

EVIDENCE FOR SEQUENTIAL SIGNALS IN THE INDUCTION OF THE ARACHIDONIC ACID CASCADE IN MACROPHAGES

BY ALAN A. ADEREM, WILLIAM A. SCOTT, AND ZANVIL A. COHN

From The Rockefeller University, New York 10021

The phospholipids of macrophage membranes contain unusually high concentrations of arachidonic acid (20:4)¹ (1). Stimulation of these cells by appropriate membrane-perturbing agents of both a soluble and particulate nature leads to the induction of phospholipase activity and the quantitative oxygenation of the released 20:4 via the cyclooxygenase (2, 3) and lipoxygenase pathways (4). Particulate agents, including zymosan and immune complexes, which trigger the arachidonic acid cascade, bind to specific membrane receptors to exert their effects. The sequence of events after receptor-ligand interaction that lead to the induction of phospholipase activity is poorly understood.

One of the receptors known to trigger the 20:4 cascade, the Fc receptor, has recently been shown to act as a monovalent cation channel capable of mediating Na⁺ fluxes across plasma membrane vesicles (25) or across planar lipid bilayers (27). Also, we reported previously (5) that zymosan-induced phagocytosis and 20:4 secretion are functionally uncoupled by high concentrations of extracellular K⁺. We have investigated whether an influx of Na⁺ into the cell mediated by the ligated FcR might act as a signal in the activation sequence of the phospholipase. We now report that an influx of Na⁺ and new protein synthesis are required as early steps in receptor-mediated induction of phospholipase activity and that both steps can be bypassed by a Ca²⁺ influx mediated by an ionophore.

Materials and Methods

Macrophage Cultures. Primary cultures of peritoneal macrophages were established from resident cells of specific pathogen-free female ICR mice (Trudeau Institute, Saranac Lake, NY) weighing 25–30 g, as previously described (6). Peritoneal cells (~9 × 10⁶ cells/ml) in MEM (α-MEM) (Gibco Laboratories, Grand Island, NY) containing 10% FCS were added to 35-mm-diam plastic culture dishes (1 ml/dish) or to 12-mm glass coverslips (0.1 ml/coverslip). After 2 h at 37°C in 95% air and 5% CO₂, cultures were washed three times in calcium- and magnesium-free PBS (PD) to remove nonadherent cells. Fresh α-MEM plus 10% FCS (1 ml/dish) containing 0.5 μCi of 5,6,8,9,11,12,14,15-[³H]arachidonic acid ([³H]20:4; sp act, 70 Ci/nmol; New England Nuclear, Boston, MA) was added, and the cells were incubated overnight (16 h).

Preparation of Unopsonized Zymosan. Zymosan was purchased from ICN K & K Labo-

This work was supported in part by grant AI 07012 from the National Institutes of Health. Address correspondence to A. Aderem, Box 280, The Rockefeller University, New York, NY 10021.

¹ Abbreviations used in this paper: 20:4, arachidonic acid; BIGG, derivitized glass beads coated with IgG; Hetes, hydroxyeicosatetraenoic acids; LTC, leukotriene C; PD, calcium- and magnesium-free PBS.

ratories, Inc. (Plainview, NY) and stock solutions in PD were prepared according to the method of Bonney et al. (2).

Preparation of Ligand-coated Particles. Glass beads (8 μm ; Duke Scientific Corp., Palo Alto, CA) were derivitized with DNP as described previously for glass coverslips (7) and were suspended in PBS at 50 mg/ml. IgG-coated beads (BIGG) were obtained by incubating 10 mg of DNP beads for 30 min at 20°C with 25 $\mu\text{g}/\text{ml}$ of affinity-purified IgG anti-DNP (8). The beads were then washed three times with PBS and resuspended in α -MEM at 50 mg/ml. DNP beads did not trigger the release of 20:4 from macrophages.

Assay of Total [^3H]20:4 Release. Macrophages prelabeled with [^3H]20:4 (New England Nuclear) were washed four times in PD and were then overlaid with 1 ml of serum-free α -MEM. Zymosan (160 $\mu\text{g}/\text{ml}$), BIGG (5 mg/ml), PMA (100 ng/ml), and A23187 (10 $\mu\text{g}/\text{ml}$) were added as indicated. The cells were incubated at 37°C in 95% air and 5% CO_2 , and at the times indicated, aliquots of medium were removed and counted in Hydrofluor (National Diagnostics, Inc., Somerville, NJ). The cells were washed in PD and scraped twice into 1 ml of Triton X-100. Portions of the cell lysates were assayed for radiolabel content and protein was determined by the method of Lowry et al. (9) with BSA as a standard.

Quantitation of [^3H]20:4 Metabolites. The 20:4 metabolites in the culture medium were extracted by the method of Unger et al. (10). To 1 ml of medium was added 1 ml of ethanol and 10 μl of 88% (wt/wt) formic acid, and the resulting solution was extracted twice with 1 ml of chloroform containing 0.005% (wt/vol) butylated hydroxytoluene (Sigma Chemical Co., St. Louis, MO). The lower (chloroform) phases were combined, evaporated to dryness under nitrogen, and the residue was dissolved in the appropriate starting solvent for further purification by reverse-phase HPLC.

Concentrated medium extracts were applied to a column of Ultrasphere C-18 (4.6 mm \times 25 cm) (Altex Scientific Inc.) (HPLC system 1). The column was eluted at a rate of 1 ml/min with 60 ml of solvent 1 (methanol/0.01 M H_3PO_4 , 65:35, vol/vol, adjusted to pH 5.4 with ammonium hydroxide), followed by 40 ml of solvent 2 (methanol/acetic acid, 100:0.01, vol/vol) (5, 11). For the characterization of the cyclooxygenase metabolites, a duplicate extract was applied on HPLC system 2 using a Waters Fatty Acid Analysis Column (Waters Associates, Millipore Corp., Milford, MA). Prostaglandins were eluted at a flow rate of 2 ml/min with 120 ml of solvent 3 (water/acetonitrile/benzene/acetic acid, 76.7:23:0.2:0.1, vol/vol/vol/vol), followed by 40 ml of solvent 2 (5, 12). Fractions from the HPLC were collected at 1 min intervals, and the radiolabel content of whole fractions or aliquots was measured by liquid scintillation counting in Hydrofluor. Corrections were made for counting efficiency (~40%). The recovery of 20:4 metabolites through extraction and purification procedures was monitored using radiolabeled standards (13).

