A Selective Defect in Arachidonic Acid Release from Macrophage Membranes in High Potassium Media

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ABSTRACT Murine peritoneal macrophages cultured in minimal essential medium (α-MEM; 118 mM Na^+, 5 mM K^+)[1] released arachidonic acid (20:4) from phospholipids on encountering a phagocytic stimulus of unopsonized zymosan. In high concentrations of extracellular K^+ (118 mM), ^3H release from cells prelabeled with [^3H]20:4 was inhibited 80% with minimal reduction (18%) in phagocytosis. The inhibitory effect of K^+ on 20:4 release was fully reversed on returning cells to medium containing Na^+ (118 mM). Preingestion of zymosan particles by macrophages maintained in high K^+ medium resulted in cells being "primed" for 20:4 release, which was only effected (without the further addition of particles) by changing the medium to one containing Na^+. In contrast, 20:4 release from cells stimulated with the calcium ionophore A23187 was unimpaired by the elevated K^+ medium, suggesting no direct effect of high K^+ on the phospholipase. Macrophages stimulated with zymosan in α-MEM metabolized the released 20:4 to prostacyclin, prostaglandin E_2 (PGE_2), and leukotriene C (LTC). The smaller quantity of released 20:4 in high K^+ medium was recovered as 6-Keto-$PGF_{1}\alpha$, the breakdown product of prostacyclin, and PGE_2. No LTC was synthesized. In high K^+, resting (no zymosan) macrophages synthesized hydroxyeicosatetraenoic acids from exogeneously supplied 20:4 in proportions similar to cells maintained in α-MEM. These findings and the similarity of products (including LTC) produced by A23187 stimulated cells in α-MEM and high K^+ medium indicated that the cyclooxygenase and lipoxygenase pathway enzymes were not directly inhibited by high extracellular K^+. We conclude that high concentrations of extracellular K^+ uncouple phagocytosis of unopsonized zymosan from the induction of the phospholipase responsible for the 20:4 cascade and suggest that the lesion is at the level of signal transduction between the receptor-ligand complex and the phospholipase.

Stimulation of macrophages by appropriate membrane-perturbing agents of both soluble and particulate nature results in the induction of phospholipase activity and the quantitative oxygenation of the released arachidonic acid (20:4) via the cyclooxygenase and lipoxygenase pathways (1–3). The 20:4 cascade can proceed maximally under conditions which prevent particle interiorization indicating that the triggering event is due to the interaction of the particle-bound ligands with plasma membrane receptors (4). Knowledge concerning the next step in the cascade, i.e., the signal between the receptor-ligand complex and the phospholipase is scant.

In this study we report that high concentrations of extracellular K^+ uncouples the binding and phagocytosis of zymosan from the induction of phospholipase activity. This property is used to probe the nature of signal transduction between the receptor-zymosan complex and the phospholipase enzymes.

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 (5). Peritoneal cells (~8 × 10^6/ml) in minimal essential medium (α-MEM Grand Island Biological Co., Grand Island, NY) containing 10% fetal calf serum were added to 35-mm diameter plastic culture dishes (1 ml/dish) or to

[1] *Abbreviations used in this paper:* 20:4, arachidonic acid; α-MEM, alpha-minimal essential medium; PBS, calcium- and magnesium-free phosphate-buffered saline; HETEs, hydroxyeicosatetraenoic acids; HHT, 12-Hydroxyheptadecanoic acid; LTC, leukotriene C; 6-Keto-$PGF_{1}\alpha$, 6-Keto prostaglandin $F1\alpha$; PGE_2, prostaglandin E_2; HPLC, reverse-phase high performance liquid chromatography; TXB_2, Thromboxane B_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

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12-mm glass coverslips (0.1 ml/coverslip). After 2 h at 37°C in 5% CO_2/95% air, cultures were washed three times in calcium and magnesium-free phosphate-buffered saline (PBS) to remove nonadherent cells. Fresh α-MEM plus 10% fetal calf serum (1 ml/dish) containing 0.5 μCi of [5, 6, 8, 9, 11, 12, 14, 15-^{3}H] arachidonic acid ([^{3}H] 20:4; specific activity, 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 (Plainview, NY) and stock solutions in PBS were prepared according to the method of Bonney et al. (2). Zymosan was washed twice by centrifugation in 100 vol of high K^+ medium to displace any bound Na^+ before it was added to cultures in high K^+ medium.

High Potassium and High Sodium Medium: 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, Grand Island, NY) and 100 mg/liter L-cysteine. Amino acids were Na^+ free and the pH was adjusted to 7.4 using KOH or HCl. 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/liter L-cysteine. The pH was adjusted to 7.4 using NaOH and HCl.

Assay of Total ^{3}H 20:4 Release: Macrophages prelabelled with ^{3}H 20:4 were washed three times in PBS, a fourth time in the medium to be used and then were overlaid with 1 ml of serum-free medium and zymosan (160 μg/ml) or calcium ionophore A23187 (10 μg/ml, Calbiochem-Behring Corp., San Diego, CA). The cells were incubated at 37°C in 95% air/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 PBS or medium and scraped twice into 1 ml of 0.05% Triton X-100. Portions of the cell lysates were assayed for radiolabel content and protein was determined by the method of Lowry et al. (6) with bovine serum albumin as a standard.

