CALCIUM IONOPHORE SYNERGIZES WITH BACTERIAL LIPOPOLYSACCHARIDES IN ACTIVATING MACROPHAGE ARACHIDONIC ACID METABOLISM

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Macrophages play important roles in inflammation and host defense. The metabolites of arachidonic acid (20:4) are important modulators of these responses (1). When macrophage phagocytic receptors interact with particulate ligands such as immune complexes or bacteria, phospholipases are activated and 20:4 is released from membrane phospholipids (2, 3). The free 20:4 is then oxygenated via the cyclooxygenase pathway to prostaglandin E_2 (PGE_2)[1] and prostacyclin (PGI_2) or along the lipoxygenase pathway to leukotriene C (LTC) and hydroxyeicosatetraenoic acids (HETEs) (2–5). Little is known about the molecular events underlying the regulation of 20:4 metabolism.

We recently reported that bacterial LPS are poor triggers of the 20:4 cascade but do have the capacity to prime macrophages for greatly increased 20:4 metabolism upon subsequent stimulation (6). One second signal is phagocytosis of latex beads. Latex can only trigger a LPS-primed cell (6). This led us to postulate that activation of 20:4 metabolism in macrophages requires at least two steps acting in sequence, a priming step followed by a triggering step (6). Receptor-mediated particulate stimulation of the 20:4 cascade is capable of both priming and triggering; LPS is capable of priming; and latex particles can only generate the triggering step. We have reported data that suggest that the first, priming step is related to LPS-dependent myristoylation of proteins involved in signal-response coupling (7). We report here that the second, triggering step is regulated by calcium. We further provide data that suggest that calcium also regulates the lipoxygenase pathway.

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 ($\sim 9 \times 10^6$/ml) in MEM (Grand Island Biological Co., Grand Island, NY) containing 10% FCS were added to 35-mm diameter plastic culture dishes (1 ml per dish). After 2 h at 37°C in 95% air/5% CO_2, cultures were washed three times in calcium- and magnesium-free PBS (PD)

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[1] *Abbreviations used in this paper:* [^{3}H]20:4, 5,6,8,9,11,12,14,15-[^{3}H]arachidonic acid; LTC, leukotriene C; PD, calcium- and magnesium-free PBS; PGE_2, prostaglandin E_2; PGI_2, prostacyclin.

to remove nonadherent cells. Fresh α-MEM plus 10% FCS (1 ml per dish) containing 0.5 μCi of 5,6,8,9,11,12,14,15-[^{3}H]arachidonic acid ([^{3}H]20:4; sp act, 70 Ci/nmol; New England Nuclear, Boston, MA) was added and the cells were incubated overnight (16 h).

Assay of Total [^{3}H]20:4 Release. Macrophages, prelabeled with [^{3}H]20:4, were washed four times in PD and were then overlaid with 1 ml of serum-free MEM plus or minus 10 ng/ml *Salmonella abortus equi* LPS (gift of Dr. C. Galanos, Max Planck Institute, Freiburg, Federal Republic of Germany). After 60 min, the cells were challenged with 0.1 μM A23187 (Calbiochem-Behring Corp., La Jolla, CA) or 25 nM PMA (Sigma Chemical Co., St. Louis, MO) as indicated. 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 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. (8) with BSA as a standard.

Quantitation of [^{3}H]20:4 Metabolites. The 20:4 metabolites in the culture medium were extracted as described previously (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.). The lower (chloroform) phase 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 × 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/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) (10, 11). For the characterization of the cyclooxygenase metabolites, a duplicate extract was applied on HPLC system 2 using a Fatty Acid Analysis Column (Waters Associates, 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 (10, 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 (6).

LPS Determination. All reagents and culture material were found to contain <0.1 ng/ml contaminating LPS using the limulus amebocyte lysate assay (Cape Cod Associates, Woods Hole, MA).

Calculation of Free Ca^{2+} Concentration. Free Ca^{2+} concentration was calculated at pH 7.4 from the pK_a values of H-EGTA, and MH-EGTA, where H and M indicate bound H^+ and metal ions (both Ca^{2+} and Mg^{2+}), respectively (13).

Results

Nonactivating Concentrations of Calcium Ionophore Trigger 20:4 Metabolism in LPS-primed Macrophages. Resident peritoneal macrophages, labeled with [^{3}H]-arachidonic acid (20:4) and treated either with LPS (10 ng/ml) or Ca^{2+} ionophore at 0.1 μM did not release ^{3}H label to the medium over a 3-h period (Fig. 1*A*). On the other hand, when the cells were first primed with LPS (10 ng/ml) for 60 min and then challenged with A23187 there was a rapid release of a large amount of 20:4 metabolites (Fig. 1*A*). The order in which stimuli were added was critical. When the cells were first challenged with A23187 and subsequently treated with LPS no release of 20:4 metabolites was observed for 40 min after which time 20:4 metabolite secretion ensued (Fig. 1*B*).