Media: High Potassium, High Choline, High Sodium, and Ca^{2+} -free. High K^+ medium consisted of 15 mM Hepes, 10 mM KHCO_3 , 118 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , 5.5 mM glucose, α -MEM essential and nonessential amino acids (Gibco Laboratories), and 100 mg/l L-cysteine. Amino acids were Na^+ free and the pH was adjusted to 7.4 using KOH or HCl. High choline medium was identical to high K^+ medium except that 120 mM choline chloride replaced the KCl. High Na^+ medium contained 15 mM Hepes, 10 mM NaHCO_3 , 118 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , 5.5 mM glucose, α -MEM essential and nonessential amino acids, and 100 mg/l L-cysteine. The pH was adjusted to 7.4 using NaOH and HCl. Ca^{2+} -free medium was identical to high Na^+ medium except that 0.5 mM EGTA replaced the CaCl_2 .

Protein Synthesis. Cells were placed in either high K^+ medium or in α -MEM containing 2 μCi of [^{35}S]methionine with or without cycloheximide (5 $\mu\text{g}/\text{ml}$) and emetine (5 $\mu\text{g}/\text{ml}$) (Sigma Chemical Co.). After incubation at 37°C in 95% air and 5% CO_2 for 2 h, the cells were washed four times with the respective medium and the reaction was quenched by addition of 10% cold TCA. Acid-precipitable material was recovered on a filter and washed with 50 vol of 10% TCA. Radioactivity remaining on the filter was then determined.

Calculation of Free Ca^{2+} Concentration. Free Ca^{2+} concentration was calculated at pH

7.4 from the pK_a values of H-EGTA, M-EGTA, and MH-EGTA, where H and M indicate bound H^+ and metal ions (both Ca^{2+} and Mg^{2+}), respectively (34).

Materials. All materials used were reagent grade. Cycloheximide, emetine, actinomycin D, quinine hydrochloride, ATP, and ADP were from Sigma Chemical Co. and A23187 was obtained from Calbiochem-Behring Corp., San Diego, CA.

Results

Effect of Membrane Depolarization on Zymosan-induced 20:4 Release. We have reported previously (5) that zymosan-induced 20:4 release from mouse peritoneal macrophages is selectively inhibited by high (118 mM) concentrations of extracellular K^+ . One consequence of placing cells in high K^+ medium is the rapid depolarization of the plasma membrane. It has recently been shown (14) that millimolar concentrations of extracellular ATP similarly depolarizes peritoneal macrophages. We therefore wished to determine whether depolarization caused by extracellular ATP also inhibited 20:4 secretion.

Resident peritoneal macrophages released 24% of their cellular 20:4 content when challenged with zymosan in a medium containing 5 mM ATP (Fig. 1B). These values compared well with the 25% 20:4 released in response to zymosan in the absence of ATP (Fig. 1A). Basal release of 20:4 in the absence of zymosan was elevated in the cells exposed to ATP (5–6% released) when compared with control cells (1–2%). Macrophages in 5 mM ADP, which does not cause depolarization, released similar amounts of 20:4 in response to the zymosan stimulus (Fig. 1C). For all conditions described above, >95% of the cells were impermeable to trypan blue at the end of the experiment (data not shown). These data suggest that depolarization of resident peritoneal macrophages per se does not inhibit the release of 20:4 from the cell membrane.

Requirement for Extracellular Na^+ in 20:4 Secretion. Macrophages that had preingested zymosan in a high K^+ medium released 20:4 when placed in an Na^+ -containing environment, without the further addition of a phagocytic trigger

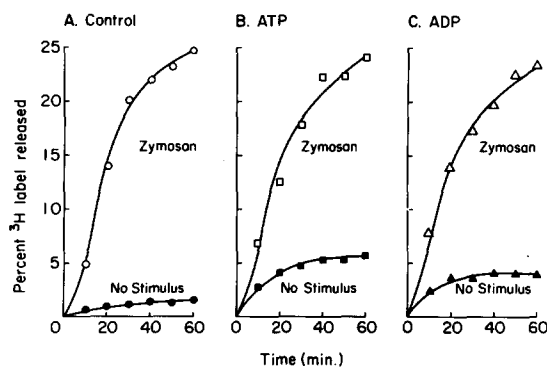


FIGURE 1. Time course of 20:4 release in media containing millimolar concentrations of adenine nucleotides. Macrophages were isolated and labeled with $[^3H]20:4$. The cells were then washed, overlaid with α -MEM (A) or α -MEM containing 5 mM ATP (B), or with 5 mM ADP (C), and challenged with zymosan (160 μ g/ml) at 37°C in 95% air and 5% CO_2 . At the specified times, duplicate aliquots of the medium were removed and counted. The cells were scraped twice in 0.05% Triton X-100 and the radiolabel and protein content of the cell lysates were determined. Values are expressed as percent of total cellular 20:4 released into the medium, and are the means of triplicate cultures.

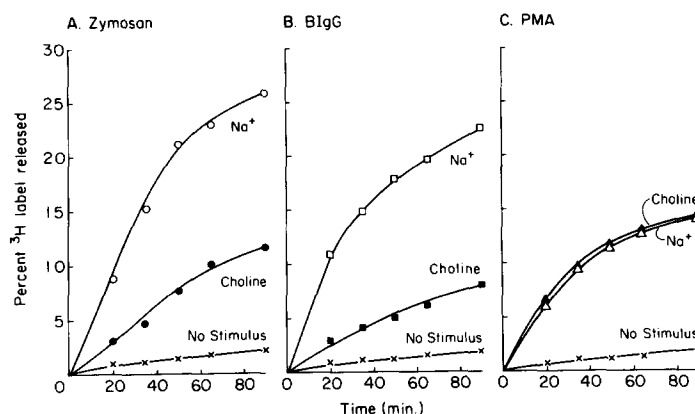


FIGURE 2. Time course of 20:4 release in high choline medium. Macrophages were isolated and labeled with [³H]20:4. The cells were washed four times in high Na⁺ or high choline medium and then challenged in the appropriate medium with (A) zymosan (160 μg/ml), (B) B1gG (5 mg/ml), and (C) PMA (100 ng/ml). At the specified times, duplicate aliquots of the medium were removed and counted. The cells were scraped twice in 0.05% Triton X-100 and the radiolabel and protein content of the cell lysates were determined. Values are expressed as percent of the total cellular 20:4 released into the medium, and are the means of triplicate cultures.

(5). Determining whether these effects were due to the absence of Na⁺ rather than the presence of high K⁺ required experiments in which Na⁺ was wholly replaced by choline.