Cell Viability and Phagocytic Index: Cell viability was assessed by trypan blue exclusion using cells cultured on glass coverslips. The extent of phagocytosis of zymosan was measured by direct phase-contrast microscopy of macrophages incubated on glass coverslips in the presence of zymosan and then fixed in 2.5% (wt/vol) gluteraldehyde. Data are expressed as the phagocytic index, defined as percent of cells that ingested zymosan times the average number of particles per cell (7).

For scanning electron microscopy, cells on coverslips were fixed in 1.25% gluteraldehyde in 0.5 M sodium cacodylate, washed, dehydrated in ethanol, transferred to amyl acetate, and critical-point-dried in CO_2. Specimens were coated with gold (Polaron EM coating unit, E5000; Polaron Instruments Inc., Boylestown, PA) and examined with a high resolution scanning electron microscope (ETEC Autoscan) at 20 kV, at a tilt angle of 30° (8).

Quantitation of ^{3}H 20:4 Metabolites: The 20:4 metabolites in the culture medium were extracted by the method of Unger et al. (9). To 1 ml of medium was added 1 ml of ethanol and 10 μl of 88% (wt/wt) formic acid, and the resultant 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 high-pressure liquid chromatography (HPLC).

Concentrated medium extracts were applied to a column of Ultrasphere C-18 (4.6 mm × 25 cm) (Altex Scientific, Inc., Berkeley, CA) (HPLC system 1). The column was eluted at a rate of 1 ml/min with 60 ml of solvent 1 (methanol/water/acetic acid, 65:34.9:0.1, vol/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) (10). For the characterization of cyclooxygenase metabolites, the material eluting at 4–16 min from HPLC system 1 was collected, evaporated to dryness under reduced pressure, and rechromatographed on HPLC system 2 using a Waters Fatty Acid Analysis Column (Waters Associates, Inc., 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 (11). 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, as described (12). The elution times on HPLC systems 1 and 2 shown in Fig. 6, *C* and *D*, were determined using tritiated 6-Keto protaglandin F1α (6-Keto $PGF_{1\alpha}$), thromboxane B_2 (TXB_2), prostaglandin 2α ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), leukotriene (LTC), and arachidonic acid (20:4) obtained from New England Nuclear (Boston, MA) and tritiated 12-Hydroxyheptadecanoic acid (HHT) and 12-Hydroxyeicosatetranoic acid (12-HETE) prepared from human platelets (a generous gift of Dr. Nicholas A. Pawlowski, The Rockefeller University [12]).

RESULTS

Effect of External Potassium on the Kinetics of Zymosan Induced 20:4 Release

In response to a phagocytic stimulus of zymosan, resident murine peritoneal macrophages cultured in α-MEM (118 mM Na^+) released ~23% of their ^{3}H-20:4 content to the extracellular medium over a 90-min time course (Fig. 1*A*). Replacing the α-MEM with a medium containing 118 mM K^+ (high K^+ medium) inhibited 20:4 release 80%. Under these conditions there was an initial, rapid phase of release in response to zymosan, which constituted 4% of the cellular phospholipid 20:4. Thereafter no further release of radiolabel occurred above basal levels of cells incubated in the absence of zymosan (Fig. 1*B*). When the K^+ was replaced by Na^+ (high [118 mM] Na^+ medium), macrophages released ^{3}H-20:4 maximally compared with cells in α-MEM (Fig. 1*C*). In high Na^+ medium, basal release of ^{3}H 20:4 appeared to be slightly enhanced compared with that in α-MEM or high K^+ medium (cf, Fig. 1, *A*, *B*, and *C*).

Cell Viability and Phagocytosis in High Potassium Medium

Greater than 96% of cells incubated in the high K^+ medium for 90 minutes were impermeant to trypan blue (Table I). The absence of toxic effects of high K^+ were also indicated by the observation that cells were capable of phagocytosis of zymosan in high K^+ medium. The phagocytic index of cells in K^+ medium (866 ± 83) was 82% that of cells in α-MEM (1,057 ± 51) (Table I).

Direct phase-contrast microscopy show that the zymosan particles attach to cells and are internalized in α-MEM (Fig. 2*B*) and in high K^+ medium (Fig. 2*A*), an observation that is confirmed by scanning electron microscopy (Fig. 2*C*).

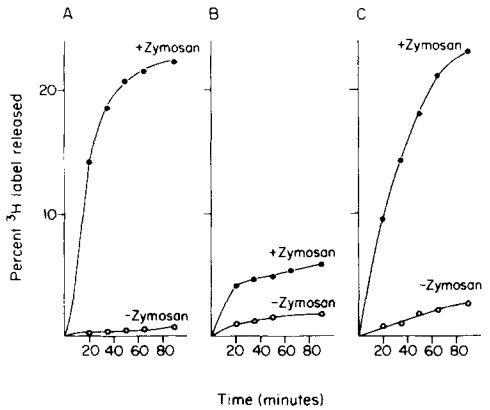

FIGURE 1 Time course of 20:4 release in high K^+ medium. Macrophages were isolated and labeled with ^{3}H 20:4. After the 16-h labeling period, the cells were washed three times in PBS and a fourth time in the appropriate experimental medium. The cells were then overlaid with (*A*) α-MEM, (*B*) high K^+ medium, and (*C*) high Na^+ medium and challenged with 160 μg zymosan at 37°C in 95% air/5% CO_2. At the specified times, duplicate aliquots of the medium were removed and counted. The cells are scraped twice in 0.05% Triton-X100 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 mean of triplicate cultures.

