Interleukin 4 Receptor Signaling in Human Monocytes and U937 Cells Involves the Activation of a Phosphatidylcholine-Specific Phospholipase C: A Comparison with Chemotactic Peptide, FMLP, Phospholipase D, and Sphingomyelinase

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Summary

Interleukin 4 (IL-4) diminishes cytokine activation of human macrophage. IL-4 binding to monocyte IL-4R is associated with protein kinase C (PKC) translocation to a nuclear fraction. The cleavage of diacylglycerol (DAG), an activator of PKC, from membrane phospholipids was investigated to define the proximal events of IL-4R signaling. IL-4 induced a statistically significant time- and dose-dependent generation of DAG. The IL-4–triggered production of DAG was not derived from phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, since neither cytosolic calcium flux nor liberation of inositol phosphates was detected in response to IL-4. Experiments were performed using [^{14}C-methyl]choline–labeled U937 cells and monocytes to determine whether IL-4R activated phospholipase C (PLC), PLD, or PLA_2 to use membrane phosphatidylcholine (PC) to form DAG. IL-4 induced a time- and dose-dependent increase of phosphocholine (pchol) with concomitant degradation of membrane PC ($p < 0.05$ compared with control). The finding that the peak reduction of PC was equivalent to peak production of pchol suggested that IL-4R signaling involved the activation of a PC-specific PLC. Changes in choline (chol) or lyso-PC and glycerolphosphocholine, the respective products of PC cleavage by PLD or PLA_2, were not detected in IL-4–treated cells. In contrast, exogenous PLD induced an increase in chol and concomitant loss of membrane PC. Additional investigation suggested that IL-4R signaling does not involve PLD. In cells labeled with L-lyso-3-PC 1-[1-^{14}C]palmitoyl, PLD but not IL-4, increased the production of phosphatidic acid (PA) and phosphatidyl-ethanol when pretreated with ethanol. Propanolol, an inhibitor of phosphatidate phosphohydrolase, and calyculin A, a phosphatase 1 and 2A inhibitor, blocked DAG production in response to FMLP but not to IL-4. In propranolol pretreated cells, PMA but not IL-4 triggered the production of PA and lowered the amount of DAG. Evidence that PLA_2 is not coupled to IL-4R is the detection of arachidonate production in response to FMLP but not to IL-4. Furthermore, IL-4R is not coupled to sphingomyelinase (SMase) since IL-4, unlike exogenous SMase, did not generate ceramide but induced the hydrolysis of PC to pchol that was comparable to exogenous PLC. In summary, IL-4R signaling in monocytes and U937 cells involves PLC and not PLD, PLA_2, or SMase, and it uses PC and not PIP_2 to form DAG.

IL-4 is a T lymphocyte–derived cytokine. Although IL-4 was originally described as a B cell growth and differentiation factor, it has pleiotropic effects on multiple cells (1–3). IL-4 plays a role in the pathogenesis of leishmaniasis (4–7), downmodulates the immune response in parasitic and retroviral infection (7), and is involved in Th-2 cell function (8). Macrophages express IL-4R, and their functions are modulated by this cytokine. Investigations reported by our laboratory and others have demonstrated that IL-4 is a potent inhibitor of macrophage activation by IFN-γ, TNF-α, IL-1, IL-3, and GM-CSF for anti-leishmanial activity and oxidative burst capacity (9). IL-4 has also been shown, in monocytes, to inhibit the induction of IL-1 and TNF-α by LPS (10, 11). Since IL-4 affects many macrophage cell functions, investigations

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were initiated to examine the early events of IL-4R signal transduction.

The interaction between an agonist, such as cytokine, and its receptor triggers a cascade of second messengers that link extracellular signals to changes in cell function. Protein kinase C (PKC)[1] is one of several second messengers involved in receptor signal transduction (12, 13). Upon agonist stimulation, PKC is activated by 1,2-diacylglycerol (DAG) (13). DAG is derived from hydrolysis of either phosphatidylinositol (PIP_2) or phosphatidylcholine (PC) by phospholipases (14–17), or from exchange of phosphocholine (pchol) between PC and ceramide (18, 19). Activated PKC transfers PO_4 from ATP to threonine and serine residues on proteins, which alters their function (13).

Although IL-4R has been cloned, the signal transduction pathway linking the IL-4R is not well defined (20). PKC has been reported in signal transduction of epidermal growth factor, platelet-derived growth factor, insulin-like growth factor 1, TNF-α, IL-1, -2, -3, IFN-γ and -α, and M-CSF or CSF-1 (for reviews see references 21 and 22). The mechanism by which some PKC is activated remains incompletely defined, whereas others have been shown to involve PC-specific phospholipase C (PLC; 21, 22). We have recently demonstrated that IL-4 binding to its receptor on human monocytes involves PKC activation and translocation to a nuclear fraction (22). To characterize further the association of PKC activation with IL-4R signaling, we investigated whether DAG was derived from PIP_2 or PC hydrolysis, and determined which phospholipase was activated by IL-4R.

Materials and Methods

Cells and their Isolation. Human peripheral blood cells obtained by Hypaque-Ficoll density centrifugation and adherence to plastic are ~99% monocytes (9). Human PMN from healthy donors were isolated as previously described, by Ficoll-Hypaque density centrifugation and dextran sedimentation (23). Human monomyelocytic cells, U937, were obtained from the American Type Culture Collection (Rockville, MD) (CRL 1593), and maintained in complete medium (RPMI 1640 containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 10 mM L-glutamine, and Hepes, pH 7.2). Murine CTLL cells, stably transfected with human (hu)-IL-4R and propagated in complete medium containing IL-4 (25 ng/ml), were a generous gift of Dr. K. Grabstein (Immunex Research Corporation, Seattle, WA; 9, 20).

1,2-DAG Radioenzymatic Assay. Cells, treated with medium or medium containing stimuli and quickly chilled (4°C) on ice with added normal saline, were pelleted by centrifugation (300 *g* for 10 min). The cell pellets were extracted with chloroform/methanol (1:1 vol/vol) to which 1/4 volume of 10 mM EDTA was added to break phase, and the lower phase (total vol, 1.2 ml) containing DAG and phospholipids (PL) was used to quantitate DAG and PLs. For DAG quantitation, 800 μl of the lower phase was evaporated under nitrogen (24). *Escherichia coli* DAG kinase (Lipidex, Westfield, NJ) was used to catalyze the transfer of $^{32}PO_4$ from γ-[^{32}P]ATP to endogenous DAG, converting it to [^{32}P]phosphatidic acid (PA) at equimolar ratio (25–27). The dried samples were solubilized in 20 μl octyl-β-D-glucoside cardiolipin solution by sonication, followed by the addition of 50 μl 2× reaction buffer, 10 μl dithiothreitol, and 10 μl DAG kinase to a total volume of 90 μl. The 2× reaction buffer is composed of (in mM): 100 imidazole, 100 NaCl, 25 $MgCl_2$, and 2 EGTA, pH 6.6. The addition of 10 μl γ-[^{32}P]ATP (sp act, 30 Ci/mM; Amersham Life Sciences, Arlington Heights, IL) and ATP (0.34 mM) mixture initiated the reaction at room temperature for 30 min. The reaction was stopped by the addition of 3 ml chloroform/methanol (1:2, vol/vol). After the removal of methanol, a two-step extraction was performed by the addition of 1 ml chloroform and 0.75 ml NaCl (1 M) with vortex, followed by 1 ml chloroform and 1 ml NaCl with vortex. Phase separation occurred after centrifugation at 500 *g* for 2 min. After measuring the volume of the chloroform phase, a 0.4-ml sample was removed and dried under nitrogen. The samples were dissolved in chloroform (40 μl), and 30 μl of each sample was spotted onto TLC plates (24, 26, 27) (Whatman Inc., Clifton, NJ). *E. coli* DAG kinase is stereospecific for *sn*-1,2-DAGs and does not detect 2,3- or 1,3-DAG (26).

The PL were separated using chloroform/glacial acetic acid (130:30:10 vol/vol/vol). Radioactive spots visualized by autoradiography corresponding to DAG standard were collected, dissolved in scintillation solution and [^{32}P]PA, and quantitated by β-emission using a liquid scintillation counter (model 1214 Rackbeta®; Pharmacia Inc., Gaithersburg, MD). The amount of 1,2-DAG present was calculated from a standard curve using 1,2-DAG (in nM) 10, 3, 1, 0.3, 0.1, and 0.03 (26, 27) (Avanti Polar Lipids Inc., Alabaster, AL).