Cells ingested zymosan in the choline medium with a phagocytic index 89% that of cells in high Na⁺ medium (data not shown), but released only 12% of their 20:4 content, compared with 26% in Na⁺-containing medium (Fig. 2A). The unimpaired phagocytic capacity of the cells in choline medium indicated cell viability. This was further confirmed by evaluating trypan blue exclusion from the cells, which was >95% after 2 h. In addition, the inhibition of 20:4 release by choline was reversed on returning cells to medium containing Na⁺ (data not shown).

Removal of Na⁺ from the extracellular medium had an even more pronounced inhibitory effect on FcR-mediated 20:4 release than was seen with zymosan. Macrophages challenged with B1gG released 8% of their cellular 20:4 in choline medium, compared with 23% secreted in high Na⁺ medium (Fig. 2B).

As reported earlier, the Ca²⁺ ionophore A23187 was able to circumvent the high K⁺ blockade, whereas receptor-ligand triggers were inhibited (5). This led us to postulate the presence of a lesion proximal to the activation of the phospholipase. As seen in Table I the Ca²⁺ ionophore A23187 triggered the release of equivalent amounts of cellular 20:4 whether the cells were challenged in choline medium (23%) or in an Na⁺ medium (25%). Similar results were obtained when the tumor promoter PMA was used as an alternative soluble trigger of the 20:4 cascade. PMA stimulated the release of 14% of the cellular 20:4 whether the cells were in choline medium or in a medium containing Na⁺ (Fig. 2C).

These data suggest that receptor-mediated 20:4 secretion is dependent on Na⁺

TABLE I
 20:4 Metabolites Synthesized by Resident Peritoneal Macrophages in Na⁺-free Medium and in the Presence of Protein Synthesis Inhibitors

Condition	Label released	6-keto-PGF _{1α}	PGE ₂	LTC	Hetes	Unreacted 20:4
α-MEM and zymosan	25.6 ± 3.1	43.4 ± 4.9	23.5 ± 3.5	18.8 ± 1.3	8.9 ± 0.7	5.4 ± 0.5
High Na ⁺ medium + A23187	26.7 ± 2.7	35.8 ± 2.5	20.2 ± 1.4	20.4 ± 1.8	18.8 ± 1.3	4.8 ± 0.2
High choline medium + A23187	23.6 ± 3.3	38.1 ± 5.7	19.0 ± 1.5	17.4 ± 2.9	17.3 ± 2.1	8.2 ± 0.6
High Na ⁺ plus cycloheximide/emetine (10 μg/ml) + A23187	24.3 ± 2.4	36.3 ± 4.4	18.7 ± 1.7	19.7 ± 1.2	19.6 ± 1.4	5.7 ± 0.8

Macrophages were isolated and labeled with [³H]20:4. The cells were washed, overlaid with the appropriate medium, and challenged with zymosan (160 μg/ml) for 90 min, or with A23187 (10 μg/ml) for 30 min. The medium was then aspirated and extracted for 20:4 metabolites. Extracts were dried under N₂ and chromatographed on HPLC systems 1 and 2 as described. Values are expressed as the percent of the total [³H]20:4 products formed and were calculated on the basis of discernible peaks above background. The data represent the mean ± SD of four determinations.

in the medium, whereas 20:4 release triggered by soluble stimuli has no Na⁺ requirement.

Effect of Choline Medium on the Uptake and Metabolism of 20:4. The amount of radioactive products released is determined by the equilibrium of 20:4 release from the cell membrane and reesterification of 20:4 into the cellular phospholipid. Furthermore, the reesterification is limited by the metabolism of the 20:4.

We therefore examined which of these processes were sensitive to choline medium. Uptake of exogenous [³H]20:4 into resident peritoneal macrophages proceeded linearly for at least 2 h and was not affected by high choline medium (see Fig. 6). These data suggest that it is not the uptake pathway that is inhibited.

Since A23187-induced 20:4 release was unimpaired by choline medium (Table I) we used this stimulus to assess the effect of Na⁺-free medium on the metabolism of 20:4. Most of the 20:4 released in response to A23187 was metabolized, and there was no significant difference in the profile of products produced by cells in the choline medium (6-keto-PGF_{1α}, 38%; PGE₂, 19%; and leukotriene C [LTC], 17%) when compared with those produced in an Na⁺ medium (6-keto-PGF_{1α}, 36%; PGE₂, 20%; and LTC, 20%) (Table I).

Since we have shown that choline did not effect either the uptake or metabolism of 20:4 we conclude that the Na⁺-requiring step is the release of 20:4 from the phospholipid. Furthermore, since choline also has no effect on PMA- or A23187-induced 20:4 release, the Na⁺-requiring step appears to be an early event in receptor-mediated activation of the phospholipase(s).

Na⁺ Dependence of Phospholipase Activation in Cells Primed with Zymosan for 20:4 Release. Macrophages were allowed to ingest suboptimal amounts of zymosan in a high K⁺ medium and then the medium was changed to one containing either choline (Fig. 3A) or Na⁺ (Fig. 3B), and 20:4 release was determined. Cells exposed to Na⁺ released an additional 9–10% of their 20:4 while those in choline medium released a further 1–2%, an equivalent amount to basal release in the absence of a stimulus (Fig. 3, A and B). While the metabolites released in the Na⁺ medium consisted of 6-keto-PGF_{1α}, PGE₂, and LTC in similar proportions to those released in response to zymosan in α-MEM (Table I), the small amount of metabolites released in the choline medium consisted only of cyclooxygenase products (data not shown).

These data suggest that the ingestion of zymosan in K⁺ medium leads to a

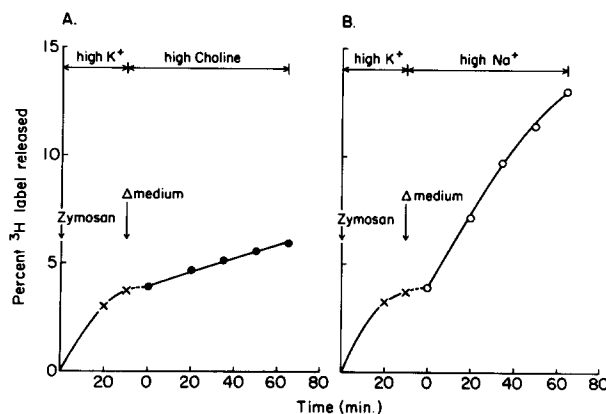


FIGURE 3. Activation of [^3H]20:4 release in cells previously given a phagocytic stimulus in high K^+ by changing the medium to one containing Na^+ . Cells were isolated, labeled, washed, and incubated with a suboptimal dose of zymosan in high K^+ medium for 30 min, resulting in the association of approximately six particles per cell. Very few particles remained unattached. Under these conditions, the cells released an initial burst of 3–4% of their [^3H]20:4 label. The dishes were washed at 4°C with high K^+ medium to remove unassociated particles. The medium was changed (indicated by \downarrow) to one containing either high choline (A) or high Na^+ (B), without any further addition of zymosan. Cells were incubated at 37°C , and at the indicated times, aliquots were removed and counted. After 65 min, the cells were scraped in 0.05% Triton X-100 and the radiolabel and protein content of the cell lysate determined. Values are expressed as percent of the total cellular 20:4 release into the medium, and are the means of triplicate cultures.

primed state with respect to 20:4 release, which is maintained in the absence of additional particle uptake and which can be expressed when Na^+ is added to the medium.