TABLE I

Effect of 118 mM Extracellular K^+ on Phagocytosis and Cell Viability

Medium	Phagocytic index	Cell viability (% of control)
α-MEM (118 mM Na^+, 5 mM K^+)	1,057 ± 51	98 ± 2
High K^+ medium (118 mM K^+)	866 ± 83	96 ± 1

Macrophages on glass coverslips were exposed to zymosan (160 μg/ml) for 90 min at 37°C. Cell viability was assessed by trypan blue exclusion, and phagocytic index was determined by direct phase-contrast microscopy after fixation with 2.5% gluteraldehyde. Results are mean ± range of duplicate determinations.

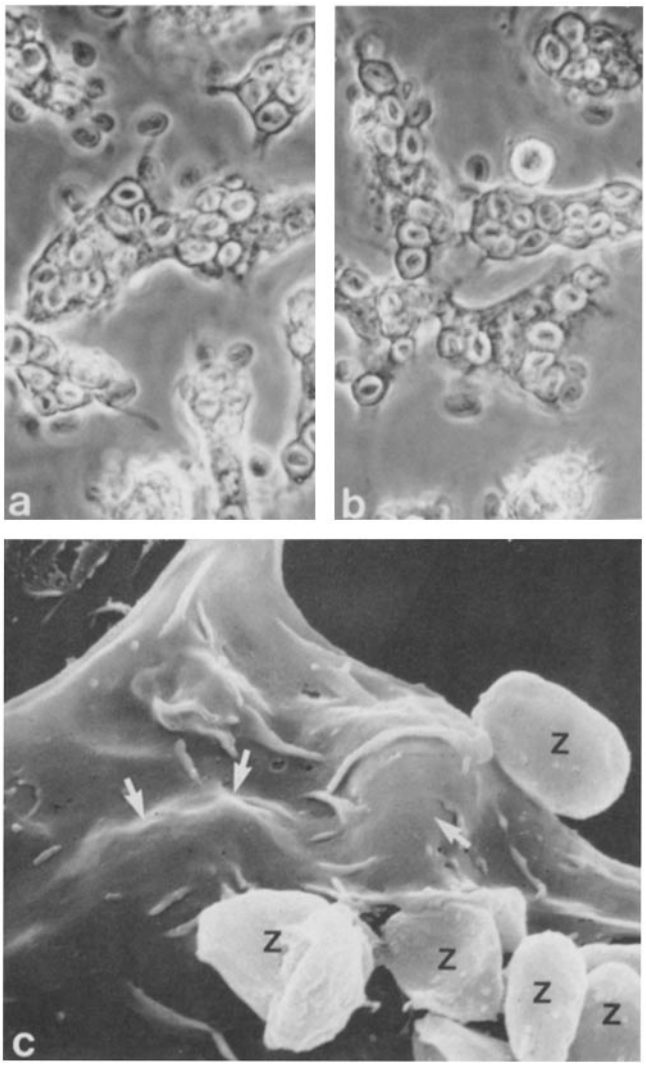

FIGURE 2 Phase-contrast and scanning electron micrographs of glutaraldehyde-fixed macrophages. Cells on glass coverslips were challenged with zymosan for 20 min at 37°C and then fixed with 2.5% glutaraldehyde. Phase-contrast micrographs of cells in high K^+ medium (a) and α-MEM (b) show macrophages containing up to 12 particles of zymosan. Also note particles associated with the membrane of the macrophages. Scanning electron micrographs of a single macrophage in α-MEM (c) show zymosan particles (z) associated with the cell and two bulges (arrows) where phagocytized particles lie just below the plasma membrane. × 8,000. Scanning electron micrographs of macrophages in high K^+ medium show similar morphological features.

Temporal Inhibition of 20:4 Release in High K^+ Medium

The effect of preincubation of macrophages for various times in high K^+ medium, followed by a stimulus with zymosan in the same medium, was determined. Increasing the preincubation time from 0 to 20 min resulted in a progressive decrease in the initial rate of ^3H 20:4 release from cells (Fig. 3*A*). A plot of the initial rate of ^3H 20:4 release vs. preincubation time was linear and showed that such a burst was ~90% inactivated after 15-min preincubation in high K^+ medium (Fig. 3*B*).

Time Course of Recovery from High K^+ Inhibition

Experiments were carried out to determine whether macrophages exposed to high K^+ could subsequently recover in α-MEM and release ^3H 20:4 in response to zymosan challenge. Cells were preincubated for 30 min in 118 mM K^+, after which the medium was changed to α-MEM containing zymosan (Fig. 4). Release of 18% of the radiolabel compared well with cells maintained in α-MEM and indicated that K^+-treated cells could not only recover but had the capacity to respond immediately to the phagocytic stimulus when the medium was replaced with α-MEM. Allowing the macrophages "recovery" time in α-MEM of up to 60 min before addition of zymosan did not enhance the response.