E. coli DAG kinase also catalyzes the phosphorylation of ceramide (26). Ceramide (Sigma Chemical Co., St. Louis, MO) in amounts of ≤10 ng, admixed with 1,2-DAG in amounts of ≤10 nM, did not compete for phosphorylation. Furthermore, the resolution of phosphorylated 1,2-DAG and ceramide was not effected and, typically, they separated from each other by at least 4 cm on TLC (data not shown).

PL was quantitated by the phosphate assay of Ames and Dubin (26–28) using 200 μl of the lower phase of the chloroform/methanol/EDTA extract of the cell pellet. The amount of DAG produced is expressed as pmol/μg of PL (26, 27). Total PL in each sample was 1 ± 0.2 μg (SD). The percentage of control in DAG was calculated as 100 × [(experimental in pmol per μg of PL)/(control in pmol per μg of PL)].

Intracellular Ionized Calcium Measurements. Monocytes, U937 cells, monocyte-depleted (mixed) B and T cells, and murine CTLL cell line stably transfected with human IL-4R (CTLL-hu-IL4-R) were loaded with the fluorescent intracellular Ca^{2+} chelator dye, Fura-2, by incubating cells with 3 μM Fura-2 acetoxymethyl ester (Fura-2-AM) (Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C in Fura buffer as previously described (29–31). Fura buffer is composed of (in mM): 140 NaCl, 2.5 KCl, 2 $CaCl_2$, 5 $MgCl_2$, 5 glucose, and 10 Hepes, pH 7.2. Fura-2-AM is metabolized to Fura-2, a membrane impermeant form, by cytosolic nonspecific esterase. After loading, the cells were washed once and resuspended in Fura buffer containing 1 mM $CaCl_2$. Ca^{2+}-dependent fluorescence was measured in a Photon Counting Spectrofluorometer (model 8000C; SLM-AMINCO, Urbana, IL), interfaced with an

[1] *Abbreviations used in this paper:* chol, choline; DAG, diacylglycerol; Fura-2-AM, fura-2 acetaxymethyl ester; GPC, glycerolphosphocholine; IP_1, inositol phosphate; IP_2, inositol biphosphate; IP_3, inositol 1,4,5-triphosphate; LPC, lyso-phosphatidylcholine; LTB_4, leukotriene B_4; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; pchol, phosphocholine; PEt, phosphatidyl-ethanol; PIP_2, phosphatidylinositol 4,5-biphosphate; PKC, protein kinase C; PL, phospholipid; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; SM, sphingomyelin; SMase, sphingomyelinase.

IBM PS/2 computer using the Fura-2 software (SLM). Experiments were performed at room temperature using 2 × 10^6 cells per sample. The excitation wavelengths were 340 and 380 nm and the emission wavelength was 510 nm. Cytosolic Ca^{2+} flux in response to IL-4 (10–50 ng/ml), ionomycin (1 μM), *N*-FMLP, or leukotriene B_2 (LTB_4) was determined by measuring the excitation ratio of 340/380 for Fura-2 (excitation wavelength for the Ca^{2+} saturated form is 340 nm, and for the Ca^{2+}-free Fura-2, 380 nm). Intracellular Ca^{2+} concentration was calculated with the following equation (29): $[Ca^{2+}]_i = K_d \times F_{sig} \times [(R - R_{min})/(R_{max} - R)]$, where K_d is the effective dissociation constant, R is the relative fluorescence ratio of 340/380 at any time point; R_{min} and R_{max} are, respectively, the minimum and maximum fluorescence ratio of 340/380 for minimum Ca^{2+} after chelation by EGTA and maximum in which all dye has bound Ca^{2+}; F_{sig} is the bound signal fluorescence ratio of 340/380.

Inositol Phosphates Measurement. Monocytes (2 × 10^6 in duplicate) were labeled with myo-2-[^{3}H]inositol (2 μCi/ml, Amersham Life Sciences) in complete medium containing 2% AB serum for 72 h. The cells (16 × 125-mm glass tubes) were resuspended in fresh medium after centrifugation at 250 *g* for 10 min, and equilibrated with 10 mM LiCl for 1 h [32], 37°C before stimulation with FMLP (50 μM), ionomycin (10 μM), and IL-4 (50 ng/ml). The reaction was stopped with ice cold methanol and the samples were placed on dry ice. Released inositols were extracted from cells with chloroform/methanol/HCl/ (100:100:1, vol/vol/vol) (33, 34). Pooled upper phase was dried with N_2 and resuspended in 3 ml ion-free H_2O. Inositol phosphates were separated by 50% (wt/vol) slurry of Dowex-1 (0.5 ml, 100–200 mesh. A-61X-5) column. After loading the sample onto the column, the sample was eluted with 5 mM myo-inositol/0.1 M formic acid. Inositol phosphates, IP_1, IP_2, and IP_3, respectively, were eluted from the column by stepwise addition (each 6 ml) of 0.2, 0.4, and 0.8 M ammonium formate (33). The eluants were dissolved in scintillant and counted by β-emission for calculation of total, and for each form of inositol phosphate.

Measurement of Membrane PL and Metabolites. U937 cells and monocytes were cultured with complete medium containing 2% AB serum and methyl-[^{14}C]choline (chol) (1 μCi/ml, sp act, 56.4 mCi/mmol; Amersham Life Sciences) as previously described (21). Cells were serum starved for 4 h in complete medium supplemented with 2% BSA. Aliquots of cells (in 12 × 75-mm glass tubes) were suspended in PBS, pH 7.2, and treated with PBS or PBS containing IL-4 at 37°C. In other experiments, the effect of IL-4 was compared with exogenously added phospholipases (Sigma Chemical Co.): PC-specific PLC (5 × 10^{-2} U/ml, *Bacillus cereus*, [P6135]) (35), sphingomyelinase (SMase; 10^{-3} U/ml, *Staphylococcus aureus*) (36, 37), and phospholipase D (PLD; 0.5 U/ml, *Streptomyces chromofuscus*).

The reaction was stopped by immersion of the tubes in methanol/dry ice (−70°C) for 10 s. Cells were centrifuged in a microfuge to obtain supernatant and pellet and processed as previously described (21). The supernatants were collected for determination of extracellular water soluble methyl-[^{14}C]chol metabolites. The pellet was resuspended in cold $CH_3OH/CHCl_3/H_2O$ (2.5:1.25:1, vol/vol/vol), vortexed, and separated into aqueous and organic phases containing the respective cell-associated [^{14}C]chol metabolites and ^{14}C-PL (21). Aliquots of the aqueous and lipid phases were dried under nitrogen and resuspended in 50% ethanol and chloroform, respectively. The water-soluble fractions were separated on thin-layer silica plates using the following separation system: CH_3OH/0.5% NaCl/NH_4OH (100:100:2, vol/vol/vol). PL were separated by TLC using a solvent system containing $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (100:60:20:5; vol/vol/vol/vol) (21, 38). Glycerolphosphocholine (GPC), pchol, chol, PC, sphingomyelin (SM) and LPC (Sigma Chemical Co.) were used as standards. The resolved products were visualized in iodine vapor. Autoradiography was performed to determine the location of each metabolite. Metabolites corresponding to the standards were cut out, dissolved in scintillant, and counted by β-emission.

Measurement of PA, Phosphatidyl-Ethanol (PEt) and DAG for PLD Activity. Serum-starved U937 cells were labeled for 2 h with L-lyso-3-PC, 1-[1-^{14}C]palmitoyl at 1 mCi/ml (sp act, 56.8 mCi/mmol, Amersham Life Sciences) as previously described (21). U937 cells, untreated or treated with 1% ethanol for 2 min or with 200 μM propranolol for 5 min were incubated with medium or medium containing 100 ng/ml IL-4 for indicated times. Cells were equally divided for quantitation of PA or PEt and DAG. ^{14}C-labeled PEt or ^{14}C-labeled PA was separated by TLC and quantitated by the method described for membrane PL and metabolites. In the split cell sample, DAG was quantitated by *E. coli* kinase.

Effect of PA Phosphohydrolase (PAP) and Protein Phosphatase Inhibitors. Human monocytes, U937 cells, and PMN were either not treated or treated with propranolol (200 μM) (39) or calyculin A (100 nM) (40, 41), and stimulated with either medium or medium containing IL-4 (10 ng/ml) or FMLP (5 μM). The generation of DAG at 10 min of stimulation was measured and expressed as a percentage of control (medium). In additional experiments, serum-starved U937 cells labeled for 2 h with L-lyso-3-PC, 1-[1-^{14}C] palmitoyl at 1 mCi/ml were either untreated or treated with 200 μM propanolol for 5 min and incubated with medium or medium containing 100 ng/ml IL-4. The ^{14}C-labeled PA produced from these conditions was quantitated.