Role of Ca^{2+} Ions in 20:4 Secretion. The role of Ca^{2+} in 20:4 secretion has been investigated. Macrophages challenged with BIGG released 18% of their cellular 20:4 in medium containing 1.8 mM CaCl_2 (Fig. 4A). In contrast, cells challenged with BIGG in a Ca^{2+} -free (EGTA) medium did not secrete 20:4 above basal levels released in the absence of a stimulus (Fig. 4A). Similarly, A23187-stimulated 20:4 release was wholly dependent on Ca^{2+} in the external medium (Fig. 4C). PMA-induced secretion of 20:4 from macrophages was inhibited $\sim 50\%$ in Ca^{2+} -free medium (Fig. 4B). Furthermore, PMA-triggered 20:4 release could be decreased to basal levels when the macrophages were preloaded with quinine, a drug known to inhibit Ca^{2+} -mediated responses in cells (26) (Fig. 4B). The quinine-induced inhibition of 20:4 release was completely reversible (Fig. 4B). It was not possible to determine the role of Ca^{2+} in zymosan-induced secretion of 20:4, since the zymosan-receptor interaction is Ca^{2+} -dependent (33).

EGTA inhibition of 20:4 release was fully reversible. Cells that had been preincubated in EGTA medium for 2 h, and which had subsequently been allowed to recover in a Ca^{2+} -containing medium, released similar amounts of 20:4 to control cells when challenged with BIGG or A23187 (data not shown). This reversibility was used to probe the nature of the Ca^{2+} dependence of 20:4 release. Cells in EGTA medium were allowed to bind and ingest BIGG for 35 min. Under these conditions, no release of 20:4 occurred (Fig. 5A). The cells were then exposed to Ca^{2+} , which resulted in the rapid release of $\sim 18\%$ of the

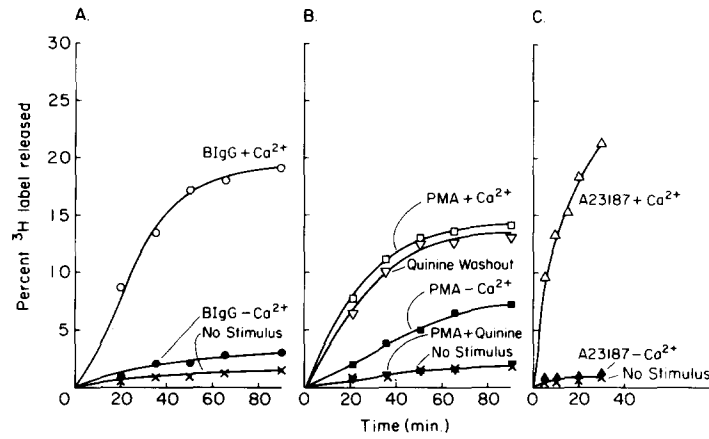


FIGURE 4. Effect of Ca^{2+} on 20:4 release from macrophages. Labeled macrophages were washed four times in high Na^+ medium or in Ca^{2+} -free (EGTA) medium. The cells were then overlaid with high Na^+ medium ($+\text{Ca}^{2+}$) or with EGTA medium ($-\text{Ca}^{2+}$) and challenged with (A) BIgG (5 mg/ml), (B) PMA (100 ng/ml), and A23187 (10 $\mu\text{g}/\text{ml}$). At the times indicated, duplicate aliquots of medium were removed and counted. The cells were scraped in Triton X-100, and the radiolabel and protein content of the cell lysates were quantitated. Cells were also preincubated in high Na^+ medium containing 250 μM quinine for 60 min, after which they were challenged in the same medium with PMA (\blacktriangledown) (B) and the time course of 20:4 release was assessed. The reversibility of the inhibition of 20:4 release by quinine is also shown in B. Cells were loaded with 250 μM quinine for 60 min, washed twice with PD, allowed to recover in high Na^+ medium for 60 min, and then challenged with PMA (∇). Values are expressed as percent of total cellular 20:4 released into the medium, and are the means of triplicate cultures.

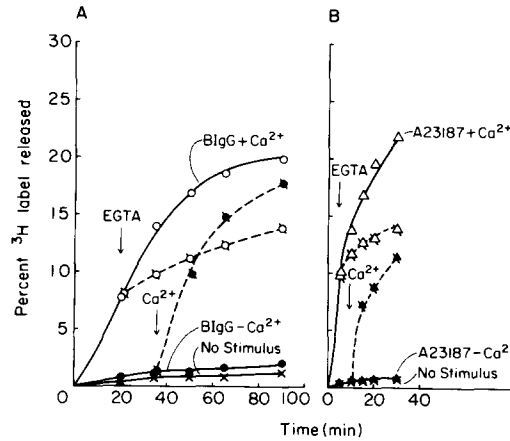


FIGURE 5. Effect of added Ca^{2+} or EGTA on 20:4 release by macrophages. Cells were labeled, washed, and overlaid with Ca^{2+} (open symbols) or EGTA medium (closed symbols) as described in the legend to Fig. 5, and then challenged with (A) BIgG (5 mg/ml) or (B) A23187 (10 $\mu\text{g}/\text{ml}$). At the times indicated, Ca^{2+} (2.3 mM; free $[\text{Ca}^{2+}]$, 1.8×10^{-3} M) was added to cells in EGTA medium (\bullet , \blacktriangle), and EGTA (2.5 mM; free $[\text{Ca}^{2+}]$, 9.6×10^{-8} M) was added to cells in Ca^{2+} medium (\circ , \triangle). Protons liberated during these additions did not significantly alter the pH of the medium. 20:4 release was assessed as described in the legend to Fig. 5. Values are expressed as the percent of total cellular 20:4 release into the medium, and are the means of triplicate cultures.