Activation of ^3H 20:4 Release in Cells Previously Given a Phagocytic Stimulus in High K^+ by Changing the Medium to One Containing Na^+

The ability of high K^+ medium to dissociate particle uptake from 20:4 release allowed us to examine further the nature of the zymosan stimulus. The results presented in Fig. 5 indicate that the cell ingesting zymosan in high K^+ has a "memory" for this trigger and will release 20:4 when the medium is replaced with high Na^+ at a later time (Fig. 5*B*). However, replacement with high K^+ fails to stimulate 20:4 release above the basal level of 1.5%/h (Fig. 5*A*). The ingestion of zymosan

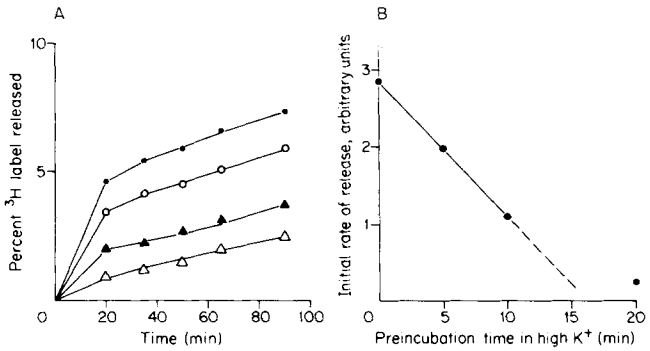

FIGURE 3 (*A*) Time-course of inactivation of 20:4 release by preincubation of macrophages in high K^+ medium. Cells were isolated, labeled with ^3H 20:4, washed, and preincubated for 0 (●), 5 (○), 10 (▲), and 20 (△) min in high K^+ medium at 37°C in 95% air/5% CO_2. Cells were then challenged with 160 μg zymosan and medium aliquots were counted at the indicated times. After 90-min, the cells were washed and scraped into 0.05% Triton X-100. The radiolabel and protein content of the cell lysates were then determined. Values expressed as percent of total cellular 20:4 released into the medium and are the mean of triplicate cultures. (*B*) Shows the initial rate of release (in arbitrary units) plotted against the preincubation time in high K^+ medium.

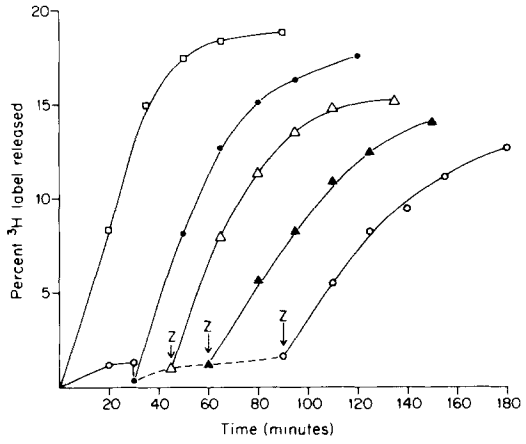

FIGURE 4 Time course of recovery from high K^+ inhibition. Cells were isolated, labeled, washed, and preincubated in high K^+ medium for 30 min. The medium was changed to α-MEM and zymosan (z) (160 μg) was added immediately (●) or after a 15- (△), 30- (▲), or 60-min (○) recovery period. Medium aliquots were removed at times indicated, the cells were scraped and ^{3}H-20:4 quantitated as described in Fig. 1. Zymosan stimulated release of 20:4 in α-MEM by macrophages that had not been preincubated in high K^+ is also shown (□). Values are expressed as percent of total cellular 20:4 released and are the mean of triplicate cultures.

in the presence of high K^+ leads to a priming event in terms of 20:4 release, which is maintained in the absence of additional particle uptake, and can be expressed when Na^+ is added to the medium.

Arachidonate Metabolism by Cells Cultured in Medium of Different Ionic Composition

Macrophages cultured in α-MEM released 21% of their esterified ^{3}H 20:4 in response to a zymosan challenge and the fatty acid was subsequently metabolized via cyclooxygenase and lipoxygenase pathways. Chromatographic separation of medium extracts showed 44% of the released 20:4 appeared as 6-Keto $PGF_1\alpha$, the stable breakdown product of prostacyclin and 24% as PGE_2 (Fig. 6*B*, Table II). Lipoxygenase metabolites, recovered as LTC, represented 18% of the released 20:4 (Fig. 6*A*). Similar proportions of 20:4 metabolites were formed by cells incubated in the presence of high Na^+ (6-keto-$PGF_1\alpha$ 43%; PGE_2 26%, and LTC 17%) (Table II).

The much diminished 20:4 response to zymosan in high K^+ medium occurred within the first 30 min. Extracts of pooled media from these macrophages were examined and revealed a different ratio of metabolites. While the profile of the major cyclooxygenase products were similar (6-Keto-$PGF_1\alpha$, 49%; PGE_2, 29%), only 3% of the 20:4 metabolites was present as LTC (Table II).

We determined the arachidonic acid metabolites produced by cells first primed with zymosan in high K^+ medium and then changed to medium of differing ionic composition. Cells transferred to high K^+ medium released a further 1.7% of ^{3}H-20:4 per hour, a value similar to basal release in the absence of a stimulus. The products consisted of 59% 6-Keto-$PGF_1\alpha$ and 20% PGE_2 while LTC was undetectable (Table III). Macrophages returned to high Na^+ medium released an extra 9% of the label comprising 6-Keto-$PGF_1\alpha$ (42%); PGE_2 (25%), and LTC (17%). Cells returned to α-MEM liberated a further 8% of label consisting of 6-Keto-$PGF_1\alpha$ (39%), PGE_2 (29%), and LTC (17%) (Table III). It appears, therefore, that the transient exposure of cells to high K^+ medium does not influence the relative amounts of 20:4 metabolized via the cyclooxygenase and lipoxygenase pathways.