The experiments reported here with 10 ng/ml LPS and 0.1 μM A23187 were representative for LPS concentrations of 5–500 ng/ml and for A23187 concen-

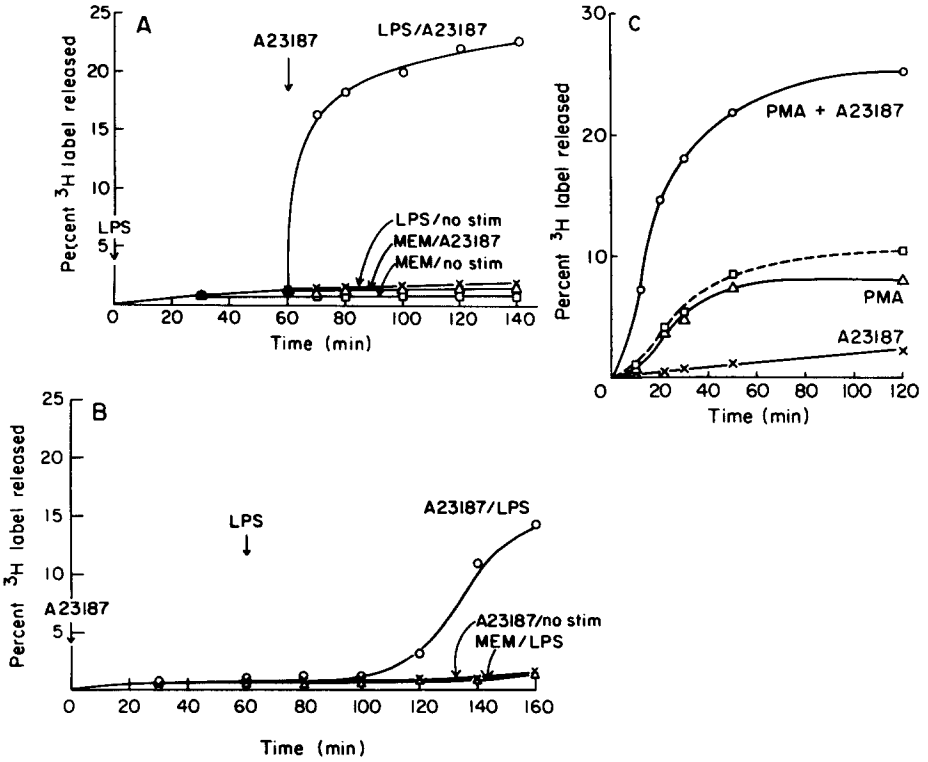

FIGURE 1. Effect of Ca^{2+} ionophore A23187 on 20:4 secretion in LPS-primed and control macrophages. Murine resident peritoneal macrophages were isolated by peritoneal lavage and labeled overnight with [^{3}H]20:4 in α-MEM and 10% FCS (6). (*A*) The cells were washed four times with PBS and overlaid with α-MEM containing 10 ng/ml LPS (○, ×) or with α-MEM alone (△, □). After 60 min at 37°C in 95% air/5% CO_2, the cells were treated with 0.1 μM A23187 in DMSO (○, △) or with DMSO alone (×, □). The final concentration of A23187 or DMSO had no effect on cellular morphology, protein content, or on the capacity of the macrophages to exclude trypan blue. At the specified times, duplicate aliquots of the medium were removed and counted. The cells were scraped twice in 0.5% Triton X-100, and the radiolabel and protein content of the cell lysates were determined (6). Values are expressed as percent of total cellular 20:4 released into the medium, and are means of triplicate cultures (SEM <10%). The profile of 20:4 metabolites secreted after 120 min are shown in Table I. (*B*) The experiment was done exactly as described for *A*, except that A23187 was added at 0 min and LPS was added after 60 min. (*C*) Macrophages were washed four times with PBS, overlaid with α-MEM, and challenged with 25 nM PMA (△), 0.1 μM A23187 (×) or with a combination of 25 nM PMA and 0.1 μM A23187 (○). The time course of 20:4 metabolite release was assayed and expressed as described in *A* and the profile of 20:4 metabolites secreted after 120 min is shown in Table I. The dashed line (□) is the calculated value of the sum of PMA and A23187–induced 20:4 secretion.

trations of 0.05–0.5 μM (data not shown). Concentrations of LPS >1 μg/ml triggered macrophages to secrete ~8% of their cellular 20:4 as cyclooxygenase products (Table I and reference 6) and concentrations of A23187 of 1 μM or higher killed the cells and promoted the rapid release of 20:4 metabolites from both LPS-primed and control macrophages (6).