TABLE II
Effect of High K⁺ Medium on Cellular Protein Synthesis

Culture condition	Percent TCA-precipitable [³⁵ S]methionine
α -MEM	100
α -MEM and cycloheximide/emetine	3 \pm 3
High K ⁺ medium	16 \pm 4
High K ⁺ medium and cycloheximide/emetine	3 \pm 2
Preincubate in high K ⁺ for 30 min, then change to α -MEM	90 \pm 6

Cells were placed in either high K⁺ medium or α -MEM containing 2 μ Ci/ml of [³⁵S]methionine with or without cycloheximide and emetine (5 μ g/ml). After incubation at 37°C for 2 h, the cells were washed, and acid-precipitable ³⁵S was determined as described. The data are expressed as a percentage (\pm SD; $n = 4$) of the TCA-precipitable [³⁵S]methionine incorporated by cells in α -MEM.

cellular 20:4 (Fig. 5A). Similarly, cells treated with A23187 in EGTA medium released 20:4 only when Ca²⁺ was added to the medium, although in this case only 13% of radiolabel was released (Fig. 5B). Furthermore, 20:4 release in response to BIGG or A23187 was inhibited when the extracellular free Ca²⁺ concentration was decreased to micromolar levels by addition of EGTA to the medium after the cells had been challenged with the stimulus (Fig. 5). These data indicate that, irrespective of the stimulus, Ca²⁺ is required for the secretion of 20:4 metabolites from macrophages.

A Requirement for Protein Synthesis in Signal Response Coupling in the 20:4 Cascade. The inhibition of 20:4 secretion by K⁺ showed complex biphasic kinetics, and complete inhibition was only obtained after preincubating the cells for 15 min in high K⁺ medium (5). This suggested that the K⁺ effect was more complicated than the mere displacement of necessary extracellular Na⁺. Protein synthesis inhibitors had previously been shown to inhibit PGE₂ release from macrophages (15) and from fibroblasts (16), and we wondered whether high K⁺ media might also be causing a decrease in 20:4 release by this mechanism.

High K⁺ medium decreased the incorporation of [³⁵S]methionine into the TCA-precipitable fraction by 85% (Table II). This effect was reversible, and returning the cells to α -MEM resulted in normal rates of protein synthesis (Table II). The high K⁺ medium had no effect on the uptake of the [³⁵S]methionine label into the cells (data not shown). We further explored the effects of protein and RNA synthesis inhibitors on 20:4 secretion in macrophages. A combination of cycloheximide (5 μ g/ml) and emetine (5 μ g/ml) that inhibited protein synthesis in macrophages by 97% (Table II) decreased zymosan-, BIGG-, and PMA-induced secretion of 20:4 by ~80–90% (Table III). Preincubation of cells in actinomycin D (1 μ g/ml) for 70 min resulted in an ~85% inhibition of 20:4 release in response to zymosan, BIGG, and PMA (Table III). Cells treated with the protein synthesis or RNA synthesis inhibitors as described above showed unimpaired phagocytosis of zymosan and BIGG, indicating that not all receptor-ligand-mediated functions

TABLE III
Effect of Protein Synthesis Inhibitors on 20:4 Release from Macrophages

Stimulus	Control	Cycloheximide/emetine	Actinomycin D
None	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
Zymosan	25.2 ± 1.9	5.8 ± 0.6	4.2 ± 0.3
BIgG	23.7 ± 2.5	4.1 ± 0.3	3.6 ± 0.4
PMA	13.2 ± 1.1	2.6 ± 0.5	2.9 ± 0.3
A23187	25.5 ± 3.1	23.2 ± 2.8	25.7 ± 2.3

Macrophages were isolated and labeled with [³H]20:4. The cells were washed and preincubated for 30 min in α -MEM containing cycloheximide/emetine (5 μ g/ml), or for 70 min in α -MEM containing actinomycin D (1 μ g/ml). The cells were then challenged in the same media with zymosan (160 μ g/ml), BIgG (5 mg/ml), or PMA (100 ng/ml) for 120 min, or with A23187 (10 μ g/ml) for 30 min. At the specified time, duplicate aliquots of the medium were removed and counted. The cells were scraped in Triton X-100 and the radiolabel and protein content of the cell lysates were determined. Values are expressed as percent \pm SD ($n = 4$) of total cellular 20:4 released into the medium.

were inhibited (data not shown). Competent receptor-ligand triggering was also indicated by the observation that these inhibitors did not affect the secretion of H₂O₂ triggered by zymosan, BIgG, or PMA (data not shown).

Interestingly, the A23187-stimulated and basal release of 20:4 from macrophages was unaffected by cycloheximide/emetine and actinomycin D (Table III). Macrophages, pretreated with cycloheximide and emetine and stimulated with A23187, secreted a similar profile of 20:4 metabolites (6-keto-PGF_{1 α} , 36%; PGE₂, 19%; LTC, 20%; and Hetes, 20%) to control cells triggered in the absence of protein synthesis inhibitors (6-keto-PGF_{1 α} , 36%; PGE₂, 20%; LTC, 20%; and Hetes, 19%) (Table I).

We investigated the effect of protein synthesis inhibitors on the rate of esterification of exogenously added 20:4 into membrane phospholipid. Uptake of [³H]20:4 into resident macrophages proceeded linearly for at least 2 h and was not affected by the presence of cycloheximide and emetine (Fig. 6).

The time course of zymosan-induced 20:4 release in the presence of cycloheximide and emetine is shown in Fig. 7 and resulted in an 80% inhibition within 15 min. Inhibition of zymosan-triggered 20:4 release by actinomycin D was slower, with maximal inhibition occurring after ~40 min (Fig. 7).

These data suggest the presence of a rapid turnover-protein that regulates the release of 20:4 from macrophage membranes in response to PMA, zymosan, and BIgG, and that such a modulatory step is proximal to the phospholipase activity.

Discussion

Occupancy of macrophage FcR by immune complexes triggers both the secretion of 20:4 metabolites and phagocytosis. The nature of the signals generated by this ligand-receptor complex are poorly understood. Recent studies from this laboratory have shown that the ligated FcR functions as a monovalent

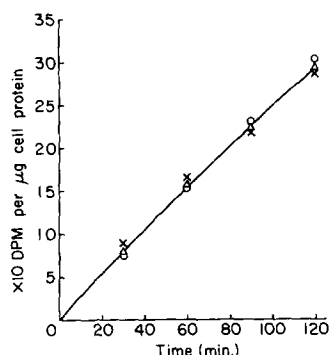


FIGURE 6. Time course of incorporation of exogenously added 20:4 by macrophages in Na^+ -free medium or in the presence of protein synthesis inhibitors. Macrophages were isolated and cultured in MEM containing 10% FCS for 16 h. Cells were then washed and overlaid with MEM (O), cycloheximide plus emetine ($5 \mu\text{g}/\text{ml}$) (Δ), or high choline medium (X). [^3H]20:4 ($0.5 \mu\text{Ci}$, 8.3 nM) was added to the medium, and the cells were incubated at 37°C . At the times indicated, triplicate culture dishes were washed four times with PD, scraped into Triton X-100, and radiolabel and protein content were determined. Data shown are the means of triplicate cultures (SEM $<10\%$).