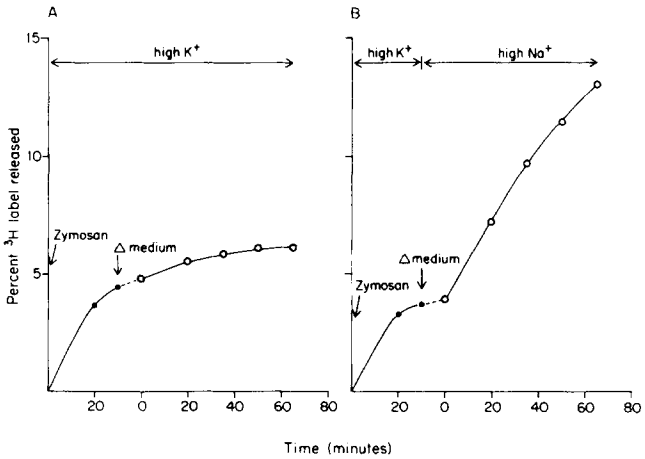

FIGURE 5 Activation of ^{3}H 20:4 release in cells previously given a phagocytic stimulus in high K^+ by changing medium to one containing Na^+. Cells were isolated, labeled, washed, and incubated with a suboptimal dose of zymosan in high K^+ medium for 30 min, which resulted in the association of approximately six particles per cell. Few particles remained unattached. Under these conditions, the cells released an initial burst of 3–4% of their [^{3}H]20:4 label. The dishes were washed at 4°C with high K^+ medium to remove unassociated particles. The medium was changed (indicated by ↓) to one containing either high K^+ (*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 released into the medium and are the mean of triplicate cultures.

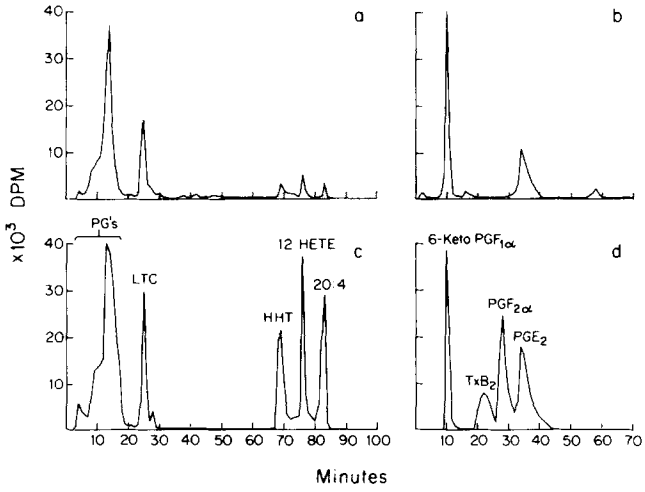

FIGURE 6 HPLC chromatograms of arachidonic acid metabolites. Macrophages were labeled with ^{3}H-20:4, challenged with zymosan in α-MEM and the 20:4 metabolites were extracted as described in Table II. Extracts were chromatographed on HPLC System I (*a*) as described in Materials and Methods and the prostaglandins (pooled fractions 4–17) were further characterized on HPLC System II (*b*). HPLC profiles for known tritiated standards are shown for System I (*c*) and System II (*d*).

Effect of High K^+ on the Production of Metabolites from Exogenous 20:4

Resident peritoneal macrophages will also metabolize exogenously added 20:4 in the absence of a stimulus (13). Exposure of macrophages to 0.5 μCi (8.3 nM) ^{3}H 20:4 in serum-free α-MEM for 20 min results in the esterification of ~36% of the fatty acid into cellular phospholipids (Table IV). The remaining radiolabel in the medium (64%) consisted of 53% cyclooxygenase products (major species 6-Keto $PGF_{1\alpha}$), 38% hydroxyeicosatetraenoic acids (HETEs) and 9% unreacted 20:4. When the experiment was carried out in high K^+ medium, 41% of the 20:4 was esterified into the cell membrane while 44% of the remaining label had been metabolized to cyclooxygenase products (mainly 6-Keto-$PGF_{1\alpha}$), 49% to HETEs, and 7% was unreacted 20:4 (Table IV). Preincubation of macrophages for 30 min in high K^+ medium did not significantly alter the exogenous activity when the cells were subsequently assayed in high K^+.

The data suggest that exposure to high K^+ has little effect on the macrophages' capacity to metabolize exogenous 20:4 via the cyclooxygenase or lipoxygenase pathways and that the extent and rate of esterification of 20:4 into the cellular phospholipid is unaffected.

Effect of K^+ on Ionophore-induced Release of 20:4

We wished next to examine the influence of high K^+ on a soluble pharmacological stimulus to 20:4 release. Fig. 7 indicates that the exposure of macrophages to the calcium ionophore A23187 in high Na^+ medium leads to the rapid release of 20% of the ^{3}H 20:4 into the medium. The exposure of cells in high K^+ medium to A23187 leads to the mobilization of ~21% of ^{3}H 20:4 from the membrane. Preincubation of cells in high K^+ for 30 min before addition of A23187 in the same medium had little effect on release that was 20% of the cellular 20:4 content (Fig. 7). It can be concluded therefore that

TABLE II

20:4 Metabolites Synthesized by Resident Peritoneal Macrophages in Response to Zymosan in Various Media

