Tumor Necrosis Factor Induces Rapid Production of 1'2'Diacylglycerol by a Phosphatidylcholine-specific Phospholipase C

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Summary

Tumor necrosis factor (TNF) is a proinflammatory polypeptide that is able to induce a great diversity of cellular responses via modulating the expression of a number of different genes. One major pathway by which TNF receptors communicate signals from the membrane to the cell nucleus involves protein kinase C (PKC). In the present study, we have addressed the molecular mechanism of TNF-induced PKC activation. To this, membrane lipids of the human histiocytic cell line U937 were labeled by incubation with various radioactive precursors, and TNF-induced changes in phospholipid, neutral lipid, and water-soluble metabolites were analyzed by thin layer chromatography. TNF treatment of U937 cells resulted in a rapid and transient increase of 1'2'diacylglycerol (DAG), a well-known activator of PKC. The increase in DAG was detectable as early as 15 s after TNF treatment and peaked at 60 s. DAG increments were most pronounced (~360% of basal levels) when cells were preincubated with [^{14}C]lysophosphatidylcholine, which was predominantly incorporated into the phosphatidylcholine (PC) pool of the plasmamembranes. Further extensive examination of changes in metabolically labeled phospholipids indicated that TNF-stimulated hydrolysis of PC is accompanied by the generation of phosphorylcholine and DAG. These results suggest the operation of a PC-specific phospholipase C. Since no changes in phosphatidic acid (PA) and choline were observed and the production of DAG by TNF could not be blocked by either propranolol or ethanol, a combined activation of phospholipase D and PA-phosphohydrolase in DAG production appears unlikely. TNF-stimulated DAG production as well as PKC activation could be blocked by the phospholipase inhibitor *p*-bromophenacylbromide (BPB). Since BPB did not inactivate PKC directly, these findings underscore that TNF activates PKC via formation of DAG. TNF stimulation of DAG production could be inhibited by preincubation of cells with a monoclonal anti-TNF receptor (p55-60) antibody, indicating that activation of a PC-specific phospholipase C is a TNF receptor–mediated event.

TNF-α (referred to as TNF) is one of the earliest soluble mediators secreted in response to infectious stimuli (1, 2). TNF can be produced by a variety of tissues, which points to important autocrine and paracrine functions. TNF has been shown to induce numerous physiologic effects upon diverse tissues. Immunostimulatory capacities of TNF may be beneficial to the host. However, protracted secretion of large amounts of TNF has also been implicated in the pathogenesis of clinical syndromes like septic shock, cachexia, and lethal outcome of cerebral malaria (for review see reference 3). In addition, TNF appears to play a pivotal role in the activation of the HIV-long terminal repeat enhancer, thereby promoting virus replication and dissemination in the host (4, 5). TNF-induced cellular responses are brought about by TNF's striking ability to stimulate or downmodulate the expression of a large number of genes (for review see reference 6). Despite the plethora of TNF's bioactivities, the pathways by which TNF signals are communicated to the transcriptional apparatus in the nucleus are poorly understood. Two membrane-associated molecules with molecular masses of 55–60 kD (termed p60) and 75–80 kD (termed p80), respectively, have been identified that bind TNF with high affinity and may function as specific cell surface TNF receptors (7–12). Recently, both TNF receptor chains have been molecularly cloned (13–18). Evidence that p60 or p80 TNF receptor chains are directly involved in TNF signaling came from studies using

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mAbs recognizing either p60 or p80 (19, 20). Anti-p80 antibodies that compete for binding apparently do not inhibit TNF action. In contrast, most of the anti-p60 antibodies reported exhibit agonistic activity, that is, are able to elicit TNF responses (20). Recently, Thoma et al. (21) described a monoclonal anti-p60 antibody, H398, that competed for TNF binding and inhibited cellular responses to TNF. While the functional significance of p80 has yet to become resolved, these observations clearly established that the TNF receptor chain p60 is crucially involved in triggering certain TNF signal transfer reactions. Sequence analysis of both receptor chains revealed no homologies to functional domains of tyrosine or serine/threonine-specific protein kinases. Thus, TNF receptor signaling across the membrane is still unclear.

One of the membrane-associated events triggered by TNF receptors is the activation of phospholipase A_2 (PLA_2)[1] (22–26), which releases arachidonic acid (AA) and lysophosphatidates. AA can be metabolized to leukotrienes and prostaglandins, potent mediators of inflammatory processes. In addition, protein kinases (PK) appear to play a crucial role in TNF signal transduction pathways (27–31), and a number of cellular substrates for TNF-responsive PK have been detected (32–39). We have recently shown that TNF stimulates translocation and activation of PKC (30). The mechanism of PKC activation, however, remained unclear. Certain polypeptide growth factors activate PKC via stimulating a phospholipase C (PLC) that hydrolyzes phosphatidyl-inositol 4,5 biphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (see reference 40 for review). Strikingly, TNF appears to stimulate PKC in the absence of PIP_2 turnover. We show here that TNF can stimulate a phosphatidylcholine (PC)-specific PLC that hydrolyzes PC instead to generate phosphorylcholine and DAG, which is probably responsible for subsequent activation of PKC.

Materials and Methods

Cell Culture and Biological Reagents. U937 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained under standard conditions as described (32). Highly purified human rTNF-α (sp act 5×10^7 U/mg) was provided by Dr. G. Adolf (Boehringer Research Institute, Vienna, Austria). The mAb clone H398, directed against the TNF receptor chain (p60), has been described recently by Thoma et al. (21).

Intracellular Ca^{2+} Measurements. Loading of U937 cells with the fluorescent intracellular Ca^{2+} chelator dye Quin2 was performed essentially as described by Charest et al. (41). Ca^{2+}-dependent Quin2 fluorescence was determined in a Farrand fluorimeter (model A-3; LKB, Freiburg, Germany) which has been modified to allow continuous stirring of the sample. The excitation wave length was 334 nm and the emission wave length was 492 nm. Cytosolic Ca^{2+} concentrations were determined according to the method described by Tsien et al. (42).

Preparation of [^{14}C] Choline-labeled Lyso-phosphatidylcholine. 10^7 U937 cells were incubated with 200 μCi [methyl^{14}C]choline (Amersham Corp., Arlington Heights, IL) for 48 h. Phospholipids were extracted as described by Bligh and Dyer (43) and separated by thin layer chromatography on preparative silica 60 plates (Merck, Darmstadt, Germany), using a $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (100:60:20:5) solvent system. [^{14}C]PC was identified by autoradiography, the spots were scraped off, and [^{14}C]PC was extracted according to Bligh and Dyer (43). Purified [^{14}C]PC was then subjected to 50 U phospholipase A_2 from Naya Naya Venom (Sigma Chemical Co., St. Louis, MO) in 100 nM Tris, pH 8.9, 40 mM $CaCl_2$ for 60 h at 37°C, followed by $CHCl_3$ extraction. The $CHCl_3$ phase was then loaded onto silica 60 mini-columns, and L-lyso-3-phosphatidyl [methyl^{14}C]choline was eluted with $CHCl_3/CH_3OH/H_2O$ (65:35:5). The recovery of L-lyso-3-phosphatidyl-[methyl^{14}C]choline was 70–80%.

Metabolic Labeling. For labeling of neutral lipids and phospholipids, U937 cells were either labeled for 16 h with ^{14}C-oleic acid (0.5 μCi/ml) (Amersham Corp.) (sp act 54.9 mCi/mmol), or [^{14}C]arachidonic acid (0.5 μCi/ml) (Amersham Corp.) (sp act 54.2 mCi/mmol), or labeled for 2 h with L-lyso-3-phosphatidylcholine, 1-[^{14}C]palmitoyl at 1 μCi/ml (sp act 56.8 mCi/mmol) in medium containing 5% FCS. For detection of water-soluble choline metabolites, cells were either labeled for 48 h with [methyl^{14}C]choline (1 μCi/ml, sp act 56.4 mCi/mmol) or for 2 h with L-lyso-3-phosphatidyl [methyl^{14}C]choline (1 μCi/ml, sp act 22 mCi/mmol).