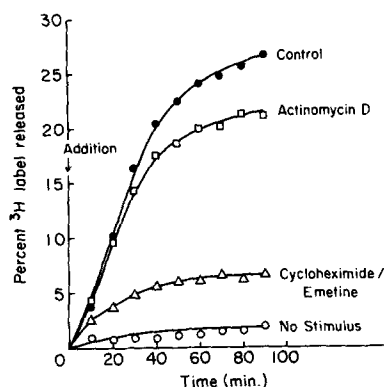


FIGURE 7. Time course of 20:4 release in the presence of protein synthesis and RNA synthesis inhibitors. Macrophages were isolated, labeled, washed, and overlaid with MEM. Cells were challenged with zymosan ($160 \mu\text{g}/\text{ml}$), and at the same time, actinomycin D ($1 \mu\text{g}/\text{ml}$) (\square) or cycloheximide and emetine ($5 \mu\text{g}/\text{ml}$) (Δ) were added. Zymosan-induced 20:4 release in the absence of inhibitors (\bullet) and basal release in the absence of a stimulus (\circ) are also shown. At the specified times, duplicate aliquots of medium were removed and counted. The cells were scraped in Triton X-100 and the radiolabel and protein content were determined. Values are expressed as percent of total cellular 20:4 released, and are the means of triplicate cultures.

cation channel when reconstituted into plasma membrane vesicles (25) and planar lipid bilayers (27). We have conducted ion-replacement studies to determine whether this potential monovalent cation flux is involved in activating the 20:4 cascade in macrophages.

We have shown previously (5) that high potassium media selectively inhibit zymosan-induced 20:4 release from peritoneal macrophages, while phagocytosis is relatively unaffected. In this paper we further explore the nature of this uncoupling. An important consequence of placing cells in a high potassium medium is the depolarization of the plasma membrane. Our data suggest that

depolarization of the cell membrane per se does not result in the inhibition of 20:4 release, for the following reasons. (a) Secretion of 20:4 in response to zymosan is unimpaired by the millimolar concentrations of extracellular ATP that depolarize macrophage membranes (14). (b) Treatment of macrophages with ouabain, which results in the partial depolarization of the cells (14, 17), does not inhibit zymosan-induced release of 20:4 (data not shown). (c) The requirement of a 15 min preincubation in K^+ medium to completely inhibit 20:4 release (5) argues against K^+ acting by depolarizing the cells, which occurs very rapidly. Therefore, while changes in membrane potential have been shown to precede O_2^- production in neutrophils (18–20), they do not appear to affect 20:4 release in macrophages.

The observation that cells pretreated in high K^+ medium regain the ability to release 20:4 in response to zymosan when returned to a medium containing Na^+ suggests that it might be the absence of Na^+ and not the presence of K^+ that inhibits the signal to the phospholipase (5). In support of this, we show here that the spontaneous release of 20:4 from cells that have been primed by preingestion of zymosan in high K^+ medium is absolutely dependent on Na^+ in the external medium (Fig. 3). This requirement for external Na^+ is consistent with the hypothesis that an influx of Na^+ into the cell acts as a primary signal for 20:4 release. However, an increase of intracellular Na^+ is not in itself sufficient to trigger the 20:4 cascade, since treatment of the cells with ouabain or monensin do not cause the release of 20:4 metabolites (our unpublished observations). Na^+ influxes have been shown to be an early event when neutrophils are stimulated with the chemotactic peptide FMLP (21, 22). Furthermore, removal of Na^+ from the medium decreases chemotactic responsiveness in neutrophils (23) as well as FMLP-stimulated lysosomal enzyme secretion (24) and O_2^- generation (21). Immune complex- and Con A-stimulated O_2^- production and lysosomal enzyme secretion in human neutrophils also require external Na^+ (18).

The localization of the Na^+ -requiring step(s) in the 20:4 cascade was examined with a panel of triggers. Receptor-mediated secretion of 20:4 (BIgG and zymosan) appears to require extracellular Na^+ , while soluble stimuli such as PMA and A23187 do not (Fig. 2 and Table I). The observation that A23187-induced release and metabolism of 20:4 is unimpaired in choline medium (Table I) indicates that the phospholipase(s), cyclooxygenase, and lipoxygenase enzymes are active in this Na^+ -free medium, and that Na^+ is required at some point proximal to the phospholipase. Together, these data suggest that an influx of Na^+ acts as an early signal for receptor-mediated induction of 20:4 release.

Recent studies in our laboratory (28) have shown that ligation of the FcR causes a transient rise in intracellular Ca^{2+} , the magnitude of which depends on the extent of receptor aggregation. Our current studies showing that FcR-mediated 20:4 secretion is inhibited in Ca^{2+} -depleted (EGTA-containing) medium (Figs. 4A and 5A) suggest that the increased intracellular Ca^{2+} triggered by the ligated FcR might activate the cellular phospholipases. The phospholipases described in macrophage lysates have been shown to be Ca^{2+} -dependent enzymes (29, 30). An alternative explanation might be that Ca^{2+} is required on the outside of the cell for some associated process. We consider this alternative unlikely, as

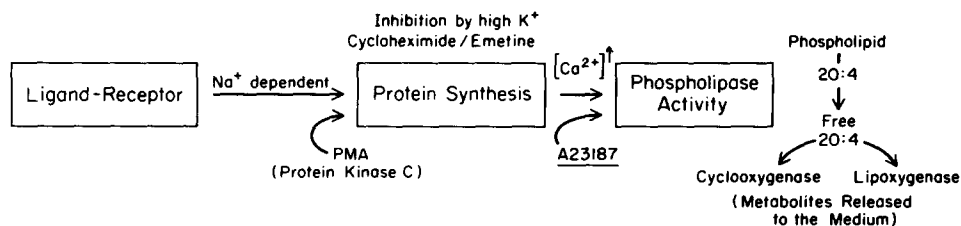
macrophages are capable of binding and ingesting IgG-coated erythrocytes in Ca^{2+} -free medium (28).