To be sure that Ca^{2+} (and not A23187) was critical for the triggering step, cells were cultured with LPS for 60 min and then exposed to A23187 in Ca^{2+}-free medium (Fig. 2*A*). In the absence of external Ca^{2+} very little 20:4 was

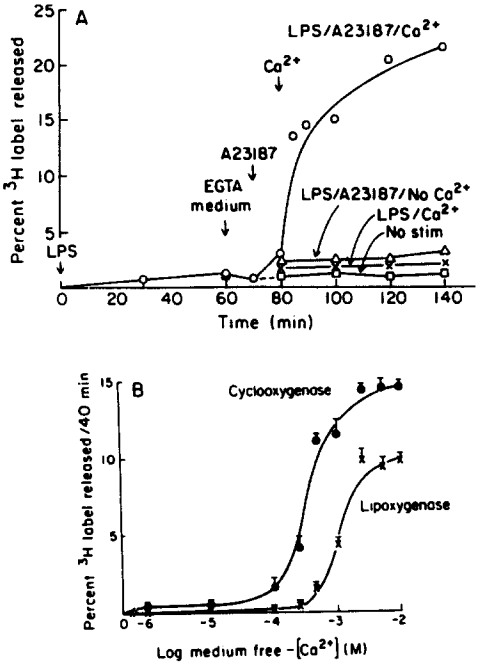

FIGURE 2. Ca^{2+} concentration dependence of cyclooxygenase and lipoxygenase metabolite secretion from LPS-primed macrophages. Macrophages were isolated, labeled with [^{3}H]20:4, and treated with 10 ng/ml LPS as described in Fig. 1*A*. After 60 min, the medium was changed to α-MEM minus Ca^{2+} containing 0.5 mM EGTA (EGTA medium, custom made by K.C. Biological, Inc., Lenexa, KS); after 70 min A23187 was added were indicated (○, △), and at 80 min Ca^{2+} was added such that the medium free Ca^{2+} concentration was 1.8×10^{-3} M (13). (△) Ca^{2+} was omitted; (x) A23187 was omitted. (*B*) Macrophages were treated in sequence with LPS, EGTA medium, A23187, and Ca^{2+} as described in *A* except that the amount of Ca^{2+} added was varied to yield the medium free Ca^{2+} concentrations indicated on the *x* axis. The cyclooxygenase metabolites (6-keto-$PGF_{1\alpha}$ and PGE_2) and the lipoxygenase products (LTC and 5-HETE) were determined as described in Table I, 40 min after addition of the Ca^{2+} (i.e., 120 min after being treated with LPS). The data represent the mean ± SD of four determinations.

secreted into the medium. On the addition of Ca^{2+} (free $[Ca^{2+}] = 1.8 \times 10^{-3}$ M) to the medium there was a rapid release of 20:4 metabolites, confirming that an increase in intracellular Ca^{2+} triggers 20:4 metabolite secretion in the LPS-primed cell.

PMA, an activator of protein kinase C, has previously been shown to synergize with A23187 in stimulating macrophage 20:4 metabolism (14, 15). We therefore compared the synergy of LPS and A23187 with that of PMA and A23187. PMA alone promoted the release of ~8% of the cell-associated [^{3}H]20:4 in 2 h, while PMA in combination with nonactivating concentrations of A23187 stimulated the secretion of ~25% of cellular 20:4 (Fig. 1*C*). The data reported in Fig. 1*C* are representative for PMA concentrations of 5–100 nM and A23187 concentrations of 0.05–0.5 μM.

Calcium Ionophore Unmasks the Lipoxygenase Pathway. An additional role for Ca^{2+} became apparent when we examined the profile of 20:4 metabolites secreted from cells that were either treated with LPS alone, or sequentially treated with LPS and A23187 (Table I). High concentrations of LPS (1 μg/ml) promoted the release of only cyclooxygenase products (6-keto-$PGF_{1\alpha}$, 54.3%; PGE_2, 43.2%) (Table I) whereas macrophages treated sequentially with LPS and A23187 secreted, in addition, large quantities of the lipoxygenase product, LTC_4 (32.3%). This represented a 150-fold increase in LTC production. A similar shift in profile of 20:4 metabolite secretion occured when macrophages were treated with PMA and A23187 (14, 15) (Table I). While PMA alone stimulated macrophages to secrete only cyclooxygenase products, the combination of PMA and A23187 promoted the release of large amounts of LTC as well (Table I). Therefore,