Measurements of Arachidonate. Monocytes obtained by differential adherence and harvested by a rubber policeman were plated onto 13-mm glass coverslips in 24-well plates (2 × 10^6/ml/well). After incubation for 20 h at 37°C in 5% CO_2, monocytes were labeled with [^{3}H]arachidonic acid (1 μCi/well, 100 Ci/mmol, New England Nuclear, Bedford, MA) for 20 h at 37°C (42, 43). Unincorporated [^{3}H]arachidonate was removed by washing with warm saline containing 1% BSA. After the addition of fresh medium, cells were equilibrated for 10 min at 37°C before the addition of medium or medium containing stimuli into duplicate or triplicate wells. Supernatant (0.5 ml) was removed at the indicated time, and centrifuged at 500 *g* for 1 min to pellet any detached cells. The amount of [^{3}H]arachidonic acid released was determined by counting for β-emission in scintillant. Stimulus-induced release of arachidonic acid was expressed as a percentage of control (medium).

Statistical Analysis. Student's *t* test of paired samples was used, and values are expressed as mean ± SEM unless otherwise indicated. *n* equals the number of individual experiments performed using different donors or cell lines from different culture dates.

Results

IL-4 Induced a Time-dependent Increase in the Production of 1,2-DAG. IL-4R signaling involves PKC translocation. Since PKC is activated by DAG, the production of DAG in response to IL-4 was examined. IL-4 induced a time-dependent increase in production of 1,2-DAG for both U937 human promonocytic cells and monocytes. Fig. 1 *A* illustrates the production of DAG from U937 promonocytic cells. Basal DAG was low and IL-4 (10 ng/ml) induced a time-dependent increase in the generation of DAG that reached 335 ± 36% of control at 10 min. Statistically significant increase in DAG

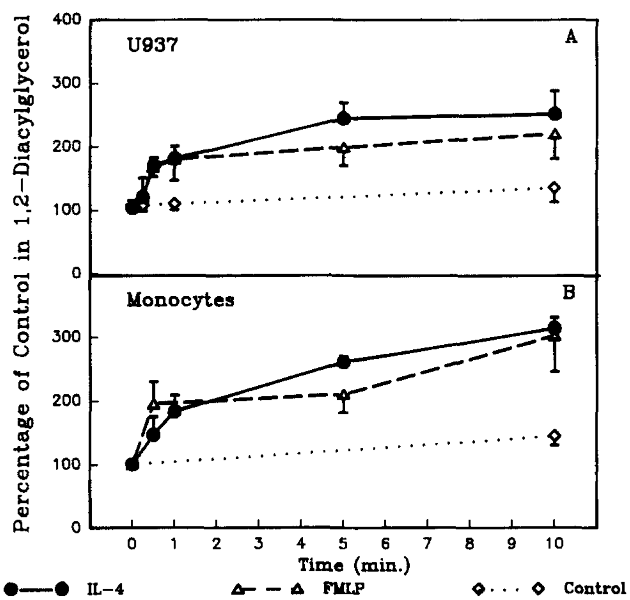

Figure 1. Production of 1,2-DAG by human U937 promonocytic cells and monocytes induced by IL-4 (10 ng/ml) and FMLP (5 μM) expressed as percentage of control. (*A*) Production of DAG by U937 cells. Basal DAG for U937 was 134 ± 44 pmol/μg of PL. Exposure to IL-4 (10 ng/ml) at 37°C for 0.25, 0.5, 1, 5, and 10 min induced DAG production, respectively, 213 ± 45, 290 ± 55*, 322 ± 50*, 420 ± 59*, 449 ± 43* pmol/μg PL, compared to basal DAG, *p <0.05 (Student's t test, n = 5). (*B*) Production of DAG by monocytes. Basal DAG was 286 ± 28 pmol/μg of PL. In response to IL-4 (10 ng/ml) DAG production at 0.5, 1, 5, and 10 min was 435 ± 62*, 538 ± 66*, 752 ± 48*, 891 ± 46* pmol/μg PL, compared to basal DAG (*p <0.05, paired Student's t test; mean ± SEM, n = 4).

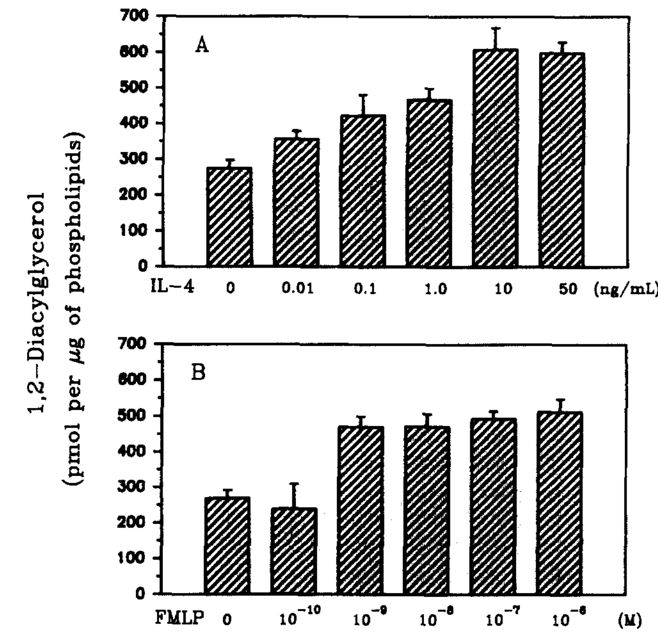

Figure 2. Production of DAG (pmol/μg of PL) by U937 cells in response to increasing concentrations of IL-4 and FMLP. IL-4 and FMLP induced a dose-dependent increase in production of DAG for 10 min at 37°C. Maximal increase in DAG observed with IL-4 at 10 ng/ml corresponds to the peak dose-effect on functional and PKC response of macrophages to IL-4. The stepwise increase in DAG was statistically significant for IL-4 doses >0.01 ng/ml and FMLP doses >10^{-10} M (p <0.05; mean ± SEM, n = 4).

was noted for stimulation of ⩾0.5 min (compared to basal DAG, $p < 0.05$, Student's t test, n = 5–8). Fig. 1 *B* illustrates the production of DAG by human monocytes. Basal DAG was 286 ± 28 pmol/μg PL, and IL-4 (10 ng/ml) induced a time-dependent increase in the amounts of DAG that rose to 314 ± 17% at 10 min. Statistically significant increase was demonstrated for an exposure time of ⩾1 min (compared to basal DAG, p <0.05, Student's t test, n = 4–6). FMLP, another receptor ligand, induced a similar time-dependent increase in DAG in U937 cells and monocytes (Fig. 1, *A* and *B*).

The production of DAG in response to increasing concentrations of IL-4 and FMLP was studied to provide further evidence for receptor-associated generation of DAG. Fig. 2 illustrates the generation of DAG by U937 cells in response to IL-4 (*A*) and FMLP (*B*). IL-4 and FMLP induced a dose-dependent increase in the production of DAG. Maximal increase in DAG of 625 ± 25 pmol/μg PL (248% above medium) was observed with IL-4 at 10 ng/ml, a dose that corresponds to the reported peak functional and PKC response in IL-4–treated macrophages (23). The stepwise increase in DAG was statistically significant for IL-4 doses >0.01 ng/ml and FMLP doses > 0.1 nM (n = 4, p < 0.05).

IL-4 R Signaling Does Not Involve Cytosolic-free Calcium Flux. Many ligand receptors on phagocytes are coupled to PLC that hydrolyzes PIP_2 to inositol triphosphate (IP_3) and DAG (14, 16–18). The generation of IP_3 leads to the release of calcium from intracellular stores or to entry of extracellular calcium from plasma membrane channels resulting in an elevation of cytosolic-free calcium (14). Exposure to IL-4 (10–50 ng/ml) over a recording time of 10 min had no effect on calcium levels in monocytes, mixed B and T cells, U937 cells, and CTLL-hu-IL-4R cells suspended in medium containing 1 mM or higher of Ca^{2+}. As illustrated in Fig. 3, monocytes, mixed B and T cells, U937 cells, and CTLL-hu-IL-4R cells remained responsive to ionomycin (1 μM) with measured peak cytosolic-free calcium ($[Ca^{2+}]_i$) flux that was respectively 500, 380, 553, and 429% above basal (n = 3–10). The peak $[Ca^{2+}]_i$ flux was not altered by pretreatment with IL-4.

To further define that Fura-2–loaded cells remained responsive to receptor stimuli, $[Ca^{2+}]_i$ flux of monocytes and U937 cells in response to FMLP, and LTB_4 were compared with ionomycin. Monocytes responded to FMLP, LTB_4, and ionomycin with a $[Ca^{2+}]_i$ flux that was 255, 230, and 451% of basal (Table 1). Similarly, stimulation of U937 cells with FMLP and ionomycin resulted in peak $[Ca^{2+}]_i$ flux of 234 and 445% of basal. Therefore, monocytes and U937 remained responsive to other receptor ligands even though IL-4 did not trigger a $[Ca^{2+}]_i$ flux.