Cell Stimulation and Extraction. U937 cells were serum-starved for 4 h in medium supplemented with 2% BSA. Aliquots of 10^7 cells were suspended in 1 ml PBS and treated for indicated times with 100 ng/ml TNF or phorbol ester (PBt_2, 40 ng/ml; OAG, 100 μM; PMA, 100 nM). Incubation of cells with inhibitors before TNF and phorbol ester treatment was as follows: *p*-bromophenacylbromide (BPB), 100 μM, 45 min; propranolol, 200 μM, 5 min; ethanol, 1%, 2 min. Treatments were stopped by immersion of the sample tubes in methanol/dry ice (−70°C) for 10 s followed by a centrifugation at 4°C in a microfuge. Supernatants were collected for determination of extracellular, water-soluble [methyl^{14}C]choline metabolites, and cell pellets were resuspended in cold $CH_3OH/CHCl_3/H_2O$ (2.5:1.25:1). Further extraction of cell pellets and separation into an aqueous phase containing intracellular [^{14}C]choline metabolites and an organic phase containing phospholipids was performed according to the procedure described by Bligh and Dyer (43).

For separation of labeled 1'2'DAG and labeled phospholipids, cell pellets were resuspended in hexane/isopropanol (3:2), sonicated for 2 min, and insoluble material was removed by centrifugation for 10 min at 2,500 *g*. Supernatants were dried under nitrogen, the material was resuspended in $CH_3Cl/CH_3OH/H_2O$ (30:60:8; vol/vol/vol), and desalted by passage through a Sephadex G-10 minicolumn. The eluates were dried under N_2. Neutral lipids were separated from phospholipids in a two-phase methanol/hexane system.

Thin Layer Chromatography. Water-soluble compounds were separated on silica gel 60 thin layer plates using the following system: CH_3OH/0.5% NaCl/NH_4OH (100:100:2; vol/vol/vol) as described by Yarvin (44). Phosphorylcholine, glycerophosphorylcholine, acetylcholine, CDP-choline, and choline (Sigma Chemical Co.) were used as standards. Samples containing equal amounts of radioactivity were loaded. Plates were exposed to Kodak XAR-films and the autoradiographs were analyzed by two-dimensional scanning using a densitometer (Ultroscan XL; Pharmacia LKB Biotechnology Inc., Freiburg, Germany). To determine the radioactivity

[1] *Abbreviations used in this paper:* AA, arachidonic acid; BPB, *p*-bromophenacylbromide; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PEt, phosphatidyl-ethanol; PIP_2, phosphatidyl-inositol 4,5 biphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D.

incorporated into specific compounds, respective spots were scraped off and extracted from the gel.

Neutral lipids were separated by TLC using in a solvent system of benzene/ethylacetate (6.5:3.5). Samples containing equal amounts of radioactivity were loaded. 1'2'DAG, 1'3'diacylglycerol, 1-monoacylglycerol, triglycerides, and cholesterol were applied as standards (Sigma Chemical Co.) and visualized by coomassie-staining according to Nakamura and Handa (45). The stained plates as well as the autoradiographs were scanned with a two-dimensional laser densitometer, the amount of cholesterol, visualized by staining, was taken as an internal control to correct for equal amount of material loaded.

Phospholipids were separated by TLC using a solvent system containing $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (100:60:20:5). Lyso-phosphatidylcholine, phosphatidyl-choline, sphingomyeline, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylethanol, and phosphatidic acid (Sigma Chemical Co.) were used as standards and visualized in iodine vapor. For separation of phosphatidylethanol from phosphatidic acid and other phospholipids, TLC was performed in a solvent system consisting of the organic phase of ethylacetate/acetic acid/water (110:20:110). Plates were autoradiographed and analyzed by two-dimensional laser scanning. Specific radioactivity of [methyl^{14}C]choline-labeled phospholipids was determined by scintillation counting of corresponding spots scraped off the gel. Mass determinations were performed by charing densitometry of TLC plates as described (46).

PKC Assay. Activation of PKC by TNF was determined by estimation of phorbol ester binding capacity of whole cells as described (30). Briefly, U937 cells (5×10^6 in 1 ml Click's/RPMI [Gibco Laboratories, Grand Island, NY) supplemented with 4 mg/ml BSA) were left untreated or treated at 37°C with 100 ng/ml TNF for various periods of time or with various TNF concentrations for 3 min. Cells were then washed with 10 ml ice-cold PBS, divided in two aliquots, and incubated for 10 min at 37°C in PBS containing 4 mg/ml BSA and 40 nM [^{3}H]PBt_2 (Amersham Corp.) with or without 10 μM of unlabeled competitor PMA (47). Cell-associated [^{3}H]PBt_2 was separated from free [^{3}H]PBt_2 by passage through glass fiber filters. Filters were washed five times with PBS and radioactivity was determined by liquid scintillation counting. Specific binding was estimated by subtracting the mean nonspecific binding in the presence of 250-fold excess of unlabeled PMA.

To determine membrane-associated PKC activity by the histone IIIS phosphorylation assay, 2×10^7 cells in 20 ml medium were washed at 4°C with homogenization buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 50 mM 2-ME, 0.1 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml aprotinin [Sigma Chemical Co.]), and homogenized in 0.8 ml of the homogenization buffer by 30 strokes at 1,000 rpm in a homogenizer (Potter-Elvehjem, Braun Diessel Biotech, Melsungen, Germany). Homogenates were first precleared by centrifugation (100 *g*, 5 min) to remove cell debris and nuclei. A second centrifugation at 100,000 *g* for 60 min was performed to separate membranes from the cytosol. Membranes were then resuspended in homogenization buffer supplemented with 0.1% Triton X-100 and stirred for 60 min at 5°C to solubilize proteins which were recovered by centrifugation at 100,000 *g* for 60 min. To separate PKC from other protein kinases, the Triton X-100 solubilized membrane fractions were subjected to DE-52 chromatography according to Kikkawa et al. (48). Samples were loaded onto small DEAE-cellulose (DE-52; Whatman, Madstone, England) columns that had been equilibrated with 20 mM Tris, pH 7.4, 20 mM EDTA, 50 mM 2-ME, 4 μg/ml leupeptin, 100 μg/ml aprotinin. After loading, columns were washed with the same buffer and PKC activity was eluted with 100 mM NaCl and stabilized with 1 mg/ml BSA. The PKC activity was assayed in a final volume of 100 μl containing 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.25 mg of histone III-S/ml (Sigma Chemical Co.), 50 μg phosphatidyl serine (PS)/ml (Sigma Chemical Co.), 5 μg/ml of diolein (Sigma Chemical Co.), and 50 μg of the PKC-containing DEAE-cellulose eluate (49). Assays without both PS and diolein served as controls of non-PKC-specific (phospholipid-independent) kinase activity. After thermoequilibration at 30°C for 5 min, the reaction was started by addition of [^{32}P]ATP (Amersham Corp.) (50 μM, 40 nCi/nmol). After 10 min, the reaction was stopped by addition of 20 μl buffer containing 50 mM potassium phosphate, pH 7, 10 mM EGTA, 2 mM EDTA, 5 mM unlabeled ATP, and 5 mg BSA/ml. Proteins were precipitated onto glass fiber filters with 25% (wt/vol) TCA, washed under vacuum with 15 ml 25% TCA, and ^{32}P incorporation was determined by liquid scintillation counting.

Results

TNF Does Not Affect Intracellular Ca^{2+} Levels. To obtain information as to the mechanism of TNF activation of PKC, we first investigated the effects of TNF on free intracellular Ca^{2+} levels. U937 cells were loaded with the Ca^{2+} chelator dye Quin2, and Ca^{2+}-dependent fluorescence was measured. As shown in Fig. 1, rapid influx of Ca^{2+} was observed after treatment with the Ca^{2+} ionophore A23187. Furthermore, stimulation of cells with the mitogen Con A resulted in the release of Ca^{2+} from internal stores. In contrast, even at very high concentrations, TNF had no measurable effect on intracellular Ca^{2+} levels. These results indicate that mechanisms other than IP_3 metabolism may be involved in TNF-mediated PKC activation. In this regard, it is now well recognized that the "classical" activator of PKC, 1'2'DAG, can also be generated in the absence of PIP_2 cleavage by PLC-mediated hydrolysis of PC to DAG and phosphorylcholine (for review see reference 50). Therefore, we next examined in U937 cells whether TNF may trigger the production of 1'2'DAG by stimulation of a PC-specific PLC.