Our data imply that Ca^{2+} from extracellular pools are required for the activation of cellular phospholipases. However, internal Ca^{2+} pools may be depleted by the EGTA medium during the relatively long time required for 20:4 secretion, and we are therefore unable to differentiate between these two Ca^{2+} pools. The observation that PMA-stimulated 20:4 release is only partially inhibited by the removal of extracellular Ca^{2+} (Fig. 4B) may reflect a synergy between protein kinase C-mediated signals and suboptimal concentrations of Ca^{2+} mobilized from intracellular pools. This hypothesis is supported by our recent observations (A. Aderem, unpublished results) that a combination of suboptimal concentrations of A23187 and PMA result in greatly enhanced release of 20:4 metabolites from the cells.

While studying the mechanism by which high K^+ media inhibit receptor-mediated 20:4 release, we found that high concentrations of extracellular K^+ reversibly inhibit protein synthesis in macrophages (Table II). High K^+ medium has also been shown (31) to inhibit protein synthesis in L cells. We therefore explored the effects of protein and RNA synthesis inhibitors on 20:4 secretion in macrophages. Zymosan-, immune complex-, and PMA-induced 20:4 release was inhibited 80–90% by cycloheximide, emetine, and actinomycin D, while A23187-induced secretion and basal secretion of 20:4 was relatively unaffected (Table III).

Protein and RNA synthesis inhibitors did not affect the cyclooxygenase or lipoxygenase pathways, as the profile of 20:4 metabolites synthesized in response to A23187 is identical in the presence or absence of the inhibitors (Table I). This is also indicated by the observation that macrophages pretreated with protein and RNA synthesis inhibitors are capable of metabolizing exogenously added 20:4 to the same extent as untreated cells (data not shown). The rate of acylation of exogenously added 20:4 into macrophage membranes was unaffected by preincubation of the cells in protein synthesis inhibitors (Fig. 6). This suggests that the decrease in 20:4 secretion in the presence of these inhibitors is not due to enhanced esterification of free 20:4. Since the cyclooxygenase and lipoxygenase pathways, as well as the esterification of 20:4 into the membrane are unaffected by protein and RNA synthesis inhibitors, the lesion must be at the level of 20:4 release from the membrane.

Kinetic analysis showed that the effect of the protein synthesis inhibitors is very rapid, with 80% inhibition occurring within 15 min (Fig. 7). This time course is similar to the temporal inhibition of 20:4 release by high K^+ medium, where macrophages had to be preincubated for 15 min in K^+ medium to completely inhibit zymosan-induced 20:4 secretion (5). Inhibition with actinomycin D occurred within 40 min (Fig. 7), suggesting a precursor-product relationship. These data suggest the synthesis of a rapid turnover-protein that regulates the release of 20:4 from macrophage membranes in response to PMA, zymosan, and IgG immune complexes. Since the A23187-induced and basal secretion of 20:4 was unimpaired, we conclude that (a) basal and ionophore-stimulated 20:4 release is mediated via a different phospholipase than that induced by PMA, zymosan, or IgG immune complexes, or (b) that a rapid



Scheme I

FIGURE 8. Possible sequence of signals in the 20:4 cascade in macrophages. Receptor-ligand complexes first generate an Na^+ -dependent signal. This is followed by the synthesis of a rapid turnover-protein. Finally, an increase in intracellular Ca^{2+} results in the activation of the phospholipase(s). If the soluble triggers activate the same pathway, PMA would stimulate the pathway at a point proximal to the protein synthetic step, while A23187 would bypass this step.

turnover-protein modulates the 20:4 cascade at a step proximal to the phospholipase.

FcR-mediated induction of phospholipase activity in macrophages clearly involves a number of steps (Fig. 8) (scheme 1). Since monovalent ligands do not trigger the 20:4 cascade (32), further events in addition to receptor binding must be required. Such events may include receptor clustering and/or crosslinking. An early signal in the 20:4 cascade has an Na^+ -dependent component, possibly reflecting the influx of Na^+ into the cell. For further transduction of the signal, there is a requirement for the synthesis of a rapid turnover-protein. If the soluble stimuli trigger the same pathway as that mediated by the receptor-ligand complex, PMA would stimulate the pathway at a point proximal to the protein synthetic step, while A23187 would bypass the step. This implies that an increase in intracellular Ca^{2+} is the terminal event in the release of 20:4 from the membrane phospholipid.

Summary

We have examined the requirement for Na^+ , Ca^{2+} , and protein synthesis in the induction of the arachidonic acid (20:4) cascade in cultured murine peritoneal macrophages. Replacement of extracellular Na^+ with choline or with K^+ inhibited receptor-mediated 20:4 release by 60–90%, but did not inhibit release stimulated by the soluble triggers PMA and A23187. Cells that had preingested zymosan particles in a K^+ medium could be induced to secrete 20:4 metabolites merely by changing the medium to one containing Na^+ . The Ca^{2+} ionophore A23187 caused cells in Na^+ -free medium to release and metabolize 20:4 to prostacyclin, PGE_2 , leukotriene C, and hydroxyeicosatetraenoic acids, suggesting that the phospholipase(s), cyclooxygenase, and lipoxygenase enzymes do not have a requirement for extracellular Na^+ . These data suggest that receptor-mediated 20:4 secretion has a requirement for extracellular Na^+ , while 20:4 release triggered by soluble stimuli do not.

Immune complex- and A23187-induced 20:4 release was absolutely dependent on extracellular Ca^{2+} . PMA-triggered 20:4 secretion was inhibited 50% in

Ca²⁺-free medium, but could be inhibited completely by preloading the cells with the Ca²⁺ antagonist quinine.

Protein and RNA synthesis was required for 20:4 release induced by zymosan, immune complex, and PMA, but not by A23187. Cycloheximide and emetine were effective within 15 min of addition, while actinomycin D was an effective inhibitor within 45 min. We suggest that receptor-mediated signal response coupling in the 20:4 cascade in macrophages comprises a sequential series of signals that includes an Na⁺ influx, synthesis of a rapid turnover-protein, and finally an increase in intracellular Ca²⁺.

We thank Dr. Nicholas A. Pawlowski for help and discussion and David Sniadack and Dan Cohen for technical assistance.

Received for publication 2 October 1985.

