further depressed the extent of enzyme release (lowest curves of Figures 1 and 2). Alternatively, EDTA may have taken calcium from PMN or chelated some other cation important in degranulation. However, magnesium did not appear to be involved in enzyme release: in the presence of 0-1.4 mM calcium, 0-2.8 mM magnesium had no appreciable influence on the magnitude of AAGPC-induced degranulation (Table 1). Thus, extracellular calcium, but not magnesium, is important in the response to AAGPC.

$^{45}Ca^{2+}$ Uptake

Duplicate 1-ml samples of a 15-ml PMN suspen-

Table 1—Effect of Magnesium on the Neutrophil Degranulation Response to AAGPC*

Ca^{2+} (mM)	Mg^{2+} (mM)	Enzyme released† Lysozyme (%)	Enzyme released† β-glucuronidase (%)
0	0	37.2 ± 4.2	27.0 ± 3.9
0	0.7	33.7 ± 3.5	21.2 ± 0.8
0	1.4	37.7 ± 1.9	26.0 ± 1.5
1.4	0	71.4 ± 4.3	64.9 ± 8.5
1.4	0.7	65.7 ± 5.8	54.9 ± 3.7
1.4	2.8	68.4 ± 6.6	50.2 ± 7.1

* Rabbit neutrophils were incubated with the indicated concentrations (mM) of calcium and magnesium for 20 minutes and then exposed to 5 μg/ml cytochalasin B plus 23 nM AAGPC for 5 minutes before assay of enzyme release.

† Percentage of total cellular enzyme released, ± SEM, for at least five experiments.

sion (preincubated with 0.5 mM calcium, micromolar amounts of $^{45}Ca^{2+}$, and no magnesium for 40 minutes) were challenged with 5 μg/ml cytochalasin B plus an indicated dose of AAGPC or BSA. Cells challenged with cytochalasin B plus BSA took up a small amount of radiolabel (Figure 3, lowest curve), and this uptake correlated with the small amount of enzyme released under these conditions (see above). AAGPC, in doses of 1–100 nM, enhanced $^{45}Ca^{2+}$ uptake (Figure 3). This response peaked 4 minutes after challenge and was, therefore, somewhat more slowly developing than degranulation response, which peaked within 1–3 minutes (see above). Nevertheless, the magnitudes of the two responses were closely correlated (Table 2); the correlation coefficients between $^{45}Ca^{2+}$ uptake and lysozyme or β-glucuronidase release, seen in Table 2, are 0.90 and 0.95, respectively.

PMNs suspended in physiologic mediums adhere to the surfaces of their containers.[18,19] Because we assay $^{45}Ca^{2+}$ from 1-ml samples of a PMN suspension, adherence of PMNs to containers could systematically influence our results. Therefore, we analyzed the PMN concentrations in our samples. We found that the PMN counts in these samples abruptly fell by 10–30% after challenge with any combination of AAGPC, BSA, and cytochalasin B. When corrected for changes in PMN number, $^{45}Ca^{2+}$ uptake still closely resembled the data shown in Figure 3, ie, except for increasing the magnitude of response, (eg, per PMN, $^{45}Ca^{2+}$ uptake doubled 4 minutes after challenge with 100 nM AAGPC plus cytochalasin B), this correction did not alter the nature of the results. We conclude that extracellular calcium rapidly associates with degranulating PMN. The relation of this association to the PMN response was probed by examining the actions of verapamil.

Effects of Verapamil

Verapamil blocks calcium influx into various cells,[20] including human PMN.[21] Table 3 shows that the compound also blocked $^{45}Ca^{2+}$ uptake in AAGPC-treated rabbit PMN. Concomitantly, verapamil blocked AAGPC-induced release of lysozyme (Figure 4) and β-glucuronidase (data not shown). Hence, calcium uptake appears necessary for the degranulation response to AAGPC.

