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CALCIUM-DEPENDENT EICOSANOID METABOLISM BY CONCANAVALIN A-STIMULATED HUMAN MONOCYTES IN VITRO

Synergism with Phorbol Ester Indicates Separate Regulation of Leukotriene B_4 Synthesis and Release

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Human monocytes obtained by counter-current centrifugal elutriation released arachidonic acid when challenged in vitro with Con A, as well as with other soluble (PMA or ionomycin) or particulate stimuli (serum-treated zymosan). Cyclo-oxygenase metabolites were the principal eicosanoids detected in the supernatants of Con A-stimulated, [^{3}H]arachidonate-labeled monocytes. 5-Lipoxygenase (5-LO) products, such as leukotriene B_4 (LTB_4), were conspicuously absent. Release of arachidonate and its metabolites in response to Con A was dependent on the presence of extracellular Ca^{2+}, but not Mg^{2+}. In contrast to serum-treated zymosan challenge, which resulted in increased inositol trisphosphate and LTB_4 release, Con A-induced inositol phospholipid hydrolysis in monocytes was limited to phosphatidylinositol or phosphatidylinositol monophosphate. Despite an inability to augment LTB_4 release, Con A or PMA induced a loss of 5-lipoxygenase from a cytosolic compartment that was similar to that achieved with a calcium ionophore (ionomycin), a potent stimulus for LTB_4 generation. When cell-associated LTB_4 was evaluated, evidence for increased LTB_4 production was obtained in response to either stimulus (PMA > Con A). In combination, however, PMA and Con A treatment resulted in monocyte LTB_4 release comparable with that observed with the calcium ionophore or STZ. LTB_4 release in response to all stimuli tested was inhibited by MK-886, a drug that binds to 5-lipoxygenase-activating protein. These results indicate the following: 1) Phospholipase A_2 activation and attendant arachidonic acid release induced by agents that increase intracellular Ca^{2+} and/or generate diacylglycerol results in increased synthesis and release of PG and increased synthesis of leukotrienes, but not necessarily leukotriene release. 2) 5-LO translocation, which may occur independently of increased intracellular Ca^{2+}, may be necessary for LTB_4 generation but is insufficient for its release. 3) 5-Lipoxygenase-activating protein activity is necessary for 5-LO activation and LTB_4 release in response to all stimuli investigated here. 4) Phorbol ester, an activator of protein kinase C, may synergize with agents such as Con A (which by themselves induce a minimal intracellular Ca^{2+} rise), so as to result in the release of LTB_4. Thus, Con A may represent a class of surface receptor-aggregating agents that initiates inflammatory changes or immunomodulation associated with liberation of PG and might predispose to release of other inflammatory mediators, such as leukotrienes, in the presence of additional signals, including protein kinase activation.

PLA_2[2] hydrolyzes fatty acid from the sn-2 position of a wide variety of phospholipids (1). In many cell types, including human monocytes, PLA_2 commonly acts on substrates containing AA. The liberation of free arachidonate is a first step in the metabolism of PG, hydroxyeicosatetraenoic acids, and leukotrienes.

Monocytes and macrophages have been shown to be rich sources of arachidonate and its metabolites (2). Some biologic properties of monocytes, notably their role as immunomodulating cells, have been attributed to eicosanoid production and release (3). Accordingly, much of the interest regarding PLA_2 in human monocytes centers on this aspect of their function.

The physiologic regulation of PLA_2 in human monocytes (4) or rodent macrophages (5, 6) has been partly elucidated only recently. In either cell type, increased intracellular calcium or direct activation of PKC is associated with PLA_2 activation. For example, in vitro treatment of monocytes with agents that result in PKC activation (e.g., phorbol esters), or others that, in addition to

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[2] Abbreviations used in this paper: PLA_2, phospholipase A_2; AA, arachidonic acid; FLAP, 5-lipoxygenase activating protein; HETE, 5-(S)-hydroxy-6,8,11,14-eicosatatraenoic acid; $InsP_3$, inositol trisphosphate; LTB_4, leukotriene B_4; MK-886, 3-(1-(4-chlorobenzyl)3-*t*-butyl-thio-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol phosphate; $PtdInsP_2$, phosphatidylinositol bisphosphate; PLC, phospholipase C; PKC, protein kinase C; STZ, serum-treated zymosan.

PKC activation, cause release of Ca^{2+} from intracellular stores (i.e., STZ), or influx of Ca^{2+} from the extracellular compartment (ionophores), results in release of AA. The discovery of additive actions of such agents has suggested a potential "dual" regulation of PLA_2 by PKC and/or Ca^{2+} (4). A common denominator for PKC and Ca^{2+} mobilization may be the activation of inositol phospholipid-specific PLC. Cleavage of inositol phospholipids by PLC produces diacylglycerol, an endogenous PKC activator, and certain inositol phosphates, including 1,4,5-$InsP_3$, that may act as Ca^{2+}-mobilizing agents (7).

Human monocyte function may be initiated in vitro by many physiologic and pharmacologic stimuli. Con A, which induces mitogenesis in lymphocytes, has also been shown to stimulate arachidonate release from murine macrophages (8) and from human neutrophils (9). However, relatively little information regarding Con A-stimulated arachidonate metabolism in monocytes is available. No studies have specifically addressed the possible release of 5-LO products subsequent to Con A stimulation. In an attempt to further understand the molecular regulation of PLA_2 and 5-LO in human monocytes, we compared aspects of arachidonate metabolism in response to Con A treatment with that observed after exposure to other agents. By elucidating the effects of diverse stimuli on these related but distinct pathways, we intended to gain insight into alternative ways of activating and regulating monocyte function.

MATERIALS AND METHODS

Cells. Peripheral blood monocytes were prepared at the National Institutes of Health Clinical Center by countercurrent centrifugal elutriation using techniques that are designed to yield large numbers of purified monocytes suitable for clinical use. Briefly, PBMC from leukapheresis were isolated by using an automated Ficoll-Hypaque density gradient centrifugation (10). Elutriation was performed in a model J-6M centrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JE-5.0 rotor operating at 1440 × *g*. Monocytes were collected in HBSS without Ca^{2+} and Mg^{2+} at a flow rate of 160 ml/min. Monocytes obtained by this method were greater than 90% pure as assessed by histochemical staining. Contaminating cells consisted mainly of lymphocytes and some (<2%) granulocytes; platelets were not a source of contamination.