TNF Induces Generation of DAG. To assess TNF-induced changes of DAG levels, the phospholipid pool of U937 cells was labeled with [^{14}C]oleic acid. After TNF treatment of cells for various periods of time, the amount of 1'2'DAG produced was analyzed by TLC. As shown in Fig. 2 *a*, TNF transiently stimulates the rapid production of 1'2'DAG, reaching within 60 s a maximum of 135 ± 2.4% of the amount of untreated cells. When U937 cells were labeled instead with ^{14}C-arachidonic acid, which is incorporated predominantly at the sn-2 position of the phospholipids, maximal stimulation could be increased to 164 ± 8% of basal (unstimulated) levels (Fig. 2 *a* and Table 1). Changes in other ^{14}C-labeled neutral lipid components (1-monoacylglycerol, 1'3'DAG, and triglycerides) were not significant (Table I). Analysis of ^{14}C-fatty acid incorporation in phospholipids revealed that arachidonic acid was predominantly incorporated into PC (43% of total; Table 1). Since PC makes up a large proportion of total membrane phospholipids, possible TNF-induced changes of PC levels might well be below the level of detection.

The membrane PC pool could be labeled more selectively

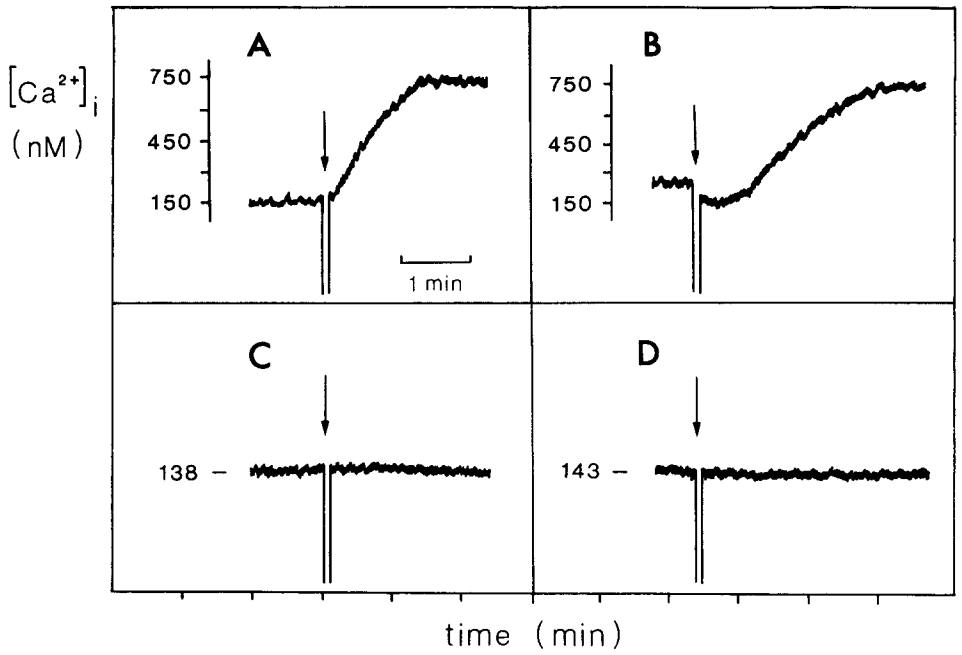

Figure 1. TNF does not stimulate increase of free intracellular Ca^{2+}. U937 cells were loaded with Quin2, and Ca^{2+}-dependent fluorescence was measured as described in Materials and Methods. 40 μM A23187 (*A*), 30 μg/ml Con A (*B*), or TNF at 10 ng/ml (*C*) or 100 ng/ml (*D*) were added to cells at 37°C, and the changes in fluorescence were monitored continuously.

when U937 cells were incubated with L-lyso-3-phosphatidylcholine, 1-[^{14}C]palmitoyl. This compound easily inserts into the cell membrane and is reacylated by acyltransferases within the cell membrane and subsequently integrated into the PC pool. As depicted in Table 1, the fraction of radioactive PC made up 65% of ^{14}C-labeled phospholipids, when U937 cells were incubated for 2 h with L-lyso-3-phosphatidylcholine, 1-[^{14}C]palmitoyl. TNF treatment of prelabeled U937 cells resulted in the production of ^{14}C-labeled 1'2'DAG with identical time kinetics compared to labeling regimes using either [^{14}C]oleic acid or [^{14}C]arachidonic acid as metabolic precursors (Fig. 2 *a*). Compared to the latter labels, the use

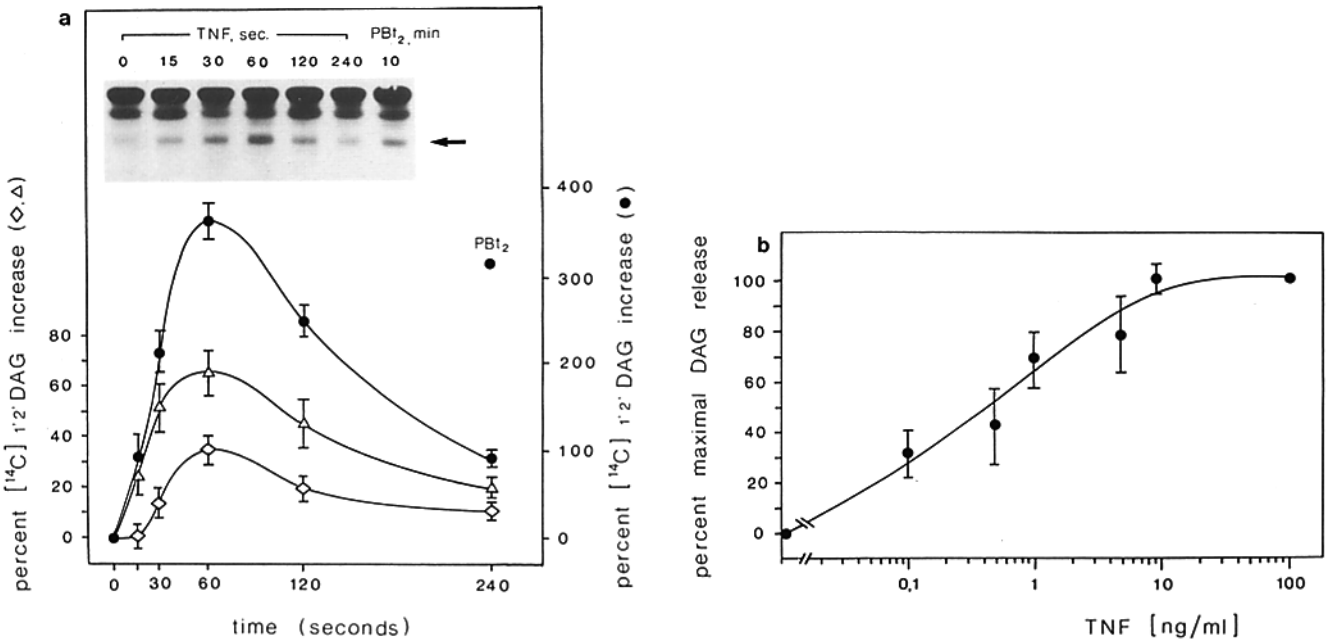

Figure 2. (*a*) TNF induces 1'2'DAG production in U937 cells. U937 cells were preincubated for either 16 h with [^{14}C]oleic acid (◇), [^{14}C]arachidonic acid (△), or for 2 h with L-lyso-3-phosphatidylcholine, 1-[1^{14}C]palmitoyl (●). After serum starvation for 4 h, cells were incubated with 100 mg/ml TNF for indicated times. For positive control, U937 cells labeled for 2 h with L-lyso-3-phosphatidylcholine, 1-[1^{14}C]palmitoyl were incubated with 20 ng/ml PBt_2 for 10 min (●). Neutral lipid metabolites were extracted and analyzed by TLC as described in Materials and Methods. The content of labeled 1'2'DAG is expressed as percent of untreated controls as estimated by two-dimensional scanning of the autoradiographs (*inset*). The arrow indicates the position of 1'2'DAG. (*b*) TNF dose-dependent production of 1'2'DAG. [^{14}C]Arachidonic acid–labeled U937 cells were serum-starved and incubated with various TNF concentrations for 1 min at 37°C. 1'2'DAG was quantitated as described in *a*. Maximal TNF-stimulated DAG production was taken as 100%. The results of three experiments (± SD) are shown.