Medium	6-Keto-$PGF_{1\alpha}$	PGE_2	LTC	Unidentified*	6-Keto-$PGF_{1\alpha}$:PGE_2	Prostaglandins: LTC
α-MEM	44.3 ± 8.0	23.7 ± 3.6	18.1 ± 3.5	13.9 ± 2.6	1.8	3.8
High Na^+	43.4 ± 6.5	25.6 ± 4.5	16.9 ± 4.6	14.1 ± 3.2	1.7	4.1
High K^+	48.9 ± 5.0	29.1 ± 4.4	3.2 ± 0.5	18.8 ± 3.8	1.7	26.0

Macrophages were isolated and labeled with ^{3}H-20:4. The cells were washed, overlaid with the appropriate medium, challenged with zymosan (160 μg/ml) and after 90 min the medium was aspirated and extracted for 20:4 metabolites. Extracts were dried under N_2 and chromatographed on HPLC systems 1 and 2 as described. Representative HPLC chromatograms are shown for products synthesized by cells in α-MEM (Fig. 6, *a* and *b*). Values are expressed as the percent of the total ^{3}H-20:4 products formed and was calculated on the basis of discernable peaks above background. The data represent the mean ± SD of four determinations.

* Includes HETEs, HHT, unreacted 20:4 and polar metabolites that coelute with prostaglandins (PGs) in HPLC system 1 but not in HPLC system 2.

TABLE III

20:4 Metabolites Synthesized by Macrophages Previously "Primed" with Zymosan in High K^+ Medium on Being Overlaid with Media of Differing Ionic Composition

Medium	6-Keto-$PGF_{1\alpha}$	PGE_2	LTC	Unidentified*	6-Keto-$PGF_{1\alpha}$:PGE_2	Prostaglandins: LTC
α-MEM	39.1 ± 5.5	28.6 ± 5.7	16.6 ± 3.3	15.7 ± 2.8	1.3	4.0
High Na^+	41.9 ± 4.6	25.1 ± 3.6	17.4 ± 3.6	15.6 ± 2.5	1.7	3.0
High K^+	59.0 ± 7.7	19.6 ± 3.5	<0.5	21.4 ± 3.2	3.0	—

Macrophages were isolated and labeled with ^{3}H-20:4. The cells were washed and given a suboptimal dose of zymosan in high K^+ medium for 30 min as described in the legend to Fig. 5. After all unassociated particles had been removed by repeated washing in high K^+ the cells were overlaid with either α-MEM, high Na^+, or high K^+ medium, and after 90 min at 37°C in 95% air/5% CO_2 the medium was extracted for 20:4 metabolites as described for Table II. The 20:4 metabolites were separated by HPLC systems I and II as described in Materials and Methods. The data represent the mean ± SD of three determinations.

* Defined as in Table II.

TABLE IV

Effect of High K^+ on Production of Metabolites from Exogenous 20:4

Condition	% [^{3}H]20:4 incorporated by macrophages	% of radiolabel in medium		
		Prostaglandins	HETEs	20:4
α-MEM	36.3 ± 4.2	52.5 ± 9.9	38.0 ± 6.8	9.5 ± 1.5
High K^+	40.6 ± 1.7	43.7 ± 7.4	49.1 ± 8.8	7.2 ± 1.3
Preincubated in high K^+; then add 20:4 in high K^+	37.3 ± 1.4	47.9 ± 6.2	39.3 ± 6.3	12.8 ± 2.3

Macrophages were isolated and incubated overnight in a α-MEM plus 10% fetal calf serum. Cultures were washed, and overlaid with α-MEM, or high K^+ medium, or else preincubated for 30 min in high K^+ medium and then overlaid with high K medium. ^{3}H-20:4 (0.5 μCi, 8.3 nm) was added to the medium and after 20 min at 37°C the medium was removed and an aliquot counted. Macrophages were scraped into 1 ml of 0.05% Triton X-100 and the radiolabel content determined. Data are reported as duplicate determination on four dishes (mean ± SD) of the total recovered ^{3}H. The medium was extracted for 20:4 metabolites as described under Materials and Methods. Medium extracts of duplicate 35-mm culture dishes were pooled and subjected to HPLC. The data represent the mean ± SD of four determinations and are presented as the percent of the total ^{3}H recovered in HPLC effluents. The ^{3}H in fractions 3–17 is listed as prostaglandins, fractions 40–80 as HETEs, and fractions 81–100 as 20:4.

phospholipase(s) are active in high K^+ and that the K^+ effect on zymosan induction is exerted at a step proximal to the release of 20:4 from the phospholipid.

Arachidonic Acid Metabolites Synthesized by Macrophages Treated with A23187

The 20:4 products formed by macrophages in response to A23187 in various media were determined (Table V). Cells in Na^+ and K^+ released ~20–25% of their 20:4 label and synthesized a similar distribution of 20:4 metabolites: high Na^+ medium (52% cyclooxygenase products; 16% LTC; 25% HETEs) and high K^+ medium (55% cyclooxygenase metabolites; 14% LTC, 23% HETEs) (Table V).

These data indicate that both the cyclooxygenase and lipoxygenase pathways are active in high K^+ and further support the contention that the K^+ effect is proximal to the phospholipase.