Reagents. AA, Con A (type IV), cytochrome *c*, zymosan, EGTA, BSA, and PMA were purchased from Sigma Chemical Co. (St. Louis, MO). Ionomycin and staurosporine were purchased from Calbiochem (San Diego, CA). Earle's balanced salt solution was obtained from GIBCO (Grand Island, NY). Iscove's medium was from Whittaker M. A. Bioproducts (Walkersville, MD). [5,6,8,9,11,12,14,15-^{3}H(N)]AA (60 to 100 Ci/mmol) was bought from New England Nuclear (Boston, MA). MK-886 (11) was a generous gift from Dr. A. Ford-Hutchinson, Merck Frosst, Canada.

STZ was prepared by incubating 5 mg of washed, sonicated zymosan particles in 5 ml of human AB serum (GIBCO) for 30 min at 37°C. After three washes with Earle's balanced salt solution, particles were resuspended in 10 ml of medium and stored at 4°C before use.

Arachidonate release. Peripheral blood monocytes (4×10^6) were incubated for 4 h at 37°C in 24-well tissue culture plates (Costar, Cambridge, MA) containing 2 ml of Iscove's medium (BSA, 1 mg/ml) with 2 μCi of [^{3}H]AA. At the end of the incubation, they were washed three times with Earle's balanced salt solution and the medium was replaced with 500 μl of fresh medium containing stimuli at the concentrations indicated. After an additional 2 h at 37°C, the medium was removed and its radioactivity determined by liquid scintillation counting in an LKB-Wallace 1218 Rackbeta counter calibrated for dpm measurement by external standard ratio. In some cases, an aliquot was analyzed for labeled eicosanoids by reverse-phase HPLC and in-line radioactivity detection (4).

Separation of inositol phosphates by HPLC. Radiolabeled inositol phosphate isomers and derivatives were separated from the TCA-soluble fraction of stimulated monocytes that had been labeled overnight with ^{3}H-*myo*-inositol, as described previously (12). HPLC was performed with a Waters Millipore system (Milford, MA) employing a 250 × 4.6-mm, 5-μm Supelcosil LC-8-DB (Supelco, Bellefonte, PA) column and a mobile phase containing hexadecyltrimethylammonium hydroxide as a surfactant (12).

RIA. Monocyte supernatants were prepared in 24-well plates as described above except for omission of labeled arachidonate and incubation in 1.0 ml of medium. Samples were stored at −20°C before analysis. PGE_2 or LTB_4 assays were carried out by using RIA kits obtained from New England Nuclear. Assays were performed according to the manufacturer's instructions using standard curves constructed in Iscove's medium/BSA.

Assay of 5-LO. 5-LO activity was determined in 0.5 ml-reaction volumes containing 0.1 M Tris-HCl, 3 mM $CaCl_2$, 1.6 mM EDTA, 2 mM ATP, 1 mM dithiothreitol, 100 μM AA, 2 μM 15-hydroperoxyeicosadienoic acid, 30 mM potassium phosphate, 2.5% glycerol, and the protein sample at a final pH of 7.5. After incubation at 37°C for 10 min, an equal volume of internal standard (13-hydroxy-linolenic acid) in ethanol was added to stop the reaction, the samples were centrifuged for 5 min at 12,000 × *g* to remove precipitated protein, and the supernatants were analyzed by reverse-phase HPLC, as described previously (13), with a flow rate of 1.2 ml/min. Under these conditions, 5-HETE and 5-(S)-hydroperoxy-6,8,11,14-eicosatatraenoic acid are the major 5-LO products. For these experiments, the activity is expressed as nmol of product produced per mg of protein.

Statistical analysis. Analysis of variance was performed by using Duncan's multiple range test of the "means" utility of Bright Stat Pak (Bright Software, New Brunswick, NJ). In comparing values from different donors, log-transformed data were always used.

RESULTS

AA release from peripheral blood monocytes in response to different stimuli. Monocytes incubated with [^{3}H]AA, took up 90% of the label within 4 h. After thorough washing, monocytes released a fraction of the incorporated label over a 2-h period. Previous studies (data not shown) indicated that spontaneous or stimulated release was maximal at this time point. In a fashion similar to PMA, ionomycin, or STZ, Con A (30 μg/ml) enhanced labeled AA release (data not shown). As demonstrated previously, monocytes stimulated with PMA, ionomycin, or STZ release most of the incorporated label as unmetabolized arachidonate (4). To assess whether Con A activated the same or different pathways of AA metabolism as did these other stimuli, the supernatants of Con A-treated monocytes were analyzed by HPLC. Chromatograms from Con A-stimulated monocytes demonstrated the presence of cyclo-oxygenase products, including 12-L-hydroxy-5,8,10-heptadecatrienoic acid, thromboxane B_2, 6-keto-$PGF_{1\alpha}$, and PGE_2 as the principal metabolites. 15-HETE was also detected, but 5-lipoxygenase products, including 5-HETE and leukotrienes, were not seen (Fig. 1). This profile was indistinguishable from those obtained with supernatants of PMA-stimulated monocytes and was in contrast to those that included 5-HETE, LTB_4, and LTC_4 when ionomycin or STZ were used as stimuli (4).

To verify the pattern of cyclo-oxygenase or lipoxygenase stimulation, representative products of each pathway were assessed by RIA (Fig. 2). All stimuli including Con A, which induced [^{3}H]AA release, also induced abundant PGE_2 release, ranging from 10- to 30-fold over that seen in unchallenged cells. Ionomycin or STZ stimulated formation and release of the greatest amounts of LTB_4 (83- and 18-fold over unstimulated cells, respectively). PMA or Con A stimulation resulted in minimal, albeit statistically significant, LTB_4 release (five- and fourfold, respectively; $p < 0.05$).

Role of extracellular Ca^{2+} in AA release in response to Con A stimulation. Con A can induce AA release and, as published previously (14), induces increased intracel-