Discussion

For over a decade, investigators have studied the role of extracellular calcium in PMN responses to C5a and formylated oligopeptides. Their studies show that many PMN responses to these agents require extracellular calcium, are inhibited by agents that block calcium uptake, and occur concomitantly with calcium uptake. Responses studied in this manner are chemotaxis,[12,13,22,23] degranulation,[11,14,16,17] oxidative metabolism,[24,25] and aggregation.[15,18,26] Here, we studied the role of extracellular calcium in the degranulation response to AAGPC. We found that this response depends on, and is proportionate to, the level of extracellular calcium (Figures 1 and 2) but not the level of magnesium (Table 1). It is inhibited by the calcium channel blocker verapamil (Figure 4). And, it occurs concomitantly with $^{45}Ca^{2+}$ uptake (Figure 3). A fair but imperfect correlation exists between the kinetics of $^{45}Ca^{2+}$ uptake and enzyme release, and an excellent correlation exists between the extent of $^{45}Ca^{2+}$ uptake and degranulation (Table 2). Like other stimuli, then, AAGPC causes PMNs to take up calcium, and this uptake appears

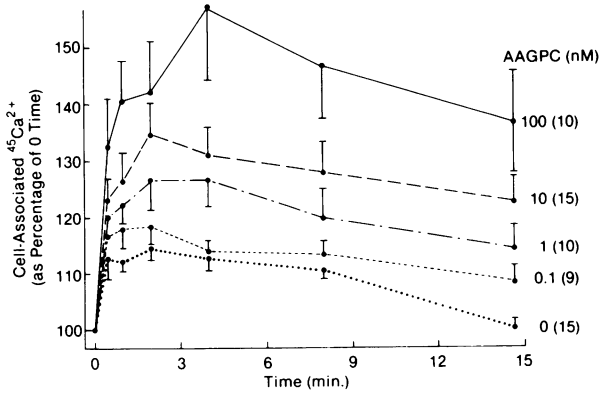

Figure 3—$^{45}Ca^{2+}$ associated with neutrophils after exposure to varying doses of AAGPC. The ordinate is expressed as a percentage of the prestimulation value. The numbers in parentheses are the number of experiments performed at each point on the indicated curves.

Table 2—Degranulation and $^{45}Ca^{2+}$ Uptake in Neutrophils Under Various Reaction Conditions*

Reaction conditions	Response		
	$^{45}C^{2+}$ uptake† (%)	Lysozyme release‡ (%)	β-glucuronidase release‡ (%)
No cytochalasin B (cyto B) or stimulus	99.0 ± 0.8§	6.0 ± 1.6	5.0 ± 0.9
Cyto B, BSA	114.7 ± 4.1	26.0 ± 2.9	15.5 ± 2.1
Cyto B, 0.1 nM AAGPC	112.5 ± 4.2	20.0 ± 2.6	17.1 ± 3.0
Cyto B, 1 nM AAGPC	129.4 ± 4.0	36.3 ± 3.3	30.0 ± 3.3
Cyto B, 10 nM AAGPC	133.0 ± 5.0	51.2 ± 3.4	42.0 ± 1.9
Cyto B, 100 nM AAGPC	158.4 ± 12.3	60.4 ± 4.0	50.6 ± 3.2
Verapamil (200 μg/ml), cyto B, 10 nM AAGPC	95.9 ± 4.2	30.6 ± 3.9	18.0 ± 2.8

* Neutrophil suspensions were incubated with 0.5 mM calcium for 40 minutes (for calcium uptake) or 20 minutes (for degranulation) and then exposed to cytochalasin B, BSA, or AAGPC, as indicated. Verapamil was incubated with the cells for 5-10 minutes before stimulation.
† Cell-associated $^{45}Ca^{2+}$ as percentage of prestimulation values, found 4 minutes after stimulation.
‡ Percentage of total cellular enzyme released 4 minutes after challenge.
§ Mean ± SEM.

necessary for the degranulating action of the phospholipid. These studies gain importance because they deal with a newly described physiologic stimulus that occurs naturally,[1,2,6,7,27-29] activates various cell types,[1-9] and is involved pathogenically in disease states such as anaphylaxis.[3,4,8,10] Furthermore, PMNs[6,7] and platelets[29] rapidly produce AAGPC when stimulated. This endogenously produced AAGPC may be responsible for the respective PMN and platelet responses to the stimuli that elicit its formation.[6,7,29] If so, our data suggest that intracellularly formed AAGPC may mediate function, in part, by influencing calcium uptake.