DISCUSSION

The data presented here indicate that high concentrations of extracellular potassium uncouple phagocytosis of zymosan from the release of esterified 20:4 from cellular membranes. Kinetic analysis of uncoupling in high K^+ medium indicates that after an initial phase of 20:4 mobilization, release decrease rapidly to basal levels observed in the absence of a stimulus. The initial release of 20:4 can be inhibited by preincubation of the cells in high K^+ for 15 min indicating a lag-phase before total inhibition is exerted. Furthermore, the immediate recovery of 20:4 release when the cells are transferred to a medium containing sodium suggest that high concentrations of K^+ are not toxic and that extracellular sodium is essential in the transduction process. As net release of 20:4 is a composite of release from and esterification into membrane pools the question arises as to where K^+ exerts its effect. One possibility is that K^+ inhibits metabolism of released 20:4, thereby increasing the pool of free 20:4 available for uptake into the membrane and resulting in a decrease in ^3H label assayed in the medium (the metabolites cannot, in general, be esterified). We have shown that K^+ does not inhibit the A23187-induced release of 20:4 or its metabolism via either the cyclooxygenase or lipoxygenase pathways. Exogenously added 20:4 is readily metabolized to both cyclooxygenase and lipoxygenase products in high K^+ medium with rates similar to those in α-MEM. Furthermore exogeneously supplied 20:4 is esterified into macrophage membranes at similar rates in both high K^+ medium and in α-MEM. Therefore, since the cells appear capable of metabolizing 20:4 in high K^+ medium and as esterification rates are similar, the K^+ inhibitable or Na^+ requiring sites must be related to release of 20:4 from the membrane. As the zymosan-induced release is inhibited by K^+ while the A23187 stimulated release is unimpaired, it is likely that the lesion is at the level of zymosan induced signal transduction at or across the macrophage plasma membrane.

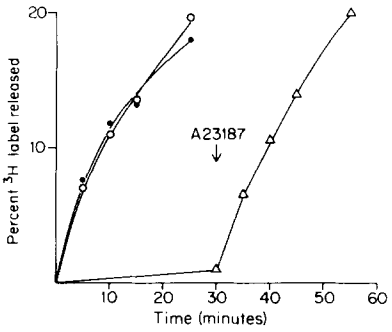

FIGURE 7 Effect of K^+ on A23187 induced release of 20:4. Cells were isolated, labeled, washed, overlaid with high K^+ (○) or high Na^+ medium (●), and challenged with the ionophore A23187 (10 μg/ml). At the times indicated duplicate aliquots of medium were removed and counted. After 25 min, the cells were washed, scraped into Triton X-100 and the radiolabel and protein content of the cell lysates was quantitated. Cells were also preincubated for 30 min in high K^+ medium (△), treated with A23187, and the time course of ^3H-20:4 release was assessed. Values are expressed as percent of total cellular 20:4 released into the medium and are the mean of triplicate cultures.

It is not clear exactly which receptor zymosan binds, though it is generally thought to be the mannosyl, fucosyl receptor (14). It has recently been shown that the mouse macrophage receptor that binds the constant region of IgG acts as a monovalent cation channel when it interacts with immune complexes or the divalent monoclonal antibody directed against its active sites (15, 16). The requirement of extracellular Na^+ for 20:4 release in response to zymosan may reflect a role for Na^+ influx as a primary message in the transduction mechanism. Sodium influxes have been shown to be an early event when neutrophils are stimulated with the chemotactic peptide fmet-leu-phe (17–19). Furthermore, removal of Na^+ from the medium decreases chemotactic responsiveness in neutrophils (20) as well as fmet-leu-phe–stimulated lysosomal enzyme secretion (21) and superoxide generation (17). Immune complex and concanavalin A-stimulated O_2^- production and lysosomal enzyme secretion in human neutrophils also have a requirement for extracellular Na^+ (22).

Interaction of zymosan with the appropriate receptor(s) appears to generate at least two distinct signals. One initiates receptor mediated phagocytosis of the particle, while the other results in the induction of phospholipase activity and the subsequent release of 20:4 from the cell membranes. It has previously been reported that ligand-receptor binding is suf-

TABLE V

20:4 Metabolites Synthesized by Macrophages Treated with A23187

Medium	^3H label released	Total cyclooxygenase	LTC	HETEs*	Unreacted 20:4
	%				
High Na^+	25.2 ± 4.8	51.9 ± 9.8	16.4 ± 2.5	24.7 ± 3.2	7.0 ± 1.5
High K^+	23.4 ± 3.5	54.7 ± 8.2	14.8 ± 2.8	23.0 ± 4.1	7.5 ± 1.4

Macrophages were isolated and labeled with ^3H-20:4. The cells were washed, overlaid with the appropriate medium, and challenged with A23187 (10 μg/ml). After 25 min at 37°C, the medium was aspirated, an aliquot counted, and the remainder extracted for 20:4 metabolites. Extracts were dried under N_2 and chromatographed on HPLC systems 1 and 2, as described. Values are expressed as percent of total ^3H-20:4 products formed. The data represent the mean ± SD of four determinations.

* HETEs include mono- and di-HETEs.

ficient to trigger 20:4 release and that the later events constituting phagocytosis are not required (4). Here we show that receptor-mediated phagocytosis can occur in the absence of 20:4 release implying that the signals for these two events are distinct.

The two signals generated by zymosan binding do interact, however, Cells that have ingested zymosan particles in high K^+ medium become primed for the release of 20:4 from the cell membranes, an event that occurs without further addition of a stimulus when the medium is changed to one containing Na^+. The specific requirement for Na^+ has been confirmed by showing that release does not occur spontaneously in a medium containing choline (manuscript in preparation).