TABLE I

20:4 Metabolites Synthesized by LPS-primed and Control Macrophages in Response to a Variety of Stimuli

Condition	Percent ^{3}H label released	Cyclooxygenase		Lipoxygenase		Unreacted 20:4
		6-Keto-$PGF_{1\alpha}$	PGE_2	LTC	HETEs	
LPS (1 μg/ml)	8.6	54.3	43.2	<0.2	1.2	1.3
LPS (10 ng/ml)	2.0	51.9	44.6	<0.2	1.4	2.1
A23187 (0.1 μM)	2.3	42.4	45.7	<0.2	4.8	7.1
LPS (10 ng/ml)/ A23187 (0.1 μM)	25.0	22.5	36.8	32.3	5.4	3.0
PMA (25 nM)	8.1	36.2	57.6	<0.2	1.6	4.6
PMA (25 nM)/ A23187 (0.1 μM)	26.0	24.9	33.7	31.9	6.6	2.9

Macrophages were isolated and labeled overnight with [^{3}H]20:4. The cells were washed, overlaid with α-MEM, and then incubated with the following: LPS (1 μg/ml); LPS (10 ng/ml); A23187 (0.1 μM); LPS (10 ng/ml) for 60 min followed by A23187 (0.1 μM) for 60 min; PMA (25 nM); and a combination of PMA (25 nM) and A23187 (0.1 μM). After 120 min, 20:4 metabolites were extracted, identified and quantitated by HPLC as described in Materials and Methods. Values are expressed as the percent of total [^{3}H]20:4 secreted into the medium calculated on the basis of discernible peaks above background. The data represent the mean of four determinations and the SEM was <10%.

increasing the Ca^{2+} concentration within macrophages with A23187 resulted in the activation of the lipoxygenase pathway.

These data implied that the cyclooxygenase and lipoxygenase pathways had different Ca^{2+} requirements. We therefore used LPS-primed macrophages that had been treated with A23187 in the absence of external Ca^{2+} as a model system in which to titrate the Ca^{2+} concentration dependence of each pathway (Fig. 2*B*). Neither cyclooxygenase nor lipoxygenase metabolites were secreted in the absence of Ca^{2+} in the medium. When Ca^{2+} was titrated into the system, cyclooxygenase products were detected at lower medium Ca^{2+} concentrations than were lipoxygenase metabolites (Fig. 2*B*). The $K_{0.5}$ (apparent) for extracellular free Ca^{2+} was 3.6×10^{-4} M for cyclooxygenase metabolite secretion and 1.5×10^{-3} M for lipoxygenase product release. These data do not represent exact $K_{0.5}$ since the ionophore only renders the cells partially permeable to Ca^{2+}, but do give a good comparative assessment of the Ca^{2+} requirements of the two pathways.

Discussion

Macrophages secrete prodigious quantities of 20:4 metabolites when stimulated with a variety of particulate and soluble agonists (2–5). The molecular mechanism(s) leading to the activation of the 20:4 cascade and those underlying the differential synthesis of cyclooxygenase and lipoxygenase metabolites remain obscure. In this paper we show that nonactivating concentrations of Ca^{2+} ionophore trigger the rapid release of 20:4 metabolites from LPS-primed macrophages but not from nonprimed cells (Fig. 1*A*). Macrophages must be incubated with LPS for at least 40 min before A23187 becomes effective as a trigger (Fig. 1*B*). This correlates exactly with the amount of time required to fully prime macrophages for enhanced 20:4 metabolism with LPS (6). In effect, a complex stimulus like latex uptake behaves in a similar way to A23187 in our system (6).

It is therefore of interest that phagocytosis of latex particles is accompanied by a two- to threefold increase in the intracellular Ca^{2+} concentration from resting levels of ~100 nM (Aderem, A., unpublished observation). Regulation of triggering by Ca^{2+} is also consistent with our previous finding that 20:4 metabolite secretion in macrophages is completely dependent on extracellular Ca^{2+} (16) and with the requirements of macrophage phospholipases for Ca^{2+} (17, 18).