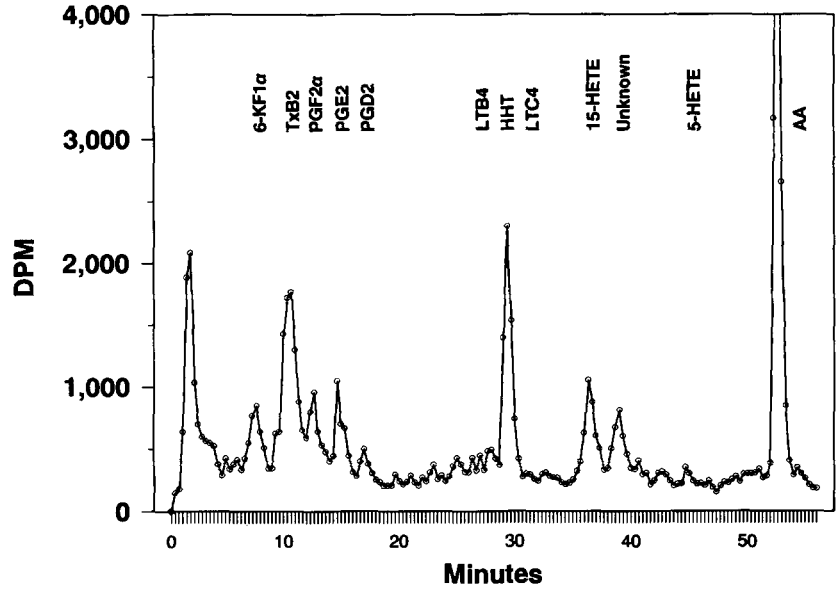


Figure 1. Chromatogram from supernatants of monocytes stimulated with Con A. Monocytes (4×10^6) were incubated at 37°C in Iscove's medium containing 1 mg/ml BSA (*Isc/BSA*) and 1 μCi/ml [^{3}H]AA for 4 h. The cells were washed three times with cold Earle's balanced salt solution. Fresh medium (250 μl) was added, which contained Con A (30 μg/ml). After a 2-h incubation at 37°C, the medium was removed and 50 μl were counted in a β-counter and the remainder (200,000 dpm) injected directly onto a reverse-phase HPLC column as described previously (4). Retention times of authentic standards are as indicated. *6KF1α*, 6-keto-$PGF_{1\alpha}$; *TxB2*, thromboxane B_2; *HHT*, 12-L-hydroxy-5,8,10-heptadecatrienoic acid.

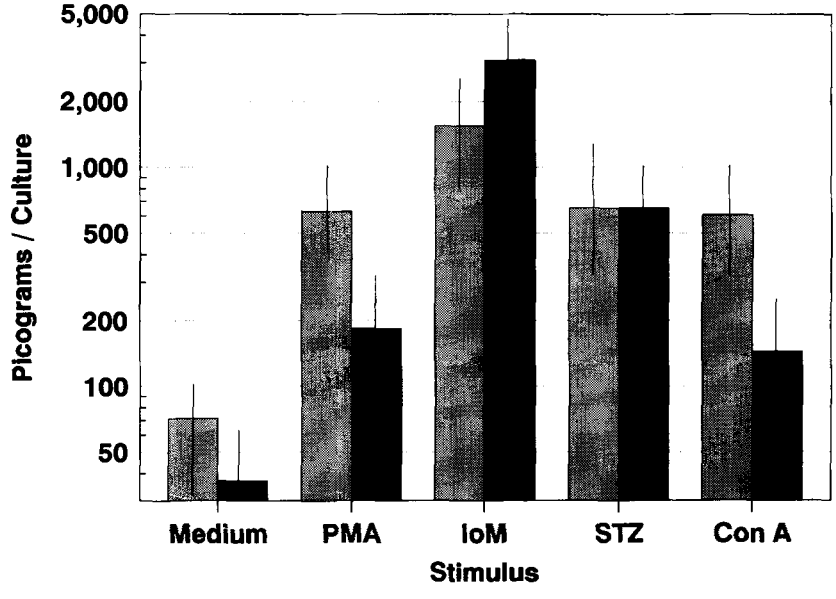


Figure 2. PGE_2 and LTB_4 release from stimulated monocytes. Monocytes were cultured for 2 h as described in *Materials and Methods* and the legend to Figure 1, in 500 μl of medium alone or with stimuli (20 nM PMA, 2 μM ionomycin, 300 μg/ml STZ, 30 μg/ml Con A) without having been labeled with [^{3}H]AA. Appropriate aliquots of supernatants, i.e., whose values for inhibition of antibody binding fell along the standard curve for PGE_2 (20 μl, *light bars*) or LTB_4 (100 μl, *dark bars*), were analyzed by RIA. The data shown represent the mean values for monocytes from 19 consecutive normal blood donors. Error bars denote the upper and lower limits of the 95% confidence interval.

lular Ca^{2+}. Therefore, it was of interest to determine whether the mechanism of action of Con A conformed to the model of PLA_2 activation derived from observations on STZ-mediated release in which increased intracellular Ca^{2+} plays a key role (4). Monocytes were incubated in medium prepared without Ca^{2+} and Mg^{2+}. Residual Ca^{2+} in the tissue culture medium used for these experiments was chelated with EGTA at doses that were nontoxic. Con A stimulation followed the same pattern seen with other stimuli except PMA, which is known to act mainly independently of Ca^{2+} (Table I). Con A stimulation of AA release depended on the presence of extracellular calcium, but not on the presence of magnesium. The reduction of AA release in the absence of extracellular calcium was not due to a failure of Con A to bind to the monocyte surface. Flow cytometry showed that fluoresceinated Con A was detected equivalently on monocytes in the presence or absence of Ca^{2+} (EGTA in the medium; data not shown).

Additional experiments evaluated the release of immunoreactive PGE_2 and LTB_4 under conditions of extracellular calcium depletion. The absence of free calcium resulted in greatly diminished PGE_2 release in response to Con A (Fig. 3). This observation was similar to that seen after STZ or ionomycin treatment, but differed from PMA-mediated release, which remained intact. As expected from previous studies (4) delineating the exquisite dependence of 5-LO activity on calcium from the extracellular medium, LTB_4 release was not augmented under conditions of extracellular calcium chelation when any of the stimuli were employed.

Con A induction of phosphatidylinositol hydrolysis by monocytes. Intracellular calcium may be raised by influx of Ca^{2+} from the extracellular compartment and mobilization from cellular stores (7). The generation of $InsP_3$ and higher inositol phosphates by the action of PLC may result in increased intracellular calcium via either mechanism. For example, exposure to STZ results in PtdIns turnover in monocytes, including hydrolysis of $PtdInsP_2$, which generates $InsP_3$ (12, 15) raising intracellular Ca^{2+} sufficiently to activate PLA_2 and 5-LO (4). In comparison, monocytes labeled with [^{3}H]inositol and

TABLE I

Effect of presence or absence of extracellular calcium or magnesium on [^{3}H]AA release by human monocytes[a]

Stimulus	Medium	Medium + EGTA	Medium + EGTA + Mg^{2+}	Medium + EGTA + Ca^{2+}
None	7,920[b]	8,798	5,543	7,317
PMA	19,901	16,501 (−36)[c]	13,802 (−31)	16,802 (−21)
Ionomycin	17,704	9,620 (−92)	5,832 (−97)	16,000 (−11)
STZ	17,361	10,997 (−77)	7,231 (−82)	17,436 (+7)
Con A	20,482	10,505 (−86)	7,317 (−86)	21,796 (+15)

[a] Monocytes were labeled with [^{3}H]AA in Iscove's medium/BSA according to the protocol described in the legend to Figure 1. After three washes in Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution, fresh medium containing stimulating agents at the concentrations noted was added. The EGTA concentration used was 0.5 mM. Calcium or magnesium chloride was added to achieve a free divalent ion concentration of 0.4 mM. Free calcium and magnesium were calculated by using the CHELATE program.