IL-4R Signaling Does Not Involve the Generation of Inositol Phosphates. To further evaluate whether PIP_2 may be used for IL-4R signaling, the generation of inositol phosphates

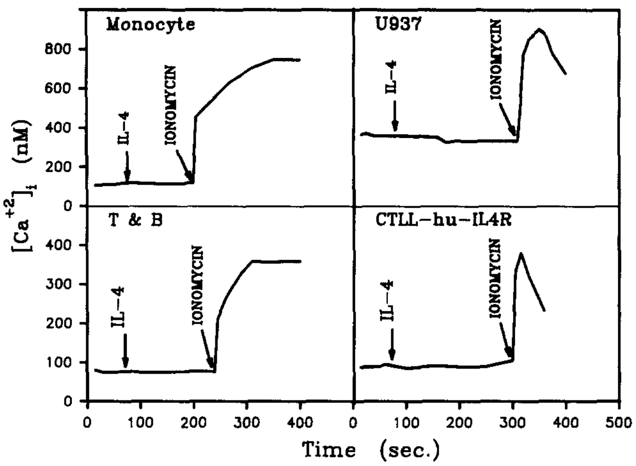

Figure 3. Intracellular calcium homeostasis in response to IL-4 and calcium ionophore, ionomycin. Representative experiments of monocytes, U937 cells, mixed B and T cells, and murine CTLL-hu-IL-4R cells loaded with Fura-2 and stimulated with medium or medium containing IL-4 and ionomycin 1 μM, are presented. Basal cytosolic ionized calcium $[Ca^{2+}]_i$ for monocytes, U937 cells, B and T cells, and murine CTLL-hu-IL-4R cells was, respectively, 144.9 ± 7.8, 90.1 ± 13.6, 113.4 ± 15.4, and 58.4 ± 5.1 nM (n = 3–10). Significant changes in $[Ca^{2+}]_i$, were not detected in response to IL-4 for each of the cell types with recordings up to 10 min (n = 3–10). For example, basal $[Ca^{2+}]_i$ for monocytes and mixed B and T cells was, respectively, 140 ± 5.5 and 91 ± 14.1. In response to IL-4 (10–50 ng/ml), increase in $[Ca^{2+}]_i$ was not observed; at 10 min the measured $[Ca^{2+}]_i$ for monocytes and mixed B and T cells was 128 ± 11.5 and 87.6 ± 17.1 (n = 9 and 5, respectively). In contrast, ionomycin (1 μM) induced a peak $[Ca^{2+}]_i$ in monocytes, B and T cells, U937 cells, and CTLL-hu-IL-4R cells that was, respectively, 720.6 ± 90.8, 342.5 ± 70.7, 625.5 ± 97.2, and 249.3 ± 29.7 nM (n = 3–10). Prior stimulation with IL-4 did not influence the detected peak calcium flux in response to ionomycin. The results are expressed as mean ± SEM.

from membrane PL was measured in monocytes prelabeled with myo-[^{3}H]inositol. FMLP (50 μM) and ionomycin (10 μM) but not IL-4 (50 ng/ml) induced the production of total inositol phosphates (Fig. 4 *A*). Similarly, treatment with FMLP and ionomycin (data not shown) resulted in a time-dependent production of IP_1, IP_2, and IP_3 but this was not observed with IL-4 (Fig. 3 *B*). The lack of detectable inositol phosphates in response to IL-4 and the detection of inositol phosphates in monocytes stimulated with FMLP, a stimulus known to elevate $[Ca^{2+}]_i$ and IP_3, suggest that PIP_2 is not a substrate for IL-4R signal transduction.

IL-4R Signaling Involves the Use of PC. Since DAG is derived from phospholipase hydrolysis of either PIP_2 or PC and cytosolic calcium flux, and inositol phosphates were not detected in response to IL-4, experiments were performed to determine whether PC is used and to define which phospholipase transduces IL-4R signal. Membrane PC was labeled with the precursor, methyl-[^{14}C]chol, to investigate whether PLC, PLD, or PLA_2 is coupled to IL-4R. If PLC is involved in IL-4R signaling, increases of pchol and unchanged amounts of chol or GPC, the respective enzymatic products of PLD and PLA_2 hydrolyses of PC would be detected upon IL-4 treatment. Similarly in response to IL-4, decreases of membrane PC and unchanged amounts of other constituents, such as SM and lyso-pchol would be observed.

U937 cells prelabeled with methyl-[^{14}C]chol were stimulated with medium or medium containing IL-4. The labeled membrane lipids and metabolites were each determined in the respective aqueous and lipid phases of cell extracts. In the aqueous phase, IL-4 induced a dose- and time-dependent increase in the production of pchol (Fig. 5, *A* and *B*). Maximal generation of pchol was observed at 5 min with IL-4 doses of 10 and 100 ng/ml, 156 and 203% of control (Fig. 5, *A* and *B*; n = 5–7, $p < 0.05$ for exposure time of ≥ 1 min). Significant changes of chol or LPC and GPC, the respective products of PLD or PLA_2 hydrolysis of PC, were not detected.

Analysis of the lipid phase of the cell extract demonstrated a concomitant dose- and time-dependent decrease in membrane PC (Fig. 6, *A* and *B*). Compared to control, the maximal changes in PC in response to IL-4 doses of 10 and 100 ng/ml were, respectively, 18 and 30%. Furthermore, IL-4 at a dose of 100 ng/ml reached a significant nadir for the maximal degradation of PC earlier than at 10 ng/ml, 15 and 60 s, respectively ($p < 0.05$ compared to control, n = 5–7). In the same cell preparations, significant changes in LPC were not demonstrated. Opposing changes in SM were observed in response to IL-4. Maximal decrease in membrane SM (26 or 74% of control) was associated with IL-4 dose of 10 ng/ml at 1 min. In contrast, treatment with 100 ng/ml of IL-4 was associated with an increase in SM (19 or 119% of control) at 1 min (Fig. 6, *A* and *B*).

Table 1. *Chemotactic Ligands Induce Cytosolic-free Calcium Flux in Human Monocytes and U937 Promonocytic Cells*

	Cytosolic free calcium (nM) in response to:			
Cells	Basal	FMLP	LTB_4	Ionomycin
Monocytes	140.6 ± 10.9	359.7 ± 24.7	334.7 ± 56.1	634 ± 90.8
U937 cells	95.0 ± 11.5	269.1 ± 84.7	ND	503.2 ± 56.3

Fura-2 loaded cells were monitored for basal calcium. A significant increase in cytosolic calcium was noted after stimulation with FMLP (0.5 μM), LTB_4 (1 μM), and ionomycin (1 μM), but not for IL-4 (50 ng/ml). The number of experiments for monocytes from different donor and U937 cells were, respectively, 4–10 and 4, p <0.05 compared with basal. The results are expressed as the mean ± SEM.