The synergy between LPS and A23187 in activating macrophage 20:4 metabolism is reminiscent of that seen with PMA and A23187 (14, 15) (Fig. 1*C*). This suggests that LPS may prime macrophages for enhanced 20:4 metabolism via a protein kinase C–dependent pathway and is consistent with the observation that LPS activates partially purified protein kinase C (19). However, there are differences in the effect of LPS and PMA on macrophage 20:4 metabolism. First, PMA alone triggers the secretion of modest amounts of 20:4 metabolites, while LPS (10 ng/ml) does not (Fig. 1, *A* and *C*). Second, it is necessary to preexpose macrophages to LPS for at least 40 min before the addition of A23187, while PMA exerts its effect immediately (Fig. 1). We have recently shown that LPS promotes the myristoylation of three macrophage proteins (7) one of which (M_r 68×10^3) is a substrate for protein kinase C (Aderem, A., K. Albert, M. Keum, J. Wang, P. Greengard, and Z. Cohn, submitted for publication). We have further shown that the myristoylated protein kinase C substrate becomes associated with the plasma membrane. Since protein kinase C is activated at the membrane (20), we hypothesize that LPS priming results in the closer association of protein kinase C and its substrate, which in turn results in a more efficient Ca^{2+}-dependent activation of 20:4 metabolism.

We have also used the LPS-primed macrophage as a model system in which to examine the regulation of the cyclooxygenase and lipoxygenase pathways. It is well known that different ligands trigger the secretion of different proportions of lipoxygenase and cyclooxygenase metabolites (6, 14, 21). Thus particulate triggers such as zymosan and immune complexes promote the secretion of large amounts of lipoxygenase and cyclooxygenase metabolites, while soluble agonists such as PMA trigger the release of cyclooxygenase products alone (6, 14, 21). These data were interpreted by Humes et al. (21) to indicate that particulate stimuli mobilize a pool of arachidonic acid that is accessible to both cyclooxygenase and lipoxygenase pathways, while soluble stimuli promote the release of a distinct pool of 20:4 that is only available to the cyclooxygenase pathway. More recently, Tripp et al. (14) showed that PMA could promote the release of LTC_4 when used in combination with low, nonactivating, concentrations of A23187. They suggested that PMA does not mobilize sufficient intracellular Ca^{2+} to activate the 5-lipoxygenase. Increasing the Ca^{2+} concentration with A23187 activates the 5-lipoxygenase and results in the secretion of both prostaglandins and leukotrienes. This interpretation is paradoxical since the phospholipase(s) also requires Ca^{2+} for activity (17, 18). This apparent paradox can be resolved if the phospholipase(s) has a higher affinity for Ca^{2+} than does the 5-lipoxygenase. The soluble stimuli may then increase the Ca^{2+} concentration sufficiently to activate the phospholipase(s) but not sufficiently to activate the 5-lipoxygenase. The data presented in Fig. 2*B* support this hypothesis; cyclooxygenase metabolites are secreted at an order of magnitude lower Ca^{2+} concentration than are

lipoxygenase metabolites. Consistent with this, the $K_{0.5}$ apparent (Ca^{2+}) of purified phospholipase C has been reported as 3 μM (22) compared with 20–100 μM for the partially purified 5-lipoxygenase (23). Thus agonists that mobilize relatively small amounts of intracellular Ca^{2+} such as PMA (24) promote the secretion of only cyclooxygenase products, while particulate agonists that stimulate rather larger increases in intracellular Ca^{2+} (25) cause the secretion of both cyclooxygenase and lipoxygenase products.

Summary

LPS, a major component of Gram-negative bacterial cell walls, prime macrophages for greatly enhanced arachidonic acid [20:4] metabolism when the cells are subsequently stimulated. The LPS-primed macrophage has been used as a model system in which to study the role of Ca^{2+} in the regulation of 20:4 metabolism. The Ca^{2+} ionophore A23187 (0.1 μM) triggered the rapid release of 20:4 metabolites from LPS-primed macrophages but not from cells not previously exposed to LPS. Macrophages required exposure to LPS for at least 40 min before A23187 became effective as a trigger. A23187 (0.1 μM) also synergized with PMA in activating macrophage 20:4 metabolism. The PMA effect could be distinguished from that of LPS since no preincubation with PMA was required. A23187 greatly increased the amount of lipoxygenase products secreted from LPS-primed macrophages, leukotriene C_4 synthesis being increased 150-fold. LPS-primed macrophages, partially permeabilized to Ca^{2+} with A23187, were used to titrate the Ca^{2+} concentration dependence of the cyclooxygenase and lipoxygenase pathways. Cyclooxygenase metabolites were detected at an order of magnitude lower Ca^{2+} concentration than were lipoxygenase products. The data suggest that Ca^{2+} regulates macrophage 20:4 metabolism at two distinct steps: an increase in intracellular Ca^{2+} regulates the triggering signal and relatively higher Ca^{2+} concentrations are required for 5-lipoxygenase activity.

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