Table 1. *TNF-induced DAG Production and PC Hydrolysis in U937 Cells*

Radioactive precursor*	Metabolite‡	Distribution of ^{14}C incorporated§	TNF-induced changes‖	Significance¶
[^{14}C]arachidonic acid	Neutral lipids (n = 4):	%	%	
	1′2′DAG	4.6 ± 0.51	+65 ± 8.2	$p < 0.01$
	TG	79.4 ± 5.80	± 4	NS
	n.i.	16.0 ± 7.2	± 12.4	NS
	Phospholipids (n = 4):			
	PC	32.5 ± 3.0	± 6.4	NS
	PE	23.2 ± 3.1	± 1.7	NS
	PA	38.0 ± 0.4	± 1.5	NS
	SM	2.5 ± 0.2	± 0.6	NS
	LPC	1.75 ± 0.25	± 0.3	NS
L-lyso-3-phosphatidylcholine, 1-[^{14}C]palmitoyl	Neutral lipids (n = 4):			
	1′2′DAG	9.5 ± 1.5	+357 ± 44	$p < 0.004$
	TG	63.8 ± 13.2	± 13.17	NS
	n.i.	26.7 ± 12.3	± 12.3	NS
	Phospholipids (n = 4):			
	PC	65 ± 7.5	−16 ± 5.2	$p < 0.01$
	LPC	9 ± 2.4	± 3.4	NS
	SM	16 ± 3.5	± 5.2	NS
	PA	10 ± 1.7	± 9.4	NS

* U937 cells were labeled for 16 h with [^{14}C]arachidonic acid (0.5 μCi/ml) or for 2 h with L-lyso-3-phosphatidylcholine, 1-[1^{14}C]palmitoyl (1 μCi/ml).
‡ Neutral lipids and phospholipids were separated and analyzed by TLC as described in Materials and Methods. Abbreviations of metabolites examined: 1′2′DAG, 1′2′diacylglycerol; TG, triglycerides; n.i., not identified neutral lipid component; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; SM, sphingomyelin; LPC, lyso-phosphatidylcholine.
§ The initial distribution of ^{14}C in each metabolite was estimated by two-dimensional scanning of the autoradiographs and is expressed in percent (±SEM) of ^{14}C in total neutral lipids or total phospholipids, respectively. Shown is the distribution before TNF treatment (t = 0).
‖ In each of the four independent experiments, the values at t = 0 were subtracted from the percent of the total ^{14}C label present in each metabolite after 60 s of TNF incubation. The means of changes (±SEM) are shown as percent of untreated controls.
¶ Statistical significance was determined by student's t test. NS, not significant ($p > 0.05$).

of L-lyso-3-phosphatidylcholine, 1-[^{14}C]palmitoyl revealed a substantially greater amplitude of DAG liberation, i.e., 360%, over control values. For positive control, U937 cells were treated for 10 min with phorbol dibutyrate (PBt_2), which has been shown to indirectly activate, that is via PKC, a PC-specific PLC (51, 52). A 320% increase in 1′2′DAG could be measured (Fig. 2 *a*), which is similar to that observed with TNF. The production of 1′2′DAG is a TNF dose-dependent event: as shown in Fig. 2 *b*, half-maximal production is achieved at 0.5–1.0 ng/ml TNF, which is sufficient to induce TNF responses such as growth arrest and differentiation (53).

TNF-induced PC Hydrolysis as Source for DAG. The enhancement of 1′2′DAG levels observed with L-lyso-3-phosphatidylcholine, 1-[1^{14}C] palmitoyl-labeled cells already suggested that PC may be a source for 1′2′DAG production. To obtain further evidence that PC is indeed the substrate of a putatively TNF-responsive phospholipase, we next labeled U937 cells with [^{14}C]choline, which allows to measure changes in water-soluble reaction products catalyzed by PLC. When cells were incubated with [^{14}C]choline for 48 h, the distribution of ^{14}C-labeled phospholipids PC, SM, and lyso-PC was 74%, 29%, and 5%, respectively (Table 2). Analysis of the water-soluble components revealed that ~79.4% of [^{14}C] was incorporated into pchol and ~7.6% into free choline. Most of the pchol labeled was located intracellularly (86%), while a minor proportion (14%) was found extracellularly (data not shown).

As shown in Fig. 3 *a*, TNF treatment of [^{14}C]choline-labeled U937 cells led to a rapid increase of the amount of intracellular pchol, reaching after 30–60 s ~141% of unstimulated values. The extracellular pchol concentration remained constant over the time period examined (not shown). Since pchol together with DAG represent the PC cleavage products generated by a PC-specific PLC, these findings strongly suggest that this phospholipase is activated by TNF/TNF receptor interactions. Similar results were obtained when

Table 2. *TNF-induced Changes of PC and Phosphorylcholine (pchol) in U937 Cells*

Radioactive precursor*	Metabolite‡	Distribution of ^{14}C incorporated§	TNF-induced changes‖	Significance¶
[methyl^{14}C]choline	Phospholipid (n = 5):	%	%	
	PC	74.2 ± 3.8	−15 ± 10.6	$p < 0.01$
	LPC	7.0 ± 2.7	± 7.7	NS
	SM	33.2 ± 7.4	± 4.0	NS
	Water-soluble (n = 7):			
	pchol	79.4 ± 13	+41.2 ± 10	$p < 0.008$
	choline	7.6 ± 4.5	± 7.0	NS
	GPC	13.2 ± 3.3	± 5.5	NS
L-lyso-3-phosphatidyl [methyl^{14}C]choline	Phospholipids (n = 4):			
	PC	85 ± 7.5	−26.3 ± 6.4	$p < 0.01$
	LPC	5 ± 1.4	± 1.3	NS
	SM	10 ± 2.3	± 2.1	NS
	Water-soluble (n = 4):			
	pchol	93.1 ± 11	+55 ± 13	$p < 0.01$
	choline	0.9 ± 0.1	± 0.25	NS
	acetylcholine	0.6 ± 0.08	± 0.2	NS
	GPC	5.4 ± 2.8	± 13.7	NS

* U937 cells were labeled for 48 h with [methyl ^{14}C]choline (1 μCi/ml) or for 2 h with L-lyso-3-phosphatidylcholine [methyl ^{14}C]choline (1 μCi/ml).
‡ Phospholipids and water-soluble components were separated and analysed by TLC as described in Materials and Methods. Abbreviations of metabolites examined: GPC, glycerophosphorylcholine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; pchol, phosphorylcholine; SM, sphingomyelin.
§ The initial distribution of ^{14}C in each metabolite was estimated by two-dimensional scanning of the autoradiographs and is expressed in percent (± SEM) of [^{14}C] in total phospholipids or total water-soluble components, respectively.
‖ The means of changes (± SEM) in each metabolite after 60 s of TNF treatment were calculated by subtraction of the values at t = 0. In each of the four to seven independent experiments, the values at t = 0 were subtracted from the percent of the total ^{14}C label present in each metabolite after 60 s of TNF incubation. The means of changes (± SEM) are shown as percent of untreated controls.
¶ Statistical significance was determined by student's t test. NS, not significant ($p > 0.05$).