References

1. Scott, W. A., J. M. Zrike, A. Hamill, J. Kempe, and Z. A. Cohn. 1980. Regulation of arachidonic acid metabolites in macrophages. *J. Exp. Med.* 152:324.
2. Bonney, R. J., P. D. Wightman, M. E. Dahlgren, P. Davies, F. A. Kuehl, and J. L. Humes. 1978. Regulation of prostaglandin synthesis and of selective release of lysosomal hydrolases by mouse peritoneal macrophages. *Biochem. J.* 176:433.
3. Bonney, R. J., P. Naruns, P. Davies, and J. L. Humes. 1979. Antigen-antibody complexes stimulate the synthesis and release of prostaglandins by mouse peritoneal macrophages. *Prostaglandins*. 18:605.
4. Rouzer, C. A., W. A. Scott, Z. A. Cohn, P. Blackburn, and J. M. Manning. 1980. Mouse peritoneal macrophages release leukotriene C in response to a phagocytic stimulus. *Proc. Natl. Acad. Sci. USA.* 77:4928.
5. Aderem, A. A., W. A. Scott, and Z. A. Cohn. 1984. A selective defect in arachidonic acid release from macrophage membranes in high potassium media. *J. Cell. Biol.* 99:1235.
6. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* 121:157.
7. Michl, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607.
8. Eisen, H. N., W. Gray, J. R. Little, and E. S. Simms. 1967. Purification of antibodies to the 2,4-dinitrophenyl (DNP) group and the 2,4,6-trinitrophenyl (TNP) group. *Methods Immunol. Immunochem.* 1:351.
9. Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193:265.
10. Unger, W. G., I. F. Stamford, and A. Bennet. 1971. Extraction of prostaglandins from human blood. *Nature (Lond.)*. 233:336.
11. Borgeat, P., and B. Samuelsson. 1979. Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. Natl. Acad. Sci. USA.* 76:2148.
12. Alam, I., K. Ohuchi, and L. Levine. 1979. Determination of cyclooxygenase products and prostaglandin metabolites using high-pressure liquid chromatography and radioimmunoassay. *Anal. Biochem.* 93:339.
13. Pawlowski, N. A., G. Kaplan, A. L. Hamill, Z. A. Cohn, and W. A. Scott. 1982. Arachidonic acid metabolism by human monocytes. *J. Exp. Med.* 158:393.
14. Sung, S. J., J. D.-E. Young, A. M. Origlio, J. M. Heiple, and S. C. Silverstein. 1985.

- Extracellular ATP perturbs transmembrane ion fluxes, elevates Ca^{2+} , and inhibits phagocytosis in mouse macrophages. *J. Biol. Chem.* 260:3442.
15. Bonney, R. J., P. D. Wightman, M. E. Dahlgren, P. Davies, F. A. Kuehl, and J. L. Humes. 1980. Effect of RNA and protein synthesis inhibitors on the release of inflammatory mediators by macrophages responding to phorbol myristate acetate. *Biochim. Biophys. Acta.* 633:410.
 16. Pong, S., S. L. Hong, and L. Levine. 1977. Prostaglandin production by methylcholanthrene-transformed mouse BALB/3T3. *J. Biol. Chem.* 252:1408.
 17. Gallin, E. K., and D. R. Livengood. 1983. Demonstration of an electrogenic $\text{Na}^+\text{-K}^+$ pump in mouse spleen macrophages. *Am. J. Physiol.* 245:C184.
 18. Korchak, H. M., and G. Weissman. 1980. Stimulus-response coupling in the human neutrophil: transmembrane potential and the role of extracellular Na^+ . *Biochim. Biophys. Acta.* 601:180.
 19. Seligmann, B. E., and J. I. Gallin. 1980. Use of lipophilic probes of membrane potential to assess human neutrophil activation. *J. Clin. Invest.* 66:493.
 20. Jones, G. S., K. van Dyke, and V. Castranova. 1981. Transmembrane potential changes associated with superoxide release from human granulocytes. *J. Cell. Physiol.* 106:75.
 21. Simchowitz, L., and I. Spilberg. 1979. Chemotactic factor induced generation of superoxide radicals by human neutrophils: evidence for the role of sodium. *J. Immunol.* 123:2428.
 22. Naccache, P. H., H. J. Showell, E. L. Becker, and R. I. Sha'afi. 1977. Transport of sodium, potassium, and calcium across rabbit polymorphonuclear leukocyte membranes. Effect of chemotactic factor. *J. Cell Biol.* 73:428.
 23. Showell, H. J., and E. L. Becker. 1976. The effects of external K^+ and Na^+ on the chemotaxis of rabbit peritoneal neutrophils. *J. Immunol.* 116:99.
 24. Showell, H. J., P. H. Naccache, R. I. Sha'afi, and E. L. Becker. 1977. The effects of extracellular K^+ , Na^+ and Ca^{2+} on lysosomal enzyme secretion from polymorphonuclear leukocytes. *J. Immunol.* 119:804.
 25. Young, J. D.-E., J. C. Unkeless, H. R. Kaback, and Z. A. Cohn. 1983. Mouse macrophage Fc receptor for $\text{IgG}_{2b/\alpha 1}$ in artificial and plasma membrane vesicles functions in a ligand-dependent ionophore. *Proc. Natl. Acad. Sci. USA.* 80:1636.
 26. Lew, V. L., and H. G. Ferreira. 1978. Calcium transport and the properties of a calcium-activated potassium channel in red cell membranes. *Curr. Top. Membr. Transport.* 10:217-277.
 27. Young, J. D.-E., J. C. Unkeless, T. M. Young, A. Mauro, and Z. A. Cohn. 1983. Role for mouse macrophage IgG Fc receptor as ligand-dependent ion channel. *Nature (Lond.)* 306:186.
 28. Young, J. D.-E., S. S. Ko, and Z. A. Cohn. 1984. The elevation of intracellular free calcium associated with $\gamma 2b/\gamma 1$ Fc receptor-ligand interactions: role in phagocytosis. *Proc. Natl. Acad. Sci. USA.* 81:5430.
 29. Wightman, P. D., J. L. Humes, P. Davies, and R. J. Bonney. 1981. Identification and characterization of two phospholipase A_2 activities in resident mouse peritoneal macrophages. *Biochem. J.* 195:427.
 30. Wightman, P. D., M. E. Dahlgren, J. C. Hall, P. Davies, and R. J. Bonney. 1981. Identification and characterization of a phospholipase C activity in mouse peritoneal macrophages. *Biochem. J.* 197:523.
 31. Christman, J. 1973. Effect of elevated potassium level and amino acid deprivation on polysome distribution and rate of protein synthesis in L cells. *Biochim. Biophys. Acta.* 294:138.
 32. Rouzer, C. A., W. A. Scott, J. Kempe, and Z. A. Cohn. 1980. Prostaglandin synthesis

- by macrophages requires a specific receptor-ligand interaction. *Proc. Natl. Acad. Sci. USA.* 77:4279.
33. Sung, S. J., R. S. Nelson, and S. C. Silverstein. 1983. Yeast mannans inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. *J. Cell. Biol.* 96:160.
 34. Vianna, A. L. 1975. Interaction of calcium and magnesium in activating and inhibiting the nucleoside triphosphatase of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta.* 410:389.