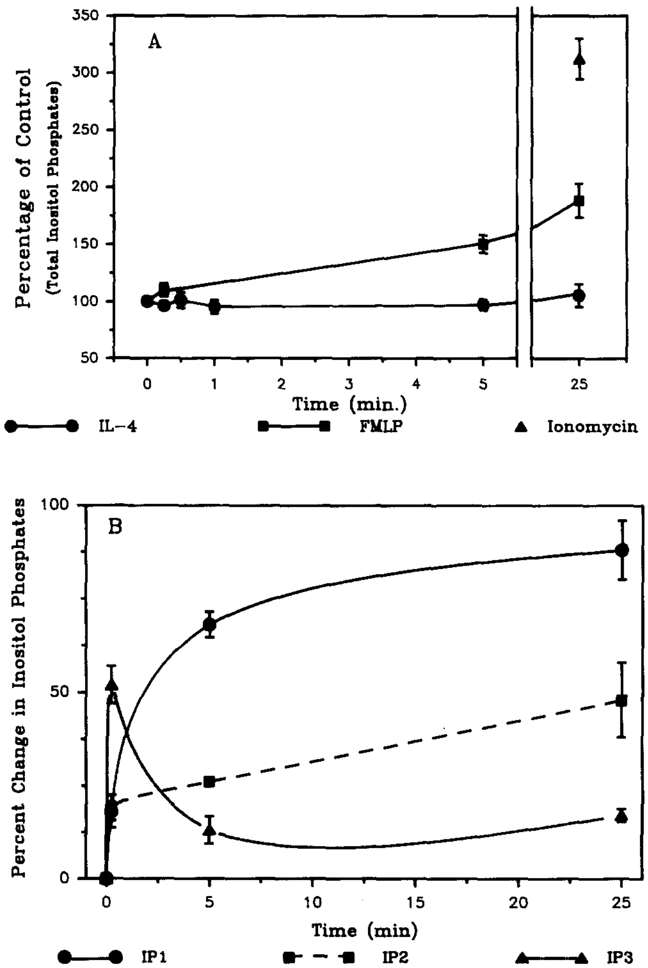

Figure 4. Production of inositol phosphates by monocytes. Monocytes previously labeled with [^{3}H]myo-inositol were stimulated with FMLP (50 μM), ionomycin (10 μM), and IL-4 (50 ng/ml). Illustrated are the generation of: (*A*) total inositol phosphates in response to three stimuli, and (*B*) IP_3, IP_2, and IP_1 in response to FMLP. Basal total inositol phosphates was 1,084 ± 175 cpm per 2 × 10^6 cells. Changes in total inositol phosphates in response to IL-4 were not detected. In contrast, the same cells generated significant increases of total inositol phosphates in response to ionomycin and FMLP at 5 and 25 min (p <0.05, mean ± SEM, n = 4). Analysis of inositol phosphates detected in response to FMLP, but not to IL-4, the initial production of IP_3, and its rapid loss was followed by an increase of IP_2 and a more sustained increase in IP_1 (mean ± SEM, n = 4).

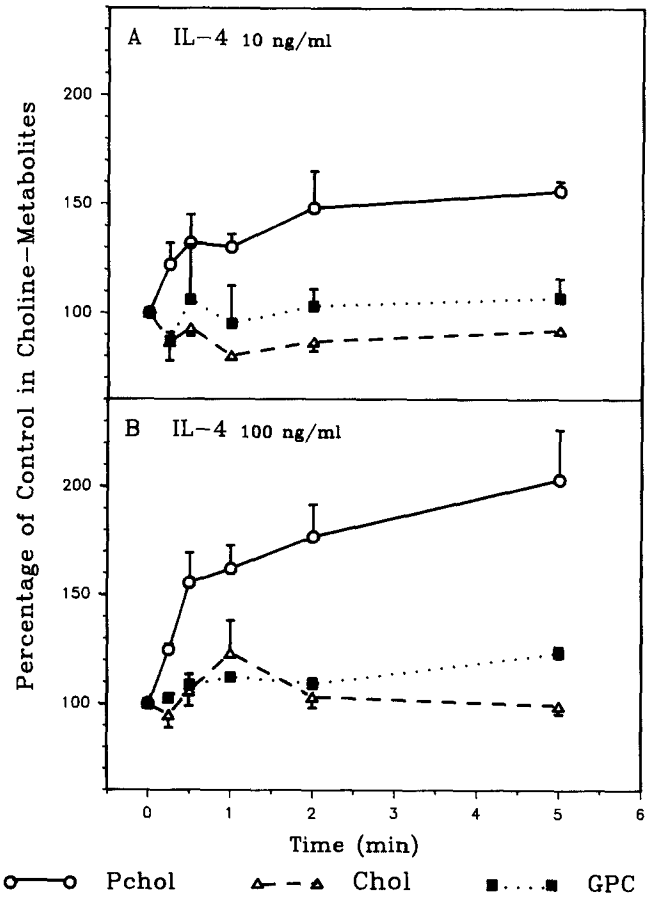

Figure 5. IL-4 induced a dose- and time-dependent increase in production of pchol. U937 cells prelabeled with methyl [^{14}C]chol (48 h) were resuspended in PBS or PBS containing IL-4. The experiment was terminated by chilling (at −70°C for 10 s), centrifugation, and extraction of the cell pellet with cold $CH_3OH/CHCl_3/H_2O$ resulting in two aqueous and one lipid phases. The aqueous phases were examined for extra- and intracellular [^{14}C]chol metabolites: pchol, chol, and GPC. Significant increases in pchol were observed for time of IL-4 exposure (10 and 100 ng/ml) of 1 min or greater (p <0.05, n = 4) but not for chol and GPC. Maximal pchol was observed at 5 min of exposure to IL-4 doses of 10 and 100 ng/ml that were, respectively, 56 and 103% change above control. The results are expressed as the mean ± SEM.

To define further whether pchol was derived from PC hydrolysis, we analyzed the incorporation of [^{14}C]chol into [^{14}C]chol lipids or metabolites and their transformation in response to IL-4. Basal incorporation of [^{14}C]chol into [^{14}C]chol lipids or metabolites in the lipid and the water soluble phases was, respectively, 167,759 ± 17,842 (± SEM) per 10^6 U937 cells and 46,178 ± 1,873 per 10^6 U937 cells. Basal PC and pchol were, respectively, 159,302 ± 1,410 and 44,155 ± 14,127 (n = 4). In response to IL-4, 10 and 100 ng/ml, peak reduction of PC (mean ± SEM) was 28,674 ± 796 and 47,790 ± 4,230 cpm/10^6 cells. The peak generation of pchol (above control) was 24,726 ± 4,796 and 45,479 ± 14,550 cpm/10^6 cells (n = 4). The amounts of PC lost being equivalent to the amount of pchol produced, suggest that pchol is derived from PC hydrolysis.

IL-4R Signaling and PLD Activity. DAG may be generated as a secondary product of PLD catabolizing PC to form PA and chol. PAP removes the phosphate group from PA, resulting in DAG (15, 17, 21). To exclude the coupling of IL-4R to PLD, we compared production of pchol and chol in response to IL-4 and exogenous PLD in [^{14}C]chol-labeled U937 cells (Table 2). PLD, but not IL-4, induced the production of chol. In contrast, IL-4 treatment led to the generation of pchol but PLD did not. Both IL-4 and PLD used PC for the generation of pchol and chol, resulting in the detected loss of PC.

To exclude further the involvement of PLD in IL-4R

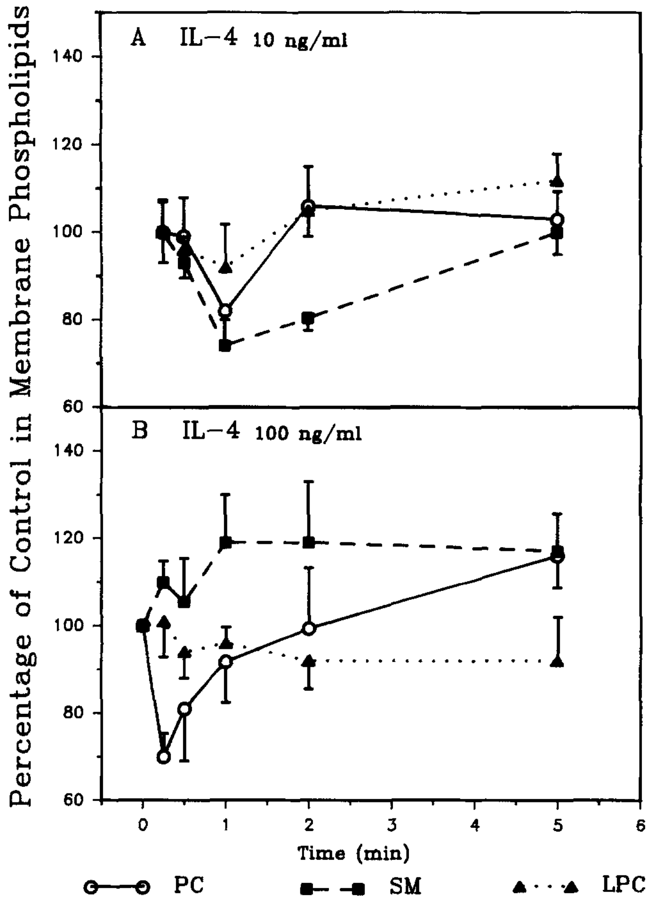

Figure 6. IL-4 induced a dose- and time-dependent decrease in membrane PC. The lipid phase of cells treated with medium or medium containing IL-4 (see Fig. 5) was examined for [14C]chol lipids, PC, LPC, and SM. Significant decreases in PC were observed for IL-4 treatment that was time- and dose-dependent. For IL-4, 10 ng/ml significant decreases in PC were observed at 1 min, and for IL-4, 100 ng/ml significant decrease was seen at 15 and 30 s ($p < 0.05$; mean ± SEM, $n = 4$). Membrane PC decrease in response to 10 and 100 ng/ml of IL-4 at 1 min and 15 s was, respectively, 18 and 30% below basal (control). Changes in LPC were not observed. In contrast, treatment with IL-4 at doses of 10 and 100 ng/ml was associated, respectively, with decrease and increase in SM.

signaling, experiments were performed using L-lyso-3-PC, 1-[1-14C]palmitoyl to label U937 cells at the glyceride portion of PC. These cells were stimulated in the presence or absence of ethanol (Table 3). Ethanol substitutes for H_2O in the PLD catalyzed cleavage of chol from PC, resulting in the formation of PEt instead of PA. As illustrated in Table 3, both IL-4 and PLD increased the population of DAG. Compared to control, treatment with PLD but not IL-4 increased PA by ~200%. Stimulating the cells with PLD and PMA in the presence of ethanol resulted in the generation of PEt and the concomitant decrease in PA. Ethanol had no effect on the production of PEt in cells stimulated with IL-4. Furthermore, ethanol reduced the amount of DAG produced in response to PMA and exogenous PLD, but had no effect on the increase in DAG triggered by IL-4.