[b] The data shown are the mean dpm/culture of three replicates that varied less than 5%. They are from one representative experiment of three performed with this protocol, which differed only in the basal level of [^{3}h]AA release.

[c] Numbers in parentheses indicate the percentage of change with respect to cells stimulated in complete medium after subtraction of values obtained from unstimulated controls.

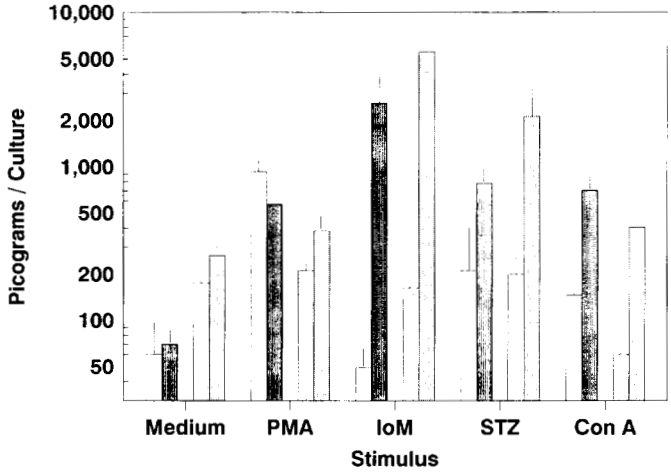


Figure 3. Effect of calcium in the extracellular medium on PGE_2 and LTB_4 release by stimulated monocytes. Monocytes were "sham labeled" in Iscove's medium/BSA according to the protocol described in the legend to Figure 1. After three washes in Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution, 1 ml of fresh medium containing stimulating agents at the concentrations noted in Figure 2 was added. RIA was carried out as described in *Materials and Methods*. *Solid bars*, PGE_2 release; *stippled bars*, LTB_4 release; *light bars*, Iscove's medium/BSA without Ca^{2+} and containing EGTA (0.5 mM); *darker bars*, Iscove's medium/BSA without Ca^{2+} and Mg^{2+}, EGTA, and added Mg^{2+} and Ca^{2+} to achieve 0.4 mM final concentration. *IoM*, ionomycin. The data shown are the means and SD from three experiments performed with this protocol.

stimulated with Con A exhibited hydrolysis of inositol phospholipids that resulted principally in the increased generation of inositol monophosphates (Fig. 4). The degree of PtdIns hydrolysis seen was substantially less than that induced by exposure to STZ. Repeated efforts to detect $InsP_3$ generation in Con A-stimulated monocytes by means of conventional anion exchange chromatography were also unsuccessful (data not shown).

Combined stimulation of monocytes with Con A and other stimuli that induce AA release. The generation of inositol phosphates subsequent to Con A treatment implied that diacylglycerols were concurrently produced. The calcium dependence of Con A effects with respect to AA release suggested that products other than $InsP_3$ or $InsP_3$-derived metabolites presumably mobilized calcium from extracellular sources. It was therefore of interest to see whether combining Con A with a potent PKC activator (PMA), or ionophore (ionomycin), or a ligand that induces PLC activation and $PtdInsP_2$ hydrolysis (STZ) might enhance eicosanoid release.

Combined treatment with Con A and STZ (Fig. 5*A*) or ionomycin (Fig. 6*A*) resulted in no additional PGE_2 release over that observed in response to either stimulus alone. Con A in combination with PMA (6 nM, Fig. 7*A*) did result in synergistic effects on PG release consistent with dual regulation of PLA_2 by Ca^{2+} and PKC activation.

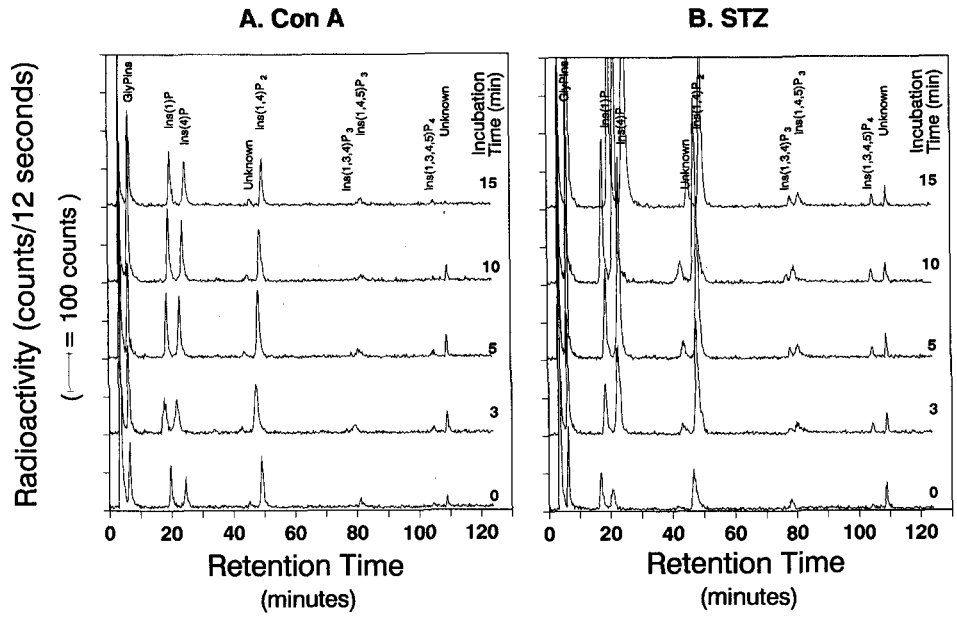
When the effect of Con A on LTB_4 release was studied in the presence of STZ (Fig. 5*B*) or ionomycin (Fig. 6*B*), each of which alone induced considerable LTB_4 release, Con A provided no additional effect (Fig. 5*B*). PMA and Con A, each of which failed to result in LTB_4 release comparable with that induced by ionomycin or STZ, resulted in full release when combined at the highest doses of each agent (20 to 60 nM PMA, 10 to 30 μg/ml Con A, Fig. 8*B*). To ensure that the ability of PMA and Con A in combination to induce 5-LO activation was not limited to monocytes from a certain subset of normal individuals, eight donors' monocytes were tested for their ability to respond to combined stimulation. In all subjects, the PMA/Con A combination was effective in inducing augmented release of LTB_4. Statistical analysis of variance indicated that although PMA or Con A alone failed to induce LTB_4 release, the combination resulted in release that was significant (Table II). The amount of LTB_4 released in response to PMA/Con A did not differ from that induced by ionophore or STZ treatment (data not shown).