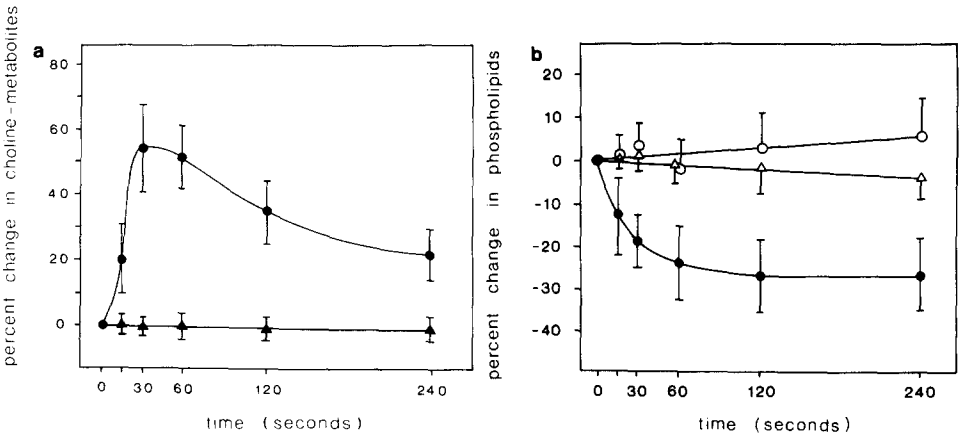

Figure 3. TNF induced changes of phospholipids and choline metabolites. (*a*) Cells were incubated with [^{14}C]-choline for 48 h, serum-starved for 4 h, and treated with 100 ng/ml TNF for indicated times. Water-soluble [^{14}C]-choline metabolites were analyzed by TLC, and the content of [^{14}C]pchol (●) and [^{14}C]choline (▲) was determined as described in Materials and Methods. Results are expressed as percent change of untreated controls and represent the mean ± SD of four experiments. (*b*) U937 cells were labeled with L-lyso-3-phosphatidyl, [methyl^{14}C]choline for 2 h, serum-starved for 4 h, and treated with 100 ng/ml TNF for indicated times. Phospholipids were separated and analyzed by TLC: (○) LPC; (△) SM; (●) PC. Results are shown as percent of untreated controls (mean ± SD of four experiments).

L-lyso-3-phosphatidyl [methyl^{14}C]choline was used as a choline-headgroup-labeled, more specific precursor for PC. When U937 cells were incubated for 2 h with this radioactive precursor, 85% of labeled phospholipid metabolites were recovered in PC, and ~93% of labeled, water-soluble components were detected in pchol, 0.9% in choline, in 0.6% in acetylcholine (Table 2). TNF treatment enhanced the levels of labeled intracellular pchol to 155% compared to untreated control values. In contrast, the levels of both labeled choline and acetylcholine remained unchanged. Of note, the rather selective labeling of PC allowed measurement of TNF-induced PC degradation in U937 cells (Fig. 3 *b*, Table 2).

The time course of PC degradation paralleled that of 1'2'DAG and pchol production. Moreover, estimation of the radioactivity extracted from the TLC plates revealed that the 26% reduction of labeled PC (39,000 ± 4,200 cpm/10^6 cells) was approximately equivalent to the amount of labeled pchol generated (32,000 ± 3,400 cpm/10^6 cells). These findings confirm the notion that the 1'2'DAG produced in TNF-treated U937 cells is derived from cleavage of PC, generating pchol as a second cleavage product.

TNF-induced DAG Production Does Not Result from Phospholipase D (PLD) Activation. Besides PC-PLC, another potential source of 1'2'DAG could be the PLD-mediated hydrolysis of PC resulting in the liberation of phosphatidic acid (PA) and choline (54–57). Subsequent dephosphorylation of PA by phosphatidate phosphohydrolase and phosphorylation of choline by a choline kinase could also account for the increase of DAG and pchol production observed in TNF-treated cells. However, after TNF treatment significant changes of intra- and extracellular choline levels could not be detected (Fig. 3 *a* and Table 2). Furthermore, PA levels remained rather constant as revealed by TLC analysis of U937 cells labeled with either [^{14}C]arachidonic acid (Table 1) or ^{32}P (data not shown). In addition, when the cells were labeled for 2 h with L-lyso-3-phosphatidylcholine, 1-[^{14}C]palmitoyl, which more selectively labeled the PC pool and allowed for optimal detection of labeled 1'2'DAG (see Fig. 2 *a*), production of labeled PA was not measurable (Table 1 and Fig. 4 *a*). To evaluate in more detail whether a PA phosphohydrolase is involved in TNF-stimulated DAG production, the effect of propanolol, a phosphohydrolase inhibitor (55, 58), on the formation of TNF-induced DAG was examined. In the absence of TNF, propanolol increased the basal levels of labeled PA by its action on constitutive PA-phosphohydrolase activity, but treatment with TNF did not result in a further accumulation of PA (Fig. 4 *a*). Notably, in the same experiment, the TNF-stimulated production of labeled DAG was not prevented by propanolol (Fig. 4 *b*). Together, these results indicate that PLD-derived PA is not the primary source of DAG formation.

To provide further evidence that PLD is not involved in DAG production, we next assessed PLD more directly by investigation of possible formation of labeled phosphatidylethanol (PEt), which would be expected from PLD's transphosphatidylation activity in the presence of ethanol (57, 59, 60). In various cell lines, activation of PLD by phorbol esters results in formation of PA and of PEt, in the presence of ethanol (61–63). As a positive control, U937 cells previously

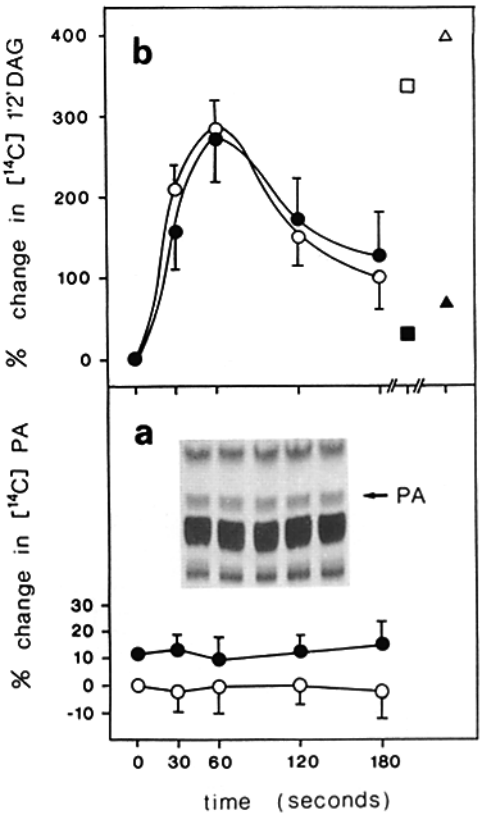

Figure 4. TNF-stimulated 1'2'DAG production is not derived from PA dephosphorylation. Serum-starved U937 cells were labeled with L-lyso-3-phosphatidylcholine, 1-[^{14}C]palmitoyl for 2 h. (*a*) Cells untreated (○) or preincubated with 200 μM propranolol for 5 min (●) were incubated with 100 ng/ml TNF for indicated times. Changes in labeled PA were estimated by two-dimensional scanning of autoradiographs of phospholipids (*inset*) after separation by TLC (see Materials and Methods). Results of three independent experiments are expressed as percent of untreated controls (± SEM). (*b*) Cells were treated with 100 ng/ml TNF for indicated times (○), or 100 nM PMA (□) or 100 μM OAG (△) for 20 min. Solid symbols represent values revealed in the presence of propanolol. Changes in labeled DAG were estimated after separation of neutral lipids as described in *a* (result from three experiments ± SEM).

labeled with L-3-lyso phosphatidylcholine, 1-[^{14}C]palmitoyl were therefore incubated for 20 min with PMA and OAG in the presence of 1% ethanol, resulting in a 85% and 100% stimulation of labeled PEt (Fig. 5 *a*), indicating activation of a PLD. In contrast, TNF treatment failed to stimulate PEt production, thus indicating that TNF does not activate PLD within the time period examined. Moreover, ethanol treatment did not prevent TNF-stimulated DAG production (Fig. 5 *b*).

Finally, to rule out the possibility that TNF stimulates a PLD acting on phospholipid pools not readily labeled with the isotopic precursors used, we estimated changes in total PA mass. Analysis of separated phospholipids by charring densitometry revealed an increase in PA in response to treatment with OAG and PMA (Fig. 6). In contrast, TNF did not significantly affect the amount of PA.