Effect of Inhibitors of PAP on the Production of DAG. To

Table 2. *Production of Pchol and Chol by U937 Promonocytic Cells*

	Percentage of control (mean ± SEM)			
	Pchol	Chol	PC	SM
IL-4 (100 ng/ml)				
15 s	130 ± 6	102 ± 5	76 ± 4	103 ± 4
5 min	183 ± 22	103 ± 4	90 ± 5	116 ± 10
PLD (0.5 U/ml)				
15 s	99 ± 1	150 ± 2	94 ± 1	113 ± 5
5 min	108 ± 3	140 ± 4	85 ± 3	95 ± 2

U937 promonocytic cells prelabeled with methyl-[14C]chol were treated with medium or medium containing IL-4 (100 ng/ml) or PLD (0.5 U/ml, Sigma Chemical Co.,) at 37°C. The aqueous and lipid layers were extracted and the specific products were separated by TLC, identified by autoradiography with comparison to standards, and counted by β-emission. The results (mean ± SEM) of three experiments are expressed as the percentage of medium (control).

Table 3. *Production of DAG, PEt, and PA by U937 Cells*

	Percentage of control (mean ± SEM)		
	1,2-DAG	PEt	PA
IL-4 (100 ng/ml)			
5 min	224 ± 44	ND	93 ± 4
10 min	294 ± 57	ND	97 ± 2
IL-4 + 1% ethanol			
5 min	206 ± 23	101 ± 15	115 ± 7
10 min	267 ± 70	71 ± 30	100 ± 1
PMA (100 nM)			
20 min	469 ± 105	ND	278 ± 15
PMA + 1% ethanol			
20 min	141 ± 10	245 ± 90	116 ± 13
PLD (0.5 U/ml)			
5 min	247 ± 65	ND	147 ± 11
10 min	276 ± 41	ND	180 ± 10
PLD + 1% ethanol			
5 min	104 ± 1	206 ± 45	109 ± 5
10 min	79 ± 4	197 ± 45	119 ± 3

Serum-starved U937 cells were labeled for 2 h with L-lyso-3-PC, 1-[1-14C]palmitoyl at 1 mCi/ml. U937 cells untreated or treated with 1% ethanol for 2 min were incubated with medium, and medium containing IL-4 (100 ng/ml), PMA (100 nM), or PLD (0.5 U/ml) for the indicated times. Cells were equally divided for quantitation of PA or PEt, and DAG. 14C-labeled PEt or 14C-labeled PA were separated by TLC and quantitated by the method described for membrane PL and metabolites. In the split cell sample, DAG was quantitated by *E. coli* kinase. The results (mean ± SEM) of four separate experiments are expressed as the percentage of control (medium).

exclude the possibility that IL-4R might be linked to a PLD with more rapid catalytic activity than exogenous PLD, we examined the effect of propranolol and calyculin A, the respective inhibitors of PAP and protein phosphatases, on IL-4–induced production of DAG in U937 cells and monocytes. Parallel experiments using human PMN were performed to serve as controls since receptors for FMLP and C5a are coupled to PLD in human PMN, and their signaling is inhibited by propranolol and calyculin (39, 40).

Illustrated in Fig. 7 *A* is the effect of propanolol on IL-4–

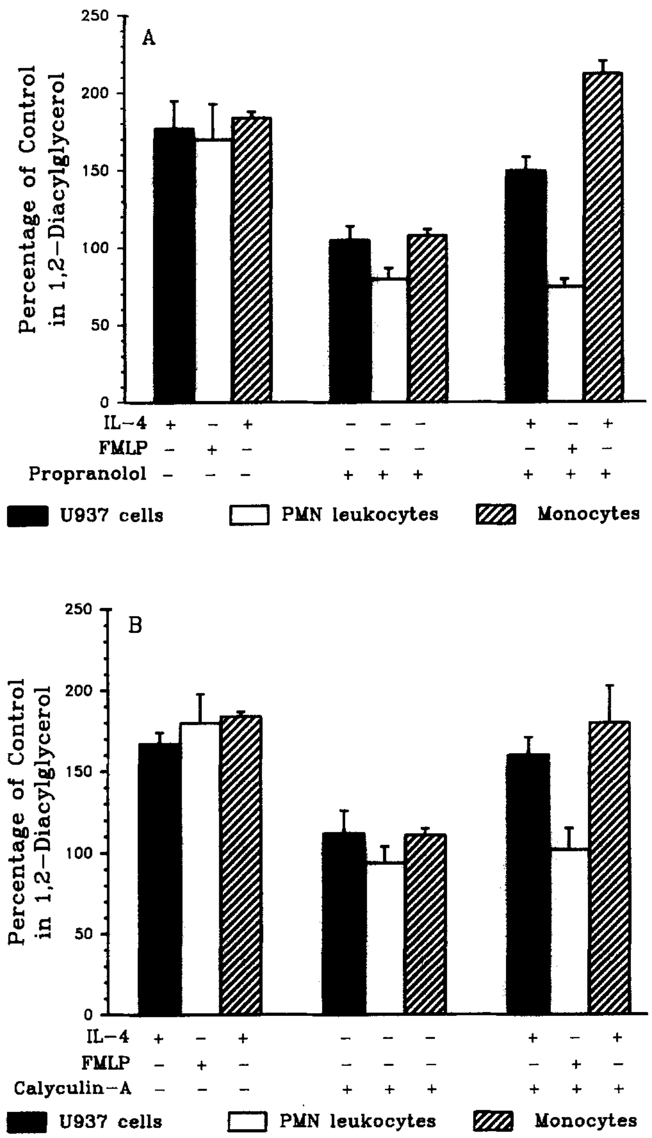

Figure 7. Effect of PAP and phosphatase 1 and 2A inhibitors on DAG production by U937 cells, PMN, and monocytes. Cells were suspended in medium or medium containing propanolol (200 μM, *A*) or calyculin A (100 nM, *B*) for 5 min at 37°C before the addition of IL-4 10 ng/ml or FMLP (5 μM) for an additional 10 min at 37°C. Propranolol and calyculin A had no effect on IL-4–induced DAG production in U937 cells and monocytes. In contrast, propranolol and calyculin A significantly inhibited PMN production of DAG in response to FMLP (p <0.05; mean ± SEM, n = 4 except monocytes n = 2).

and FMLP-stimulated production of DAG by U937 cells, monocytes, and PMN. Propanolol did not affect the IL-4–induced production of DAG by U937 cells (n = 5) and monocytes (n = 2), but abrogated the FMLP-induced generation of DAG by PMN ($p < 0.05$ propranolol with FMLP compared to FMLP, n = 5, Fig. 7 *A*). Since propranolol inhibits the activity of both PAP and PKC (39, 40), experiments were performed with calyculin A, a potent inhibitor of phosphatase 1 and 2A (40, 41) (Fig. 7 *B*). Calyculin A significantly inhibited the production of DAG induced by FMLP but not that by IL-4 ($p < 0.05$ calyculin A and FMLP compared to FMLP, n = 5).

To provide additional evidence that IL-4R is not coupled to PLD, we used a PL precursor radiolabeled at the glyceride portion of the molecule. U937 cells labeled with L-lyso-3-PC, 1-[1-^{14}C]palmitoyl were stimulated in the presence or absence of propanolol (Table 4). In the presence of propanolol, radiolabeled PA accumulated in response to PMA but not to IL-4. In addition, the amount of DAG detected was reduced in response to PMA but not to IL-4. These findings provide multiple evidence that IL-4R signaling does not involve PLD activation.