The concept that extracellular calcium moves into or on the cell to mediate enzyme release is not fully supported by the data. For instance, $^{45}Ca^{2+}$ uptake and enzyme release did not parallel one another perfectly; 1 minute after stimulation, PMNs had released most of their enzymes but continued to take up more calcium for an additional 2-3 minutes. This result indicates that at least a portion of $^{45}Ca^{2+}$ uptake occurs following degranulation. Indeed, we were unable to show that $^{45}Ca^{2+}$ uptake preceded enzyme release. In a recent study,[30] $^{45}Ca^{2+}$ uptake in cytochalasin-B-pretreated rabbit PMN (stimulated with a chemotactic factor) appeared to be a consequence, not a cause, of enzyme release. It is important, therefore, to consider the causal relations between calcium uptake and degranulation. Three points strongly argue that calcium uptake effects enzyme release. First, AAGPC-induced degranulation requires extracellular calcium and is proportional to the level of this cation (Figures 1 and 2). Second, verapamil, a drug that blocks calcium movements through membranous channels and calcium uptake in AAGPC-stimulated PMN (Table 3), inhibits degranulation (Figure 4). And third, bivalent cation ionophores such as A23187 specifically and selectively carry extracellular calcium into the PMN and concomitantly trigger degranulation.[11,14,17] Taken together, these data support the notion that calcium uptake is necessary and sufficient for the degranulation response to AAGPC. However, the way in which this uptake influences the cell response is unknown. The movement of calcium into the PMN does not necessarily alter the level of cellular calcium. Stimulated PMNs also release calcium[12-14]; the net change in cell calcium, therefore, is uncertain. Furthermore, a recent study found no change in total cellular calcium in rabbit PMN treated with cytochalasin B and a chemotactic factor.[30] Thus, changes in the level of calcium at a *subcellular* site may be important for the ensuing response. For instance, the association of calcium with cytoskeletal or contrac-

Table 3—Influence of Verapamil on $^{45}Ca^{2+}$ Uptake in Neutrophils After Exposure to AAGPC*

Inhibitor	Time (min)					
	½	1	2	4	8	15
Verapamil	87.5 ± 3.3†	92 ± 6.2	94.3 ± 6.1	104.1 ± 4.2	101.2 ± 2.9	106.2 ± 1.4
None	122.4 ± 4.9	125.6 ± 4.8	132.0 ± 5.1	129.6 ± 4.6	127.7 ± 5.4	119.0 ± 4.7

* Rabbit neutrophil suspensions were incubated with 200 μg/ml verapamil or no inhibitor for 5-10 minutes before stimulation with 10 nM AAGPC.
† Cellular $^{45}Ca^{2+}$ expressed as percentage of prestimulation value, ± SEM, for at least seven experiments. Each value for verapamil-pretreated cells is significantly ($P < 0.05$) lower than the values for cells not treated with the inhibitor.

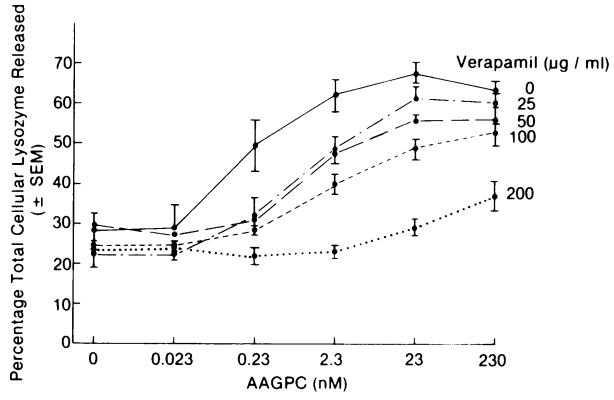

Figure 4—Influence of verapamil on neutrophil release of lysozyme stimulated by varying doses of AAGPC. β-glucuronidase release was similarly influenced by verapamil. Each point gives mean values for at least five experiments.

tile elements, cellular membranes, or enzymes involved in cyclic nucleotide or arachidonic acid metabolizing sites could trigger events leading to degranulation.[31] It is clear, then, that AAGPC, C5a, and formylated oligopeptides share similar mechanisms in activating rabbit PMN. However, the precise identity of these mechanisms remains elusive.

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