Preincubation of cells with propranolol enhanced the basal

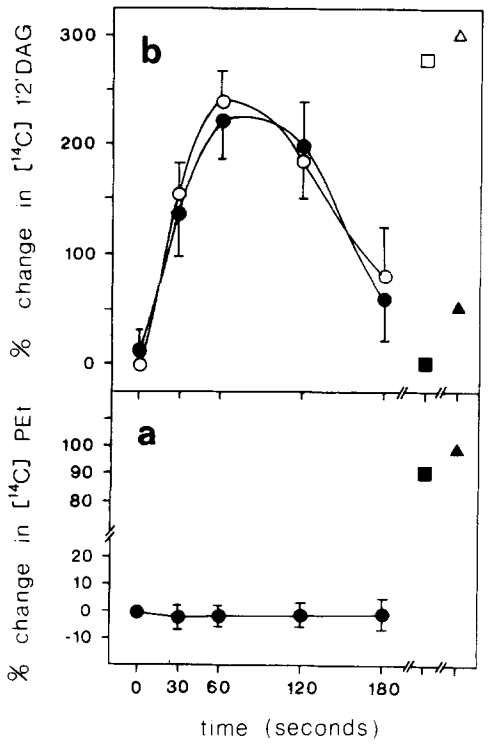

Figure 5. (*a*) TNF does not stimulate the formation of PEt. Serum-starved U937 cells were labeled as described in Fig. 4. Cells were preincubated with 1% ethanol for 2 min and then stimulated with 100 ng/ml TNF (●) for indicated times, or with 100 μM OAG (▲) or 100 nM PMA (■) for 20 min. The content of labeled PEt was estimated by two-dimensional scanning of the autoradiographs after TLC separation of phospholipids. Results from three experiments (± SEM) are shown. (*b*) Ethanol does not inhibit TNF-stimulated 1'2'DAG production. U937 cells were serum-starved and labeled as described in Fig. 4. Cells left untreated (*open symbols*) or preincubated with 1% ethanol (*solid symbols*) were stimulated with TNF (○, ●), OAG (△, ▲), and PMA (□, ■). Changes of labeled DAG were estimated by two-dimensional scanning of the autoradiographs after separation of the neutral lipids by TLC. Results from three experiments are shown (± SEM).

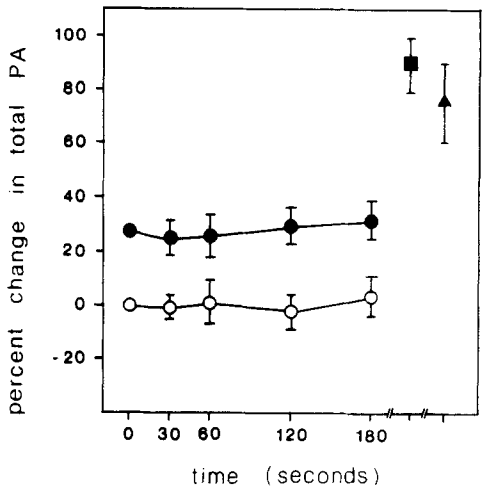

Figure 6. TNF has no effect on total PA mass. U937 cells were left untreated (○) or preincubated with propanolol for 5 min (●). Cells were stimulated with 100 ng/ml TNF for indicated times. For control, propanolol-pretreated cells were incubated with 100 nM PMA (■) or 100 μM OAG (▲) for 20 min. Phospholipids were separated by TLC, and the content of PA was determined by charing densitometry as described in Materials and Methods. Results of four experiments (± SEM) are shown.

levels of PA probably by inhibition of a constitutive PA-phosphohydrolase activity, a phenomenon that has already been observed with L-lyso-3-phosphatidylcholine, 1[1^{14}C]-palmitoyl-labeled cells (Fig. 4 *a*). Again, TNF treatment did not induce PA accumulation. Thus, several independent lines of evidence support the conclusion that TNF-induced DAG formation does not result from operation of a PLD followed by PA degradation.

PLA_2 Is Not Required for TNF Activation of PKC. Since the cleavage products of PLA_2, free arachidonic acid, and lyso-PC are potential activators of PKC (64–66), it was finally necessary to examine whether TNF might stimulate PKC via activation of PLA_2. To this, PLA_2 activity was monitored by TLC analysis of [^{14}C]-labeled lyso-PC in [^{14}C]choline-prelabeled U937 cells. TNF treatment resulted in an ~70% increase in lyso-PC, indicating that TNF, indeed, stimulates PLA_2 (Fig. 7). However, maximal PLA_2 activation occurred between 10 and 15 min, clearly preceded by the time course of DAG production (Fig. 2 *a*) and PKC activation (Fig. 8 *b*). These data were confirmed by experiments where the cells were prelabeled with [^{3}H]arachidonic acid and liberation of free arachidonic acid was observed 15 min after TNF treatment (own unpublished observation), which is in accordance with the time course of PLA_2 activation by TNF described for human synovial cells (22) and human neutrophils (26). The delayed time course of PLA_2 activation strongly argues against a role of PLA_2 in mediating TNF activation of PKC.

The formation of 1'2'DAG Is a TNF Receptor-dependent Event. We next wanted to examine whether the production of

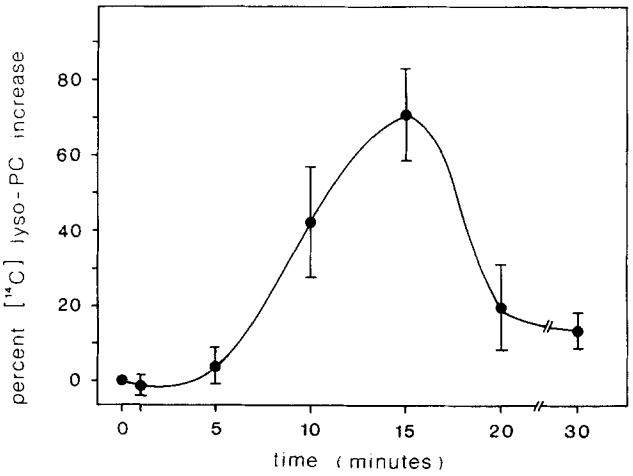

Figure 7. Activation of PLA_2 by TNF. U937 cells were prelabeled with [methyl ^{14}C]choline for 48 h, serum-starved for 3 h, and incubated with 100 ng/ml TNF for indicated times. [^{14}C]-labeled phospholipids were analyzed by TLC and the content of lyso-PC calculated as percent of untreated controls. Data from four experiments (± SD) are shown.

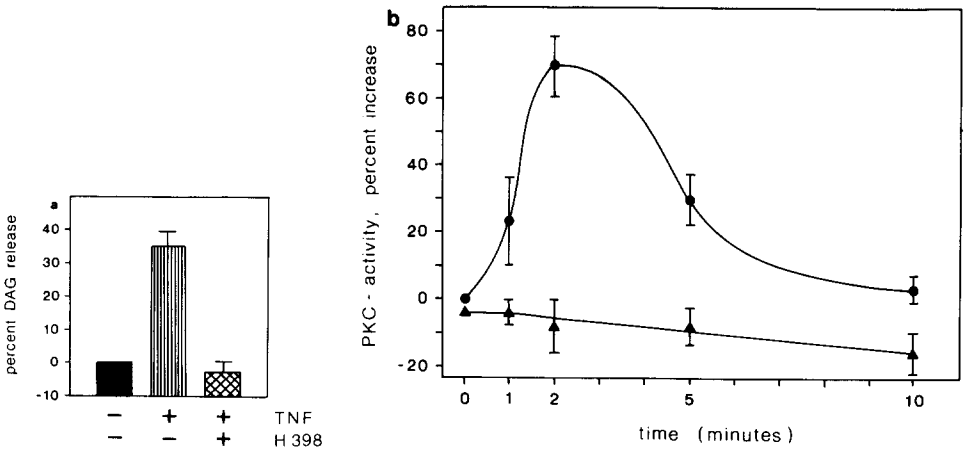

Figure 8. Monoclonal anti-TNF receptor antibody H938 inhibits both TNF-induced 1'2'DAG production and PKC activation. (*a*) U937 cells were labeled with [^{14}C]oleic acid for 16 h, serum-starved for 4 h, and incubated with 250 μg/ml anti-TNF receptor antibody H398 for 15 min at 37°C before treatment with 50 ng/ml TNF for 60 s, [^{14}C]-1'2'DAG content was analyzed by TLC and autoradiography, and the TNF-induced release in 1'2'DAG estimated as percent of untreated controls. (*b*) U937 cells left untreated (●) or pretreated with 250 μg/ml H398 for 30 min at 37°C (▲) were stimulated with 100 ng/ml TNF for indicated times. PKC activity was determined by [^{3}H]PBt$_2$ binding to whole cells as described in Materials and Methods. Percent maximal binding was calculated based on the means of quadruplicate assays (± SEM) from three experiments.