Measurement of Arachidonic Acid Generation. Receptor signaling through PLA_2 may result in the formation of DAG. PLA_2 catabolizes PC to form the intermediate metabolites GPC and LPC; PA and DAG are generated by the addition of acyl groups to glycerol 3-phosphate (15, 21). Although

Table 4. *The Effect of Propranolol on the Production of DAG and PA*

	Percentage of control (mean ± SEM)	
	1,2-DAG	PA
IL-4 (100 ng/ml)		
5 min	146 ± 15	93 ± 4
10 min	190 ± 16	97 ± 2
IL-4 + propranolol (200 μM)		
5 min	157 ± 1.4	98 ± 5
10 min	169 ± 3.5	106 ± 2
PMA (100 nM)		
20 min	205 ± 17	137 ± 2
PMA + propranolol (200 μM)		
20 min	112 ± 1	220 ± 10

Serum-starved U937 cells were labeled for 2 h with L-lyso-3-PC, 1-[1-^{14}C]palmitoyl at 1 mCi/ml. U937 cells untreated or treated with 200 μM propranolol for 5 min were incubated with medium, and medium containing 100 ng/ml IL-4 or 100 nM PMA for the indicated times. Cells were equally divided for quantitation of PA and DAG. ^{14}C-labeled PA was separated by TLC and quantitated by the method described for membrane PL and metabolites. In the split cell sample, DAG was quantitated by *E. coli* kinase. The results (mean ± SEM) of two separate experiments are expressed as the percentage of medium (control).

changes in intracellular and extracellular GPC and LPC were not detected in response to IL-4, experiments were performed to measure arachidonic acid, the first cleavage product of PLA_2. Human monocytes prelabeled with extracellular [^{3}H]arachidonate were stimulated with medium or medium containing IL-4 (50 ng/ml), FMLP (5 μM), PMA (10 μM), calcium ionophore, A23187 (5 μM), and PMA plus A23187. Fig. 8 illustrates the significant production of extracellular [^{3}H]arachidonate in response to FMLP, A23187, and PMA with A23187. In contrast, IL-4 or PMA alone had no effect on production of arachidonate. These findings suggest that IL-4R is not coupled to PLA_2 (Figs. 5, 6, and 8).

Involvement of SM Metabolism in IL-4R Signaling. That SM levels were altered in response to IL-4 led to the evaluation of SMase activity in response to IL-4 (19). Monocytes labeled with methyl-[^{14}C]chol were studied for PC and SM catabolism and pchol generation in response to IL-4 and exogenous PLC and SMase. Tables 2 and 5 indicate that IL-4 induced pchol generation and membrane PC reduction that were comparable to exogenous PC-specific PLC, without significantly reducing SM levels. Exogenous SMase reduced the amount of membrane SM and also induced slight increases of pchol and PC. We further evaluated the effect of IL-4 and exogenously added SMase on the kinetics of DAG and on ceramide generation. Exploiting the finding that *E. coli* DAC kinase phosphorylates both DAG and ceramide (27, 36, 37), the response to IL-4 and exogenously added SMase is illustrated on Table 6. IL-4 induced the generation of DAG but not ceramide. In contrast, exogenously added SMase induced the generation of ceramide that was maximal at 30 min. The

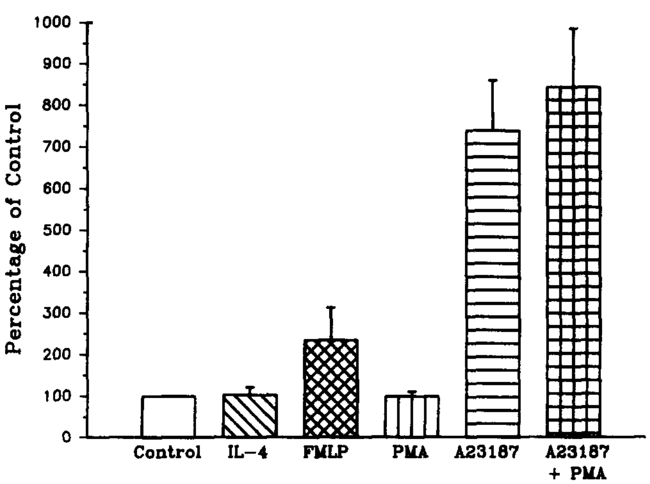

Figure 8. Stimuli-induced generation of arachidonic acid by monocytes prelabeled with [^{3}H]arachidonic acid. After washing to remove unincorporated [^{3}H]arachidonate and equilibration with fresh medium for 10 min at 37°C, medium or medium containing IL-4 (50 ng/ml), FMLP (5 μM), PMA (10 μM), and calcium ionophore (A23187, 5 μM) was added for an additional 10 min at 37°C. Release of [^{3}H]arachidonic acid into the supernatant was expressed as a percentage of medium (*Control*). Basal release of arachidonic acid from control cells was 2,041 ± 243 per well. FMLP and A23187 or A23187 with PMA, but not IL-4 and PMA, induced significant release of arachidonic acid (mean ± SEM, n = 3), $p < 0.05$.

Table 5. *Production of Pcholine from Membrane PL*

	Percentage of control (mean ± SEM)		
	Pchol	PL	SM
IL-4 (100 ng/ml)			
15 s	135.0 ± 10.0	67.6 ± 3.3	96.5 ± 2.0
5 min	201.0 ± 19.0	82.0 ± 2.0	124.0 ± 17.0
PLC			
15 s	187.0 ± 10.0	73.0 ± 6.5	95.0 ± 5.0
5 min	237.0 ± 21.0	82.0 ± 8.0	109.0 ± 12.0
SMase			
15 s	127.5 ± 1.0	115.8 ± 9.5	114.0 ± 10.0
5 min	109.5 ± 2.5	117.0 ± 10.0	89.0 ± 2.0

Monocytes prelabeled with methyl-[^{14}C]-chol and washed were treated with medium or medium containing IL-4 (100 ng/ml), PC-specific PLC (5×10^{-2} U/ml, Sigma Chemical Co.), and SMase (10^{-3} U/ml, Sigma Chemical Co.) for 15 s and 5 min at 37°C. The aqueous and lipid layers were extracted and the specific products were separated by TLC, identified by autoradiography with comparison to standards, and counted by β-emission. The results (mean ± SEM of three experiments) are expressed as the percentage of medium (control).

Table 6. *Production of DAG and Ceramide from U937 Promonocytic Cells*

	Percent of control (mean ± SEM)	
Condition and time	DAG	Ceramide
IL-4 (10 ng/ml)		
0 min	116 ± 10	96 ± 4
5 min	230 ± 2	108 ± 8
30 min	129 ± 17	89 ± 4
SMase (10^{-3} U/ml)		
0 min	119 ± 1	103 ± 3
5 min	143 ± 17	128 ± 6
30 min	120 ± 4	208 ± 13

U937 promonocytic cells were treated with medium or medium containing IL-4 (10 ng/ml), and SMase (10^{-3} U/ml) at 37°C. Termination of the assay immediately after the addition of stimuli was designated as time zero. Cells were pelleted and extracted with chloroform/methanol (1:1, vol/vol); the lower phase containing DAG, ceramide, and PL was evaporated under nitrogen. *E. coli* DAG kinase was used to catalyze the transfer of $^{32}PO_4$ from γ-[^{32}P]ATP to DAG and ceramide. The specific products and their respective standards were separated by TLC, identified by autoradiography with comparison to standards, and counted by β-emission. The results (mean ± SEM of three experiments) are expressed as the percentage of medium (control). Control DAG was 144 ± 18 pmol/μg PL, and ceramide was 53 ± 9 pmol/μg PL.

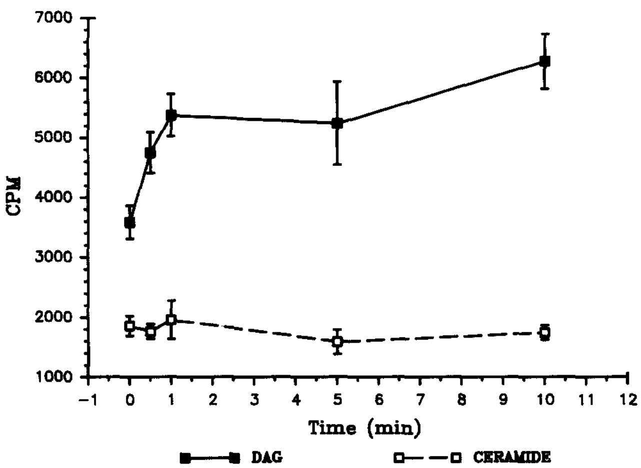

Figure 9. IL-4 (10 ng/ml) induced the generation of DAG but not ceramide in monocytes. Cells were pelleted and extracted with chloroform/methanol (1:1, vol/vol), and the lower phase containing DAG, ceramide, and phospholipids was evaporated under nitrogen. *E. coli* DAG kinase catalyzed the transfer of $^{32}PO_4$ from γ-[^{32}P]ATP to DAG and ceramide. The specific products and their respective standards were separated by TLC and identified by autoradiography with comparison to standards. The results are the mean (± SEM) of three experiments.

potential activation of SMase by IL-4R was further excluded in monocytes by the simultaneous measurements of DAG and ceramide. Illustrated in Fig. 9 is the time-dependent production of DAG induced by IL-4 (10 ng/ml) without significant changes in ceramide. The slight reduction of ceramide and the production of DAG induced by IL-4 (10 ng/ml) and exogenous SMase, respectively, suggest cross-talking between these signaling pathways.