Inhibition of PMA/Con A-induced LTB_4 release by MK-886. The observation that PMA/Con A could induce LTB_4 release raised the possibility that these stimuli were affecting pathways different from those stimulated by zymosan or ionomycin. We therefore carried out experiments employing MK-886, which inhibits 5-LO product formation in vitro and in vivo (11). This agent binds to FLAP (16). Pretreatment with 3 μM MK-886 had no effect on PGE_2 release, but it strongly inhibited PMA/Con A-induced LTB_4 release; the degree of inhibition was similar to that exerted on ionomycin- or STZ-mediated LTB_4 release (Fig. 8).

Effects of PMA/Con A on 5-LO translocation. Leukotriene synthesis has been associated with translocation of 5-LO from a cytosolic compartment to the membrane in ionophore-stimulated leukocytes (13). The failure to detect levels of LTB_4 comparable with those achieved by ionomycin or STZ treatment in supernatants of monocytes stimulated with Con A or PMA alone could be attributed to a failure to induce translocation of the enzyme. However, when resting or stimulated monocytes were analyzed for 5-LO enzyme activity (Fig. 9) or 5-LO immunoreactivity (Western analysis, data not shown), it was found that PMA or Con A, alone or in combination, induced a loss of 5-LO from the cytosol. In all cases, approximately 40 to 80% of 5-LO activity was lost from the cytosol after stimulation. Con A stimulation resulted in significantly less loss of 5-LO activity than that observed with the other stimuli.

Cell-associated PGE_2 and LTB_4 after in vitro stimulation of monocytes. The minimal release of LTB_4 from monocytes exposed to Con A or PMA, except in combi-

Figure 4. Chromatographic profile of inositol phosphates from stimulated monocytes. Monocytes (10×10^6) were incubated at 37°C in inositol-free medium containing BSA (1 mg/ml) and 16 μCi/ml ^{3}H-*myo*-inositol. After 12 h, the medium was removed and the cells were washed three times in PBS and resuspended at a concentration of 10^6/100 μl in fresh complete medium containing 10 mM LiCl. Medium (100 μl) containing Con A (60 μg/ml), STZ (600 μg/ml), or no stimulus were added and the cells incubated at 37°C for the times indicated. The reaction was terminated at the times indicated on the vertical scale by the addition of ice-cold TCA (12%, final w/v). After centrifugation at 15,000 rpm for 15 min, the supernatant was removed, TCA-extracted with a mixture of freon and tri-*N*-octylamine, dried under vacuum, reconstituted in 100 μl of deionized water, and analyzed by HPLC, as described previously (11). The chromatograms shown were obtained from injection of samples consisting of equivalent dpm and are representative of three experiments that differed only in the degree of inositol phospholipid hydrolysis induced by STZ during its time course.



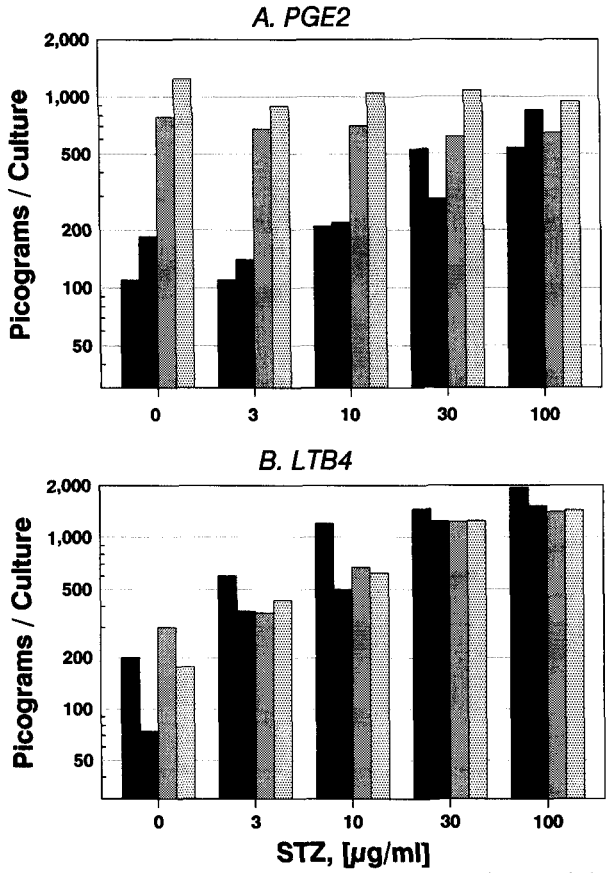


Figure 5. PGE_2 and LTB_4 release by monocytes stimulated with STZ and Con A. Monocytes were stimulated according to the protocol described in the legend to Figure 2 except that cultures were initiated by adding one-half volumes of each stimulus or its dilution to achieve the concentrations indicated. Distributions from left to right represent Con A addition at 0, 3, 10, or 30 μg/ml (final concentration). RIA was carried out as described in *Materials and Methods*. The data shown are representative of two experiments that differed solely in the basal level of PGE_2 and LTB_4 released.

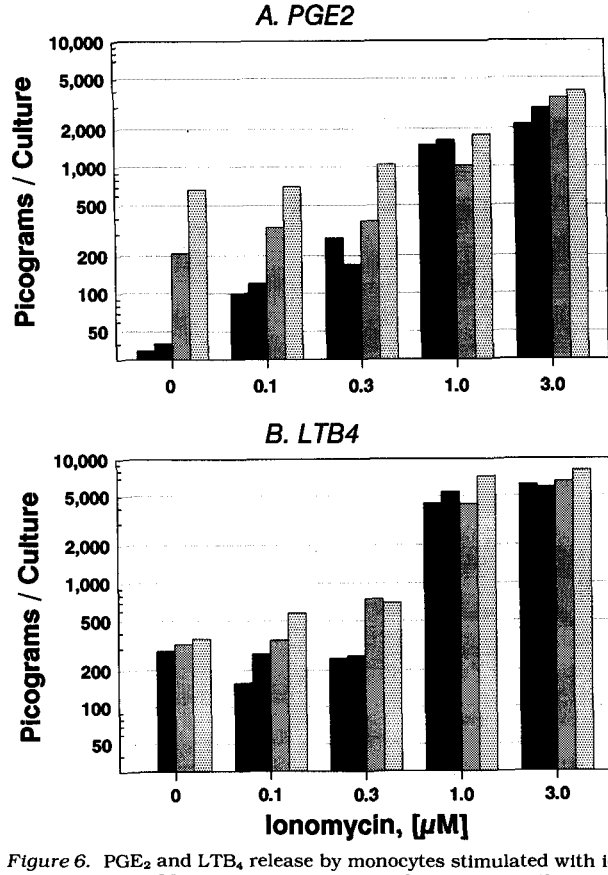


Figure 6. PGE_2 and LTB_4 release by monocytes stimulated with ionomycin and Con A. Monocytes were stimulated according to the protocol described in the legend to Figure 5 in separate experiments from those described therein. Distributions from left to right represent Con A stimulation at 0, 3, 10, or 30 μg/ml (final concentration). The data shown are representative of two that differed solely in the basal level of PGE_2 and LTB_4 released.