1'2'DAG can be linked to TNF/TNF receptor interactions. U937 cells express ~4,100 ± 870 TNF binding sites per cell (53). It has been suggested that the 60-kD chain of the TNF receptor is essential for TNF signal transfer reactions (21). A monoclonal anti-TNF receptor p60 antibody, H398, was used in our study that competes for TNF binding and can inhibit a number of cellular responses to TNF (21). As shown in Fig. 8 *a*, pretreatment with mAb H398 completely abolished the TNF-induced release of 1'2'DAG in cells labeled with [^{14}C]arachidonic acid, demonstrating that activation of the PC-specific phospholipase is a TNF receptor–dependent event. As expected, pretreatment with mAb H398 also prevented TNF-induced activation of PKC (Fig. 8 *b*), indicating that the TNF receptor p60 chain is also indispensable for PKC activation.

TNF-induced DAG Production Is Required for PKC Activation. We finally investigated whether TNF-induced DAG production is responsible for the subsequent activation of PKC. TNF-mediated activation and translocation of PKC in U937 cells has been demonstrated in a previous report (30). First, TNF doses required for either DAG production or PKC activation were compared. PKC stimulation by TNF was measured by [^{3}H]PBt$_2$ binding of whole U937 cells, which has been shown to reveal essentially identical results when compared to the analysis of PKC activation by histone III-S phosphorylation assays (30, and see Fig. 11). As shown in Fig. 9, half-maximal PKC activation was observed at 1.0 ng/ml TNF, which corresponds well with the TNF doses required for DAG production (see Fig. 2 *b*). In a second approach, we used a phospholipase inhibitor, BPB, an alkylating agent that can modify histidine residues (67) and that has been shown to inhibit, e.g., Ca^{2+}-independent, PC-specific PLC (68). This agent was used to dissect the role of DAG production for PKC activation in TNF-treated cells. As shown in Fig. 10 *a* BPB at 100 μM completely blocked the TNF-induced DAG release. Basal DAG concentrations were not affected. BPB also completely inhibited TNF-stimulated PKC activation (Fig. 10 *b*) as monitored by histone III-S phosphorylation. In contrast, BPB did not block direct PKC stimulation by PMA (Fig. 11), demonstrating that PKC itself is not sensitive to BPB. It is therefore, concluded that the activation of a PC-specific phospholipase providing DAG represents an important functional link between TNF receptors and PKC.

Discussion

Although the pleiotropic cellular effects of TNF are well established, little is known about the precise sequence of molecular events that link the interaction of TNF with its cell

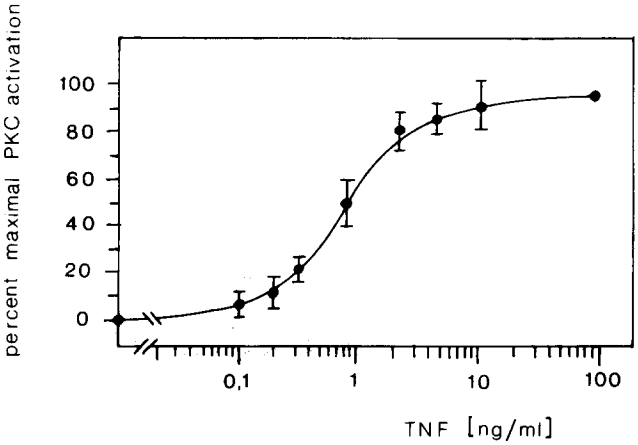

Figure 9. TNF dose-dependent activation of PKC. U937 cells were incubated with indicated TNF concentrations for 3 min at 37°C. Activation of PKC was determined by [^{3}H]PBt$_2$ binding to whole cells as described in Materials and Methods. Percent maximal binding was calculated based on the means of quadruplicate assays (± SEM) from three experiments.

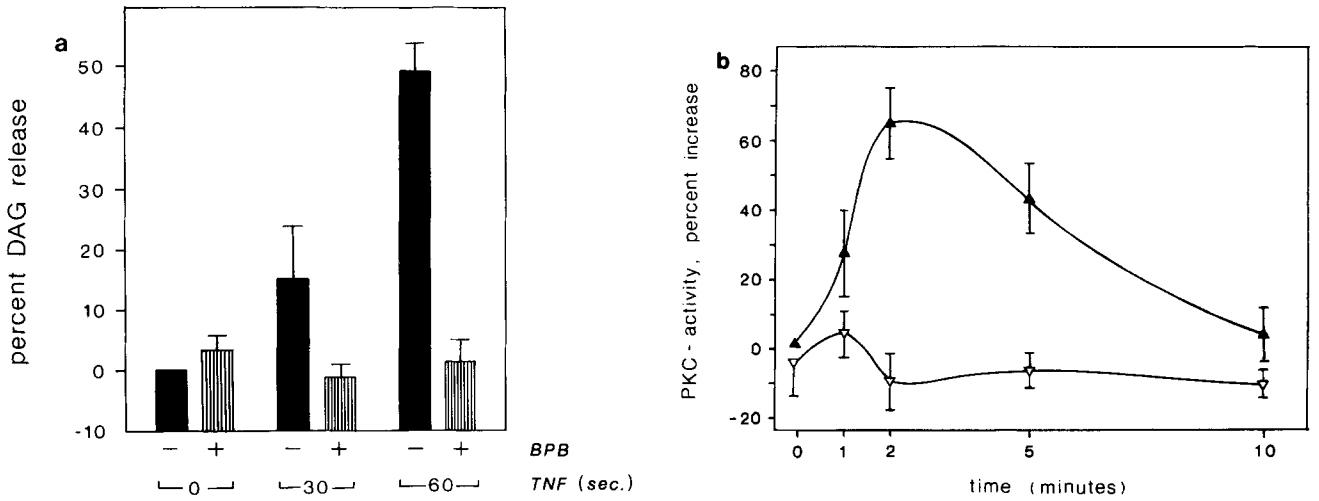

Figure 10. Inhibition of TNF-induced 1'2'DAG production and PKC activation by BPB. (*a*) U937 cells were prelabeled with [^{14}C]arachidonic acid for 16 h and treated in the absence (■) or presence of 100 μM BPB (▥) with 100 ng/ml TNF for indicated times. [^{14}C]1'2'DAG content was evaluated as described in the legend to Fig. 2 *a*. Results from three experiments (± SD) are shown. (*b*) U937 cells were treated in the absence (▲) or presence of 100 μM BPB (▽) with 100 ng/ml TNF for indicated times. PKC activity was determined by [^{3}H]PBt$_2$ binding. PKC activity is given as percent of maximal binding calculated from the means of quadruplicate assays (± SEM) from three experiments.

surface receptors to the transcriptional apparatus of the nucleus. Several lines of evidence suggest that protein phosphorylation may play an important role: first, protein kinase inhibitors could block TNF-mediated gene induction (27), and second, TNF treatment results in rapid phosphorylation of small stress or heat shock proteins (33, 35), of the receptor for epidermal growth factor (37), and of a number of unidentified cytosolic proteins in the range of 26–45 kD (32, 38). Some of these phosphorylation events may be mediated by PKC, a major kinase system involved in many hormone signal transduction pathways (see reference 69 for review).