Discussion

IL-4, initially described as a B cell growth and differentiating factor, plays an important role in Th-2 cell function and downmodulation of immune response in parasitic and HIV infection (7), and has been implicated in the pathogenesis of leishmaniasis (4–7). The role of IL-4 in leishmaniasis may be due in part to its potent inhibition of cytokine activation of macrophages (9–11). Receptor signaling has been hypothesized to provide the specific input for modulating cell function. Although the hu-IL-4R has been cloned, DNA sequence analysis revealed no homology to known tyrosine or serine/threonine protein kinases. We reported previously that IL-4R signal transduction involved PKC translocation to a nuclear fraction. The earlier IL-4R signaling events leading to PKC activation are examined in this report.

IL-4 induced a time- and dose-dependent production of DAG. The maximal dose and kinetics of DAG generation induced by IL-4 paralleled the translocation of PKC previously reported for IL-4–treated monocytes (23). As for PKC translocation, DAG production was near maximal at 5 min of IL-4 treatment and for IL-4 dose of 10 ng/ml. The source of DAG is not derived from PIP_2 hydrolysis, since neither inositol phosphates nor cytosolic calcium flux was detected. The absence of detectable calcium flux and PIP_2 hydrolysis metabolites in monocytes and U937 promonocytic cells, and the absence of detectable calcium flux in monocytic cells, mixed B and T cells and murine CTLL cell line expressing IL-4R are in agreement with studies in murine B cells (for a review see reference 22; 44, 45) but are in contrast to results in human B cells reported by Finney et al. (46, 47). Although we are not able to explain this difference, our data suggest that DAG is derived from sources other than PIP_2.

DAG may be derived from PC hydrolysis by PLC and PLD, indirectly by PLA_2, or by exchange of pchol between ceramide and PC to generate SM and DAG (15, 16–19). In methyl [^{14}C]chol-labeled U937 and monocytes, IL-4 induced a dose- and time-dependent production of pchol and degradation of PC. The finding that the amount of pchol gain equaled that of PC loss supports the suggestion that pchol is the hydrolysis product of PC. In these samples, metabolites of PLD or PLA_2, respectively chol or LPC and GPC, were not detected, suggesting that PLC activation is associated with IL-4R signaling.

Additional experiments were performed to exclude PLD or PLA_2, in IL-4R signaling. PLD hydrolysis of PC leads to the formation of chol and PA (Tables 2 and 3). Ethanol, which substitutes for H_2O in the transphosphatidylation reaction catalyzed by PLD (15, 21), decreased the generation of DAG in cells treated with PLD but not in those treated with IL-4 (Table 3). In the presence of ethanol, exogenous PLD treatment resulted in the formation of PEt whereas IL-4 treatment did not. The formation of DAG from PA is catalyzed by the enzyme, PAP, which dephosphorylates PA to form DAG. The use of propranolol and calyculin A, the respective inhibitors of PAP and phosphatase 1 and 2A (39–41), provides additional evidence that the DAG detected in response to IL-4 is not derived from PLD hydrolysis of PC. Propanolol and calyculin A inhibited DAG production in response to FMLP but not to IL-4, and propanolol inhibited the accumulation of ^{14}C-labeled PA in cells treated with PMA but not in those treated with IL-4 (Fig. 7 and Table 4).

The involvement of PLA_2 in IL-4R signaling was also excluded because neither intracellular and extracellular GPC and LPC, intermediate metabolites of PLA_2 catabolism of PC, nor extracellular [^{3}H]arachidonic acid, the direct cleavage product of PLA_2, were detected in response to IL-4 (Figs. 5, 6, and 8, Tables 3 and 4). In contrast, calcium ionophore, A23187, and FMLP, but not IL-4, induced the generation of AA in monocytes. Based on these additional findings, it is unlikely that PLD and PLA_2 are involved in IL-4R signaling.

Signaling for TNF-α and IL-1 has been reported to involve both SMase and PLC activation (35, 36). Since data using methyl [^{14}C]chol-labeled cells showed paradoxic changes in SM in response to IL-4 doses of 10 and 100 ng/ml (Fig. 6), experiments were performed to compare the effect of IL-4 and exogenously added PC-specific PLC and SMase. The generation of pchol and degradation of PC by IL-4 was similar to that of exogenous PLC (Table 2). SM levels were slightly increased as previously seen (Table 2 and Fig. 6). Only exogenous SMase induced degradation of SM. The finding that the activity of SMase occurred at 5 min whereas hydrolysis of PC associated with IL-4 and exogenous PLC occurred as early as 15 s is in agreement with previous reports (21, 36, 37).

Simultaneous measurements of DAG and ceramide were performed to exclude the possibility that the IL-4R might be linked to SMase which catalyzes ceramide production and, indirectly, DAG generation via exchange of pchol between PC and ceramide. In contrast to exogenous SMase, IL-4 induced the highest production of DAG whereas increased ceramide production (with SM degradation) was detected only for exogenous SMase. Furthermore, the maximal activity for IL-4 and SMase was 5 and 30 min, respectively. The detected changes of SM or pchol and PC in response to IL-4 and exogenous SMase probably result from the use of metabolites to regenerate different membrane lipids. The data presented suggest that IL-4R signaling involves PC-specific PLC activation and generation of DAG, which in turn activates PKC. Our findings also suggest that early IL-4R events do not involve the activation of PLD, PLA_2, or SMase.

As stated by Liscovitch (18), "Activation of PIP_2-specific PLC, PLD and PLA_2 in whatever cell type–specific sequence appears to comprise a signaling cascade which is typically utilized by most (if not all) Ca^{2+}-mobilizing agonists." The observation that IL-4R signaling involves PC-specific PLC is strengthened by the contrast with FMLP, an example of Ca^{2+}-mobilizing agonist that involves PIP_2-specific PLC, PLD, and PLA_2 (18, 43, 48–50). PIP_2-specific PLC is composed of at least 16 isoenzymes belonging to the β, γ, and δ families of PLC. Members of these families have been cloned and differ in their activation requirements by either G proteins or tyrosine kinases (16). Unlike PIP_2-specific PLC, much less is known about PC-specific PLC, although a bacterial PC-specific PLC has been cloned (51). Evidence that PC-specific PLC is activated as part of receptor signaling is reported for TNF-α, IFN-α, IL-1, IL-3, and M-CSF (21, 52–55). With the exception of TNF-α, the involvement of PLD has not been excluded. Furthermore, cytosolic PLA_2 is activated by M-CSF (56).

TNF-αR, IFN-γR, and IL-1R involve SMase activation (36, 37, 57). In addition, activation of PC-specific PLC has been reported for TNF-αR, IFNR (type I), and IL-1R (36, 37, 52, 57). M-CSF binding to the tyrosine kinase M-CSFR leads to tyrosine phosphorylation of several cytosolic proteins and the activation of PKC associated with PC hydrolysis and DAG formation (55), as well as induction of cytosolic PLA_2 activity and mRNA (56). In contrast, IL-4R activates only PC-specific PLC and not PIP_2-PLC, PLD, PLA_2, or SMase. This may provide an explanation for the fact that IL-4 downmodulates macrophages (9–11) whereas TNF-α, IFN-γ, IL-1, and M-CSF activate macrophages for killing of leishmania and for oxidative burst capacity (58, 59). PKC are reported to be involved in IL-1, IFN-α, IFN-γ, IL-3, and IL-4 receptor signaling (60–64). The specificity for modulation of macrophage activation or downmodulation may be provided also by different isoforms of PKC transducing these receptor ligands (13). One potential mechanism by which specific PKC isoforms are selectively coupled to these receptors may result from differential use of DAG isoforms that make up the many families of membrane phospholipids (65–69). Although not examined in this report, the results of Rosoff et al. (53) and Schultz et al. (21) in studies of IL-1 and TNF-α receptor signaling, respectively, suggest that the activated PLC selectively uses membrane PC with specific fatty acid side chains.

Our report did not examine whether tyrosine kinase activation may participate in IL-4R signaling. This possibility has been suggested recently in a murine myeloid cell line (70, 71). Further evaluations are needed to determine whether selective use of early signaling pathways by different cytokines provides the specificity for up- or downmodulation of macrophage cell function.

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