nation, despite evidence for translocation of 5-LO, raised the possibility that leukotriene synthesis was ongoing but that release was impaired. To address this possibility, stimulated monocytes were exposed to repeated cycles of freeze-thawing after supernatant removal and the lysates were analyzed for eicosanoids by RIA. All stimuli tested increased cell-associated PGE_2 comparably to their effects on supernatant PGE_2. Forty to 73% of the total PGE_2 was found in the supernatant. In contrast to results observed when only supernatants were analyzed, all

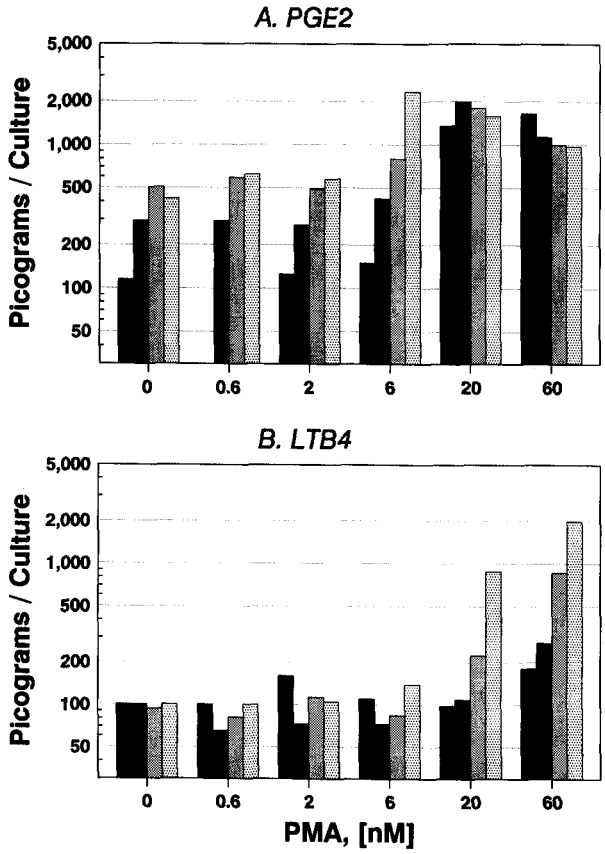


Figure 7. PGE_2 and LTB_4 release by monocytes stimulated with PMA and Con A. Monocytes were stimulated according to the protocol described in the legends to Figures 5 and 6 in separate experiments from those described therein. Distributions from left to right represent Con A addition at 0, 3, 10, or 30 μg/ml (final concentration). The data shown are representative of two that differed solely in the basal level of PGE_2 and LTB_4 released.

stimuli also induced increases in cell-associated LTB_4. Of particular note was the degree of stimulated total LTB_4 production in response to PMA. Despite this increase, only 16% was released into the supernatant, which stood in contrast to the proportions seen for PGE_2 in response to PMA. Con A also increased total LTB_4 production, although to a lesser extent than the other stimuli employed, and also resulted in a low proportion of release (22%) compared with both other stimuli and PGE_2 release induced by Con A itself. PMA and Con A in combination resulted in an additive effect on total LTB_4 synthesis, but synergized with respect to release of LTB_4 into the supernatant. This resulted in release into the supernatant comparable with that obtained with ionophore or STZ as stimuli, and in a similar proportion (Fig. 10).

When cell-associated LTB_4 was measured in the presence of MK-886, no increase was observed in response to any stimulus (data not shown), indicating that FLAP regulates 5-LO activation and is not exclusively involved in mediating release of formed product.

DISCUSSION

Con A treatment of human monocytes in vitro liberates arachidonate leading to the formation and release of cyclo-oxygenase products, including PGE_2, as do other stimuli. Arachidonate liberation induced by Con A depends on extracellular calcium, but not magnesium. Con A-mediated arachidonate release is associated with PtdIns hydrolysis, which is limited to PtdIns or PtdInsP. Con A induces translocation of 5-LO from the cytosol to the membrane of monocytes but maximal LTB_4 release only occurs in combination with PMA stimulation.

The Ca^{2+}-dependence of Con A effects on AA release is consistent with the finding that the major regulation of AA release occurs via PLA_2 activation by calcium (4–6). PLA_2 activation, arachidonate release, and PGE_2 (as an example of a cyclo-oxygenase metabolite) release are close to maximal when a relatively small calcium rise, such as that induced by Con A (14), is achieved.

Con A treatment of monocytes induces PtdIns and PtdInsP hydrolysis in human monocytes, but no evidence of $PtdInsP_2$-derived products was obtained. These results, which are similar to the effects of Con A on neutrophils (17), stand in contrast to observations made with other ligands, such as FMLP (9) or STZ (12, 15), which induce formation of $Ins(1,4,5)P_3$. The basis for this difference is presently unknown. Possibly, Con A receptors may be coupled to PLC with access to or specificity for PtdIns or