The zymosan-induced release of 20:4 from cytochalasin D–treated macrophages is accompanied by an efflux of cellular glutathione into the medium (23). Treatment of macrophages with zymosan in high K^+ medium results in a glutathione efflux (manuscript in preparation), indicating functionally competent ligand-receptor interaction and suggesting that the inhibition of 20:4 release by K^+ occurs distal to particle binding. This result also suggests the selective inhibition by K^+ of receptor-ligand induced functions.

The ionophore A23187 induces rapid release of 20:4 presumably by increasing the intracellular calcium concentration. High concentrations of extracellular K^+ has no effect on A23187–stimulated release, which indicates that the K^+-inhibitable or Na^+-requiring sites are proximal to the calcium-induced phospholipase activity. Another possibility is that different stimuli trigger distinct phospholipases. In this regard, it is of interest that lipoxygenase metabolites form a larger proportion of the 20:4 products elicited by A23187 when compared with those synthesized in response to zymosan.

It is clear that the interaction of zymosan with the macrophage membrane triggers multiple signals and that the series of events leading to the induction of the phospholipase and the subsequent release of 20:4 is extremely complex. Investigations exploring this pathway are currently being undertaken.

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REFERENCES

1. Scott, W. A., J. M. Zrike, A. L. Hamill, J. Kempe, and Z. A. Cohn. 1980. Regulation of arachidonic acid metabolites in macrophages. *J. Exp. Med.* 152:324–335.
2. Bonney, R. J., P. D. Wightman, P. Davies, S. J. Dadowski, F. A. Kuehl, Jr., and J. L. Humes. 1978. Regulation of prostaglandin synthesis and the select release of lysosomal hydrolases by mouse peritoneal macrophages. *Biochem. J.* 175:433–442.
3. Humes, J. L., P. Davies, R. J. Bonney, and F. A. Kuehl, Jr. 1978. Phorbol myristate acetate stimulated release of arachidonic acid and its cyclooxygenase products by macrophages. *Fed. Proc.* 37:1318. (Abstr.)
4. 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.* 77:4279–4282.
5. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes: Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* 121:153–170.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
7. Griffin, F. M., Jr., C. Bianco, and S. C. Silverstein. 1975. Characterization of the macrophage receptor for complement and demonstration of its functional independence from the receptor for the Fc portion of immunoglobulin G. *J. Exp. Med.* 141:1269–1277.
8. Kaplan, G., and K. Berpheussen. 1977. The morphology of echinoid phagocytes and mouse peritoneal macrophages during phagocytosis *in vitro*. *Scand. J. Immunol.* 6:1289–1296.
9. Unger, W. G., I. F. Stamford, and A. Bennett. 1971. Extraction of prostaglandins from human blood. *Nature (Lond.).* 233:336–337.
10. Borgeat, P., and B. Samuelsson. 1979. Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. Natl. Acad. Sci. USA.* 76:2148–2152.
11. Alam, I., K. Ohuchi, and L. Levine. 1979. Determination of cyclooxygenase products and prostaglandin metabolites using high-pressure liquid chromatography and radioimmunoassay. *Anal. Biochem.* 93:339–345.
12. 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.* 158:393–412.
13. Scott, W. A., N. A. Pawlowski, M. Andreach, and Z. A. Cohn. 1982. Resting macrophages produce distinct metabolites from exogenous arachidonic acid. *J. Exp. Med.* 155:535–547.
14. 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.* 96:160–166.
15. Young, J. D.-E., J. C. Unkeless, H. R. Kaback, and Z. A. Cohn. 1983. Macrophage membrane potential changes associated with Fc receptor$_{2b/a1}$ ligand binding. *Proc. Natl. Acad. Sci. USA.* 80:1357–1361.
16. Young, J. D.-E., J. C. Unkeless, H. R. Kaback, and Z. A. Cohn. 1983. Mouse macrophage Fc receptor for IgG$_{2b/a1}$ in artificial and plasma membrane vesicles functions as a ligand-dependent ionophore. *Proc. Natl. Acad. Sci. USA.* 80:1636–1640.
17. Simchowitz, L., and I. Spilberg. 1979. Chemotactic factor-induced generation of superoxide radicals by human neutrophils: evidence for the role of sodium. *J. Immunol.* 123:2428–2435.
18. Naccache, P. H., H. J. Showell, E. L. Becker, and R. I. Sha'afi. 1977. Changes in ionic movements across rabbit polymorphonuclear leukocyte membranes during lysosomal enzyme release: possible ionic basis for lysosomal enzyme release. *J. Cell Biol.* 75:635–649.
19. 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.* 73:428–444.
20. Showell, H. J., and E. L. Becker. 1976. The effects of external K^+ and Na^+ on the chemotaxis of rabbit peritoneal neutrophils. *J. Immunol.* 116:99–105.
21. Showell, H. J., P. H. Naccache, R. I. Shaf'afi, and E. L. Becker. 1977. The effects of extracellular K^+, Na^+ and Ca^{2+} on lysosomal enzymes secretion from polymorphonuclear leukocytes. *J. Immunol.* 119:804–811.
22. 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.* 601:180–194.
23. Rouzer, C. A., W. A. Scott, O. W. Griffiths, A. L. Hamill, and Z. A. Cohn. 1982. Glutathione metabolism in resting and phagocytizing peritoneal macrophages. *J. Biol. Chem.* 257:2002–2008.