Interestingly, the kinetics of TNF-induced PKC translocation were found to differ between various cell lines, suggesting a tissue-specific role for PKC in TNF signaling (30). In this report, we have addressed the molecular mechanisms that mediate TNF activation of PKC. Apparently, TNF does not induce a raise of intracellular Ca^{2+} levels through IP_3 turnover leading to the release of Ca^{2+} from internal stores. On the other hand, stimulation of TNF receptors can trigger the activation of PLA_2 (22–26), leading to the formation of arachidonic acid and lysophosphatidates. Both metabolites are potential activators of PKC (64–66). However, the time course of TNF-induced PLA_2 activation was significantly delayed when compared to that of PKC activation (Fig. 12). Thus, PLA_2 action does not seem to be required for TNF activation of PKC.

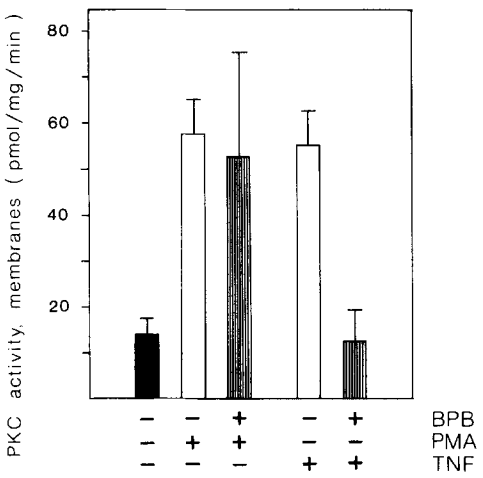

Figure 11. BPB does not inhibit PMA-induced PKC activation. U937 cells were incubated with 100 μM BPB for 45 min or left untreated. Cells were then stimulated with 20 ng/ml PMA for 2 min or with 100 ng/ml TNF for 3 min. Cells were homogenized, and membrane-associated PKC activity was determined by histone III-S phosphorylation assays. Results of three experiments (± SEM) are shown.

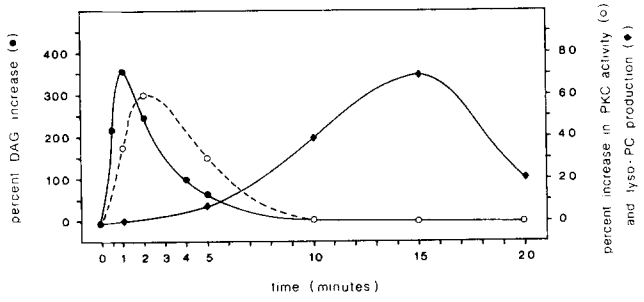

Figure 12. Sequential activation of PC-specific phospholipase, PKC, and PLA_2 by TNF in U937 cells. TNF-induced production of [^{14}C]1'2'DAG (●) was measured in [^{14}C]arachidonic acid-prelabeled cells as described in the legend to Fig. 2. PKC-activity (○ – – – ○) was determined by [^{3}H]PBt$_2$ binding assays, and activation of PLA_2 (◆) was assessed by measuring the liberation of [^{14}C]lyso-PC from [^{14}C]choline-prelabeled cells (see Fig. 7).

In the present report, we now show that TNF stimulates the rapid production of 1'2'DAG, a "classical" activator of PKC. The time course of DAG liberation precedes TNF-induced PKC activation (Fig. 12), suggesting that this component may mediate TNF stimulation of PKC. This assumption is further supported by the observation that both DAG production and PKC stimulation are initiated at similar TNF concentrations. Interestingly, DAG is liberated independent from PIP_2 cleavage, but rather results from phosphatidylcholine breakdown. PC as source for DAG production was identified by selective isotopic labeling of membrane phospholipids in U937 cells. Of note, increments of measurable DAG production depended on the radioactive precursor used for labeling the cellular phospholipids. Best results were obtained by short-term incubation of U937 cells with L-lyso-3-phosphatidylcholine, 1-[1^{14}C]palmitoyl, which revealed a 357% increase of DAG levels over untreated control. With this radioactive precursor a highly efficient labeling of the PC pool was achieved, that is, 72% of the label was incorporated in PC compared with 32.5%, when cells were equilibrated with [^{14}C]arachidonic acid label (Table 1).

The strong influence of the isotopic precursor used for metabolic labeling on the amount of detectable DAG production merits consideration. It seems as if lysophosphatidate-acyltransferase converting the radioactive precursor lyso-PC to the PC substrate is localized in close proximity to the TNF-responsive PC pool within U937 cell membranes. Indeed, differential compartmentation of labeled phosphatidylcholine pools has been documented according to the radioactive precursor used (70). In addition, hormone-responsive and -unresponsive phospholipid pools have been described in pancreatic islets (71). Thus, our observation, that use as radiolabeled precursors of either L-lyso-3-phosphatidyl [methyl ^{14}C]choline or L-lyso-3-phosphatidylcholine, 1-[1^{14}C]-palmitoyl revealed most pronounced PC decrease and increments of both pchol and DAG, may be explained by the existence of a select PC pool readily accessible and sensitive to TNF receptor–activated PLC.

In the absence of IP_3 turnover, DAG can be produced either directly by PLC or indirectly by PLD in combination with PA-phosphohydrolase. As demonstrated in this report, several observations argue against an involvement of PLD in TNF-stimulated DAG production. First, in TNF-treated cells, the levels of PLD's primary reaction products, PA and choline, remained unchanged independent of the radioactive precursor used. Second, in the presence of the PA-phosphohydrolase inhibitor propanolol, TNF-induced DAG-production was unimpeded. Since propranolol prevents dephosphorylation of PA (55, 58), an inhibition of DAG production, would have been expected if PLD and PA-phosphohydrolase were involved in TNF-induced DAG generation. Finally, in the presence of ethanol, which substitutes for H_2O in the transphosphatidylation reaction catalyzed by PLD (57, 59, 60), TNF-induced formation of PEt was not detectable. Since PA metabolization to PEt in the presence of ethanol prevents production of DAG (57), an inhibition of TNF-stimulated DAG formation would be expected, if PA was the source for DAG. Inhibition of TNF-induced DAG production, however, was not observed after treatment of cells with ethanol. In concert, these findings indicate that PLD is not activated by TNF receptors within the time frame investigated. Rather, the time courses of PC degradation and production of both DAG and pchol strongly suggest the operation of a PC-PLC. Moreover, the maximal amounts of pchol produced were equivalent to the amount of PC hydrolyzed, supporting the conclusion that TNF activates a PLC that directly hydrolyzes PC to pchol and DAG.

DAG, in turn, appears to be the main mediator of TNF activation of PKC. The phospholipase inhibitor BPB did not only block DAG formation, but also TNF-induced PKC activation. It should be noted that BPB displays a broad range of inhibitory action, which is not restricted to phospholipases. However, since BPB did not inhibit PKC directly (Fig. 8), our observations suggest that BPB inhibits TNF stimulation of PKC by blocking DAG generation.

Both DAG release and PKC activation could be completely blocked by a mAb, H398, specific for the 60-kD TNF receptor (21). Thus, the TNF receptor p60 appears to be an essential mediator of TNF-induced activation of both PC-PLC and PKC, which may account for (at least in part) the inhibitory action of mAb H398 on diverse TNF responses such as growth inhibition, HLA, or IL-2 receptor gene expression (21). Recent evidence indicates that many growth factors like epidermal growth factor, platelet-derived growth factor, insulin-like growth factor 1 induce by PC hydrolysis DAG production for prolonged periods of time and, thus, cause sustained activation of PKC (72–75). In contrast to these long-term effects, TNF mediates rapid and transient DAG generation as demonstrated in this study, resulting in rapid and transient PKC activation. Of note, TNF-induced DAG production from PC was also observed with other cell types, including the cervix carcinoma cell line HeLa and the leukemic promyelocytic cell line HL-60 (our unpublished observation), indicating that TNF-triggered hydrolysis is not restricted to U937 cells. The mechanism. The mechanism of Ca^{2+}-independent short-term DAG production by TNF via PC-PLC activation is reminiscent of the mechanisms recently described for IL-1 (76), IL-3 (77), CSF-1 (78), and IFN-α (79), indicating a possible common role for PC-PLC and PKC activation in the signaling pathways of these cytokines that may differ from that of other hormones.

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