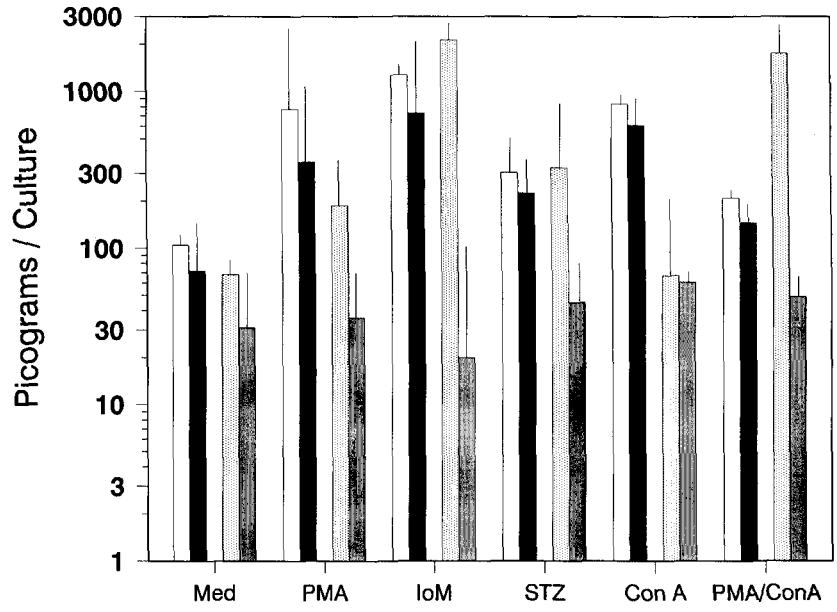


Figure 8. Effect of MK-886 on PGE_2 and LTB_4 release from stimulated monocytes. Monocytes were pre-incubated at 37°C for 16 min with 500 μl of medium containing 3 μM MK-886 (*dark bars*) or no drug (*light bars*). An additional 500 μl of medium containing stimuli at twice the desired final concentration were added and the cultures incubated for an additional 2 h. The supernatants were harvested and assayed for PGE_2 (*solid bars*) and LTB_4 (*stippled bars*) by RIA. The drug had no effect on the performance of the RIA. The data shown are the means ± 1 SD of three separate experiments.

TABLE II

PGE_2 or LTB_4 release by human monocytes stimulated by PMA and Con A

Monocytes from eight normal blood donors were elutriated and stimulated as described in *Materials and Methods*. Supernatants were harvested and analyzed for PGE_2 or LTB_4 by RIA.

Stimulus	PGE_2 (pg/culture)	LTB_4 (pg/culture)
None	32^{Aab} (14–78)[c]	34^{A} (13–91)
PMA (20 nM)	302^{B} (138–661)	141^{A} (98–200)
Con A (30 μg/ml)	259^{B} (107–617)	105^{A} (59–186)
PMA + Con A	603^{B} (224–1622)	1122^{B} (603–2042)

[a] Data are the geometric means.

[b] Superscripts indicate statistically significant differences from other groups in the same column (i. e., groups with a letter in common are not different) analysis of variance.

[c] Data in parentheses represent the 95% confidence intervals.

PtdInsP only. Another possibility that would partially explain this phenomenon might be found in inhibition or lack of concomitant stimulation of PtdInsP kinase activity attendant to Con A treatment. At this time, however, no precedent for either of these explanations exists.

Despite the absence of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ production, Con A treatment results in Ca^{2+} mobilization. Previous studies (14) and our findings regarding extracellular calcium chelation support the conclusion that increased intracellular calcium arises from influx from the extracellular compartment. By analogy to neutrophils, phosphatidic acid formed from diacylglycerol by the action of diacylglycerol kinase (18) or, alternatively, via phospholipase D activation (19), may be a candidate for mediating this increase in intracellular Ca^{2+}.

The diacylglycerol generated along with inositol phosphates (or potentially from hydrolysis of other phospholipids) upon PLC activation makes a role for PKC activation in AA release in response to Con A potentially relevant as well, particularly in view of the fact that phorbol esters per se stimulate PGE_2 release. Furthermore, chelation of extracellular Ca^{2+} did not totally abrogate labeled AA release or PGE_2 release, lending further support to the concept of "dual" regulation of PLA_2 activation by calcium-dependent and calcium-independent mechanisms (4). However, the degree of PKC involvement in eicosanoid release induced by Con A may be more limited than in the case of STZ stimulation, as suggested by the relatively greater susceptibility of the Con A effect to extracellular calcium chelation. This is consistent with the observation that Con A is a poor stimulus of monocyte superoxide release (20), a PKC-dependent process.

Despite its ability to mobilize Ca^{2+}, Con A resembles PMA in terms of inducing minimal LTB_4 release. This finding agrees with previous studies (4–6) that have demonstrated little or no release of 5-LO products from a variety of cells, unless calcium ionophores or zymosan was used as the stimulus. These findings indicated that PKC activation per se was insufficient to trigger sustained LTB_4 release and suggested that an increase in Ca^{2+}, comparable with that induced by ionomycin or STZ-mediated $InsP(1,4,5)_3$ and $Ins(1,3,4,5)P_4$ generation, was necessary. However, the finding of small, but statistically significant, amounts of LTB_4 in the supernatants of PMA- or Con A-stimulated monocytes when a relatively large number of donors were studied indicated that LTB_4 release may be controlled at multiple steps. This hypothesis was also prompted by the finding that 5-LO translocation was induced to a significant degree by either PMA or Con A, indicating that enzyme activation was sufficiently triggered by PKC activation alone. Furthermore, increased intracellular LTB_4 concentrations were found in monocytes treated with Con A or PMA, despite the small amounts released. Thus, the possibility was raised that synthesis and extrusion of LTB_4 may be controlled independently, as has been observed in neutrophils stimulated with unopsonized zymosan (21).

Combined treatment with high doses of Con A and PMA lent further credence to this hypothesis, as it induced LTB_4 release comparable with that seen when ionomycin or STZ was used. Precedents exist for synergistic effects of PKC activation by phorbol esters and increased intracellular Ca^{2+} in response to calcium ionophores in leukotriene production by mouse macrophages (22) and human neutrophils (23). The effect in these studies was ascribed to increased liberation of arachidonate, but this

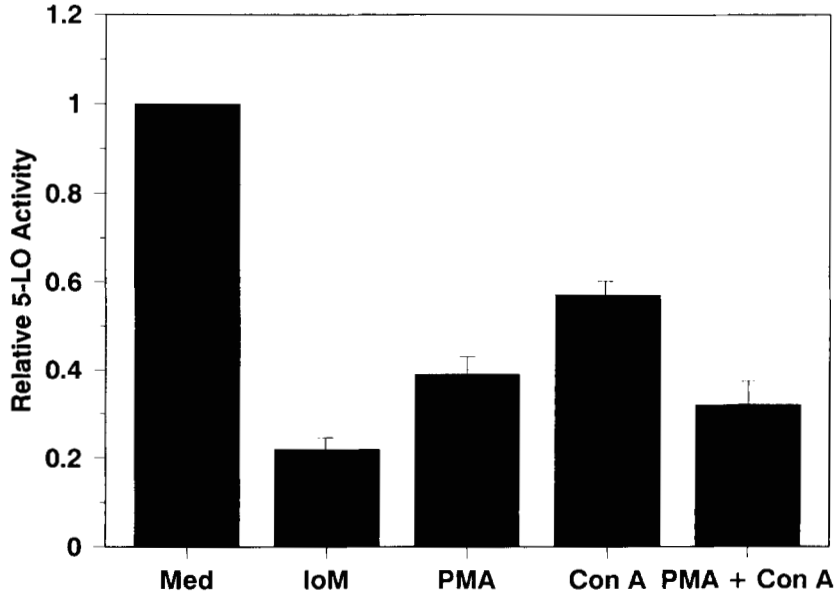


Figure 9. 5-LO analyses in cytosolic and membrane fractions from stimulated monocytes. Elutriated monocytes (30×10^6) in 10 ml Iscove's medium/BSA were stimulated with ionomycin (*IoM*, 1 μM), PMA (20 nM), Con A (30 μg/ml), or PMA/Con A combination for 30 min at 37°C in 50-ml polypropylene tubes. The reaction was stopped with 40 ml of ice-cold Earle's balanced salt solution. The cells were washed twice with cold Earle's balanced salt solution, the supernatant was discarded, and the pellet was snap-frozen in liquid nitrogen vapor. Frozen cell pellets were thawed on ice in homogenization buffer (50 mM potassium phosphate buffer (pH 7.1), 0.1 M NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, and 60 μg/ml soybean trypsin inhibitor) and were disrupted by sonication as described previously (13). Cell sonicates were centrifuged at $10,000 \times g$ for 15 min at 4°C, and the resulting supernatants were further centrifuged at $100,000 \times g$ for 60 min at 4°C. Supernatants (100S) were then aspirated and samples kept on ice until assay for 5-LO activity as described in *Materials and Methods*. Relative activity shown is compared with that of $100,000 \times g$ supernatants of unstimulated monocytes (*Med*), which was set as 1.0. Data depicted are the means ± 1 SEM of four separate experiments.

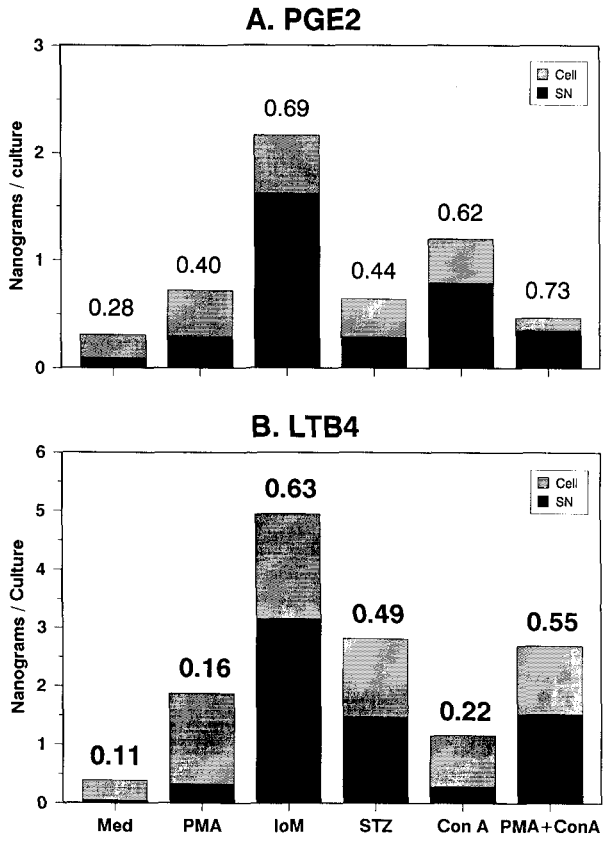


Figure 10. Cell-associated and supernatant PGE_2 and LTB_4 from stimulated monocytes. Monocytes were stimulated as described in the legends to previous figures. The supernatants were removed and aliquots stored for RIA. One ml of distilled water was added and the culture plate was snap-frozen in liquid nitrogen vapor. After 1 min, the plate was transferred to a 37°C incubator for 30 min. The cycle was repeated twice and monitored for loss of cell integrity by microscopic inspection. The lysates were subsequently stored for RIA analysis in the same assay as the supernatant samples. Data shown are the means of four experiments using monocytes from consecutive blood donors. *Bars* represent total eicosanoids detected with the darker portion denoting the amount in the supernatant, the lighter portion the amount in the cells. Numerical values denote the proportion of total eicosanoid production found in the supernatant. *IoM*, ionomycin.

explanation does not necessarily apply here since either PMA or Con A stimulation resulted in PLA_2 activation sufficient for abundant AA release and PGE_2 synthesis. The synergy between Con A and PMA does not seem to occur at the level of 5-LO since either stimulus induced translocation, and the combination resulted in minimal enhancement. An additional possibility entertained was that one or another of the two stimuli might activate or modify FLAP, since this protein has recently been shown to be necessary for LTB_4 synthesis (16). We have identified FLAP by immunoblot in the 100,000 × *g* supernatants of elutriated monocytes (our unpublished observations); however, each stimulus, alone or in combination, was similarly inhibited by MK-886. Thus, the synergistic effect of PMA and Con A appears to occur predominantly at the level of release. Possibly, the signal necessary for increased release of LTB_4 provided by Con A could be a small rise in intracellular calcium, since combined PMA/Con A stimulation failed to result in LTB_4 release when extracellular Ca^{2+} was chelated (our unpublished observations).

The results of our study are consistent with the conclusion that leukotriene formation and release may be a multi-step process. Previously, Ca^{2+}-mediated translocation of 5-LO to the membrane has been shown to accompany cellular leukotriene synthesis (13). The results reported here, that PMA induces translocation comparable with that seen with calcium ionophore and that LTB_4 is synthesized when PMA is the stimulus, indicate that non-calcium-mediated translocation may also occur, probably via activation of PKC. Furthermore, in contrast to PGE_2, a large proportion of which is released in response to all stimuli, LTB_4 is found primarily in the cell-associated fraction when the stimulus is PMA or Con A alone. Thus, the degree of PKC activation in concert with the level of intracellular Ca^{2+} rise may in turn determine the quantities of leukotrienes synthesized and eventually released. Regulation of LTB_4 release may therefore involve diverse mechanisms, including control of LTB_4 binding to cellular receptors that are associated with cytoskeletal structures (24).

Although Con A is not a "physiologic" stimulus of monocyte function, it is conceivable that foreign proteins or other ligands could aggregate surface receptors on monocytes and produce similar effects. The findings observed with Con A as an in vitro stimulus further support the regulation of PLA_2 and other enzymes of eicosanoid metabolism by a balance between PKC activation and increased intracellular calcium. It is conceivable that multiple monocyte activators could result in pathophysiologic consequences to the host that may not occur unless they are encountered in combination. For example, leukotriene production and its attendant inflammatory or allergic symptomatology may occur only after multiple diverse stimuli or agents with all the conditions required for leukotriene synthesis and release are met.

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