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Protein Kinases A and C Positively Regulate G Protein–Dependent Activation of Phosphatidylinositol-Specific Phospholipase C by Tumor Necrosis Factor-Alpha in MC3T3-E1 Osteoblasts

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Abstract The role(s) of protein kinases in the regulation of G protein-dependent activation of phosphatidylinositol-specific phospholipase C by tumor necrosis factor-alpha was investigated in the osteoblast cell line MC3T3-E1. We have previously reported the stimulatory effects of tumor necrosis factor-alpha and $A1F_4^-$, an activator of G proteins, on this phospholipase pathway documented by a decrease in mass of PI and release of diacylglycerol. In this study, we further explored the mechanism(s) by which the tumor necrosis factor or $A1F_4^-$-promoted breakdown of phosphatidylinositol and the polyphosphoinositides by phospholipase C is regulated. Tumor necrosis factor-alpha was found to elicit a 4–5-fold increase in the formation of [^{3}H]inositol-1,4-phosphate and [^{3}H]inositol-1,4,5-phosphate; and a 36% increase in [^{3}H]inositol-1-phosphate within 5 min in prelabeled cells. [^{3}H]inositol-4-phosphate, a metabolite of [^{3}H]inositol-1,4-phosphate and [^{3}H]inositol-1,4,5-phosphate, was found to be the predominant phosphoinositol product of tumor necrosis factor-alpha and $A1F_4^-$-activated phospholipase C hydrolysis after 30 min. In addition, the preincubation of cells with pertussis toxin decreased the tumor necrosis factor-induced release of inositol phosphates by 53%. Inhibitors of protein kinase C, including Et-18-OMe and H-7, dramatically decreased the formation of [^{3}H]inositol phosphates stimulated by either tumor necrosis factor-alpha or $A1F_4^-$ by 90–100% but did not affect basal formation. The activation of cAMP-dependent protein kinase, or protein kinase A, by the treatment of cells with forskolin or 8-BrcAMP augmented basal, tumor necrosis factor-alpha and $A1F_4^-$-induced [^{3}H]inositol phosphate formation. Therefore, we report that protein kinases can regulate tumor necrosis factor-alpha-initiated signalling at the cell surface in osteoblasts through effects on the coupling between receptor, G-protein and phosphatidylinositol-specific phospholipase C. J. Cell. Biochem. 65:198–208.

Key words: tumor necrosis factor-alpha; G protein; phosphatidylinositol-specific phospholipase c; protein kinases; osteoblasts

Tumor necrosis factor-alpha (TNF-alpha) is a polypeptide synthesized and secreted by activated macrophages [Beutler, 1989; Carswell et al., 1975; Matthews, 1978; Mannel, 1980] as a mediator of the inflammatory response to injury, infection or malignancy [Old, 1985; Beutler, 1986]. TNF was initially classified by its proposed role in the hemorrhagic necrosis and regression of tumors in experimental animals injected with BCG [Beutler, 1989]. The direct anti-tumor effects of TNF were later confirmed by its cytotoxic and cytostatic activities in vitro against a number of tumor cell lines [7–10]. In addition to its effects on tumor cells, TNF-alpha exhibits a diverse range of biological actions on a variety of cell types that result from its influ-

Abbreviations used: BCG, Bacillus Calmette-Guerin; I-1-P, inositol-1-phosphate; I-4-P, inositol-4-phosphate; I-1,4-P, inositol-1,4-phosphate; I-1,4,5-P, inositol-1,4,5-phosphate; PGE_2, prostaglandin E_2; PGF_{2alpha}, prostaglandin F_{2alpha}; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; TGF-beta, transforming growth factor-beta; TNF-alpha, tumor necrosis factor-alpha.

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ence on growth, differentiation, and function [for reviews, see Beutler, 1989; Old, 1985; Beutler, 1986; Sherry, 1988; Beutler, 1988]. Although TNF activates several types of immune cells, such as neutrophils [Shalaby et al., 1985] monocytes [Philip, 1986], and eosinophils [Silberstein, 1986], other effects of TNF diverge from its function(s) strictly as a local proinflammatory hormone. These include the mediation of cachexia or wasting associated with chronic diseases [Sherry, 1988], the inhibition of lipogenic gene expression [Beutler et al., 1985] and lipoprotein lipase activity [Price et al., 1986] in adipocytes, stimulation of collagenase in synovial cells [Dayer et al., 1985] and mitogenesis in fibroblasts [Palombella, 1989]. In addition, TNF exerts prominent metabolic effects in bone tissue [Beresford, et al., 1984; Gowen et al., 1984; Krakauer et al., 1985; Bertolini et al., 1986; Sato et al., 1986], where signals for bone remodeling or immunological responses to cancer, infection or autoimmune diseases such as arthritis are often responsible for accelerated bone turnover.

Recent evidence suggests that activation of sphingomyelin breakdown to release ceramide may represent an important signalling pathway for TNF in multiple cell types [Schutze et al., 1994]. Despite extensive investigation, no molecular mechanism underlying the broad physiological effects of TNF in numerous tissues has been conclusively identified. TNF's activity as a biological modifier depends upon its initial binding to specific high affinity receptors which have been demonstrated to be on the surface of every type of nucleated cell tested [Kull, 1988]. These receptors have been identified on malignantly transformed cells that are sensitive as well as resistant to the cytotoxic actions of TNF [Tsujimoto et al., 1985]. Moreover, although the sensitivity to TNF's actions can be modified by the down regulation of its receptors [Unglaub et al., 1987], the cellular responsiveness to TNF is not directly dependent on either receptor number or affinity [Lewis et al., 1987]. These observations suggest that post-receptor mechanisms regulate the cellular specificity of at least some of TNF's physiological actions. It has been suggested that cAMP [Zhang et al., 1988] and a pertussis toxin- sensitive G protein [Imamura et al., 1988] each play a role both in TNF-evoked signalling and biological effect. However, no theory has been proposed which explains how a multiplicity of cellular responses for TNF can be transduced by the limited number of distinct membrane signalling systems available as control mechanisms.

We have previously demonstrated that the effects of TNF-alpha on prostaglandin synthesis in MC3T3-E1 osteoblasts by TNF depends upon its activation of PI-specific PLC [Rapuano, 1991], which is an important source of membrane-derived second messengers [Majerus et al., 1986]. The heterogeneity of both the PLC family [Rhee et al., 1989] and that of the family of G proteins that regulate this enzyme [Simon et al., 1991] is extensive, and both signal molecules may in turn be regulated by protein phosphorylation [Rhee et al., 1989; Simon et al., 1991]. Therefore, the signalling systems that transduce the biological effects of TNF can be better understood by elucidating overall cellular mechanisms that regulate G protein-dependent release of second messenger products of PI-specific PLC. In view of these considerations, we examined the modulatory influences of protein kinases A and C on the responsiveness of PI-specific PLC to activation by TNF-alpha in osteoblasts. We show that the TNF-induced activation of phosphoinositide breakdown by PLC is largely dependent on a pertussis toxin-sensitive G protein. We also report that the TNF-alpha receptor-mediated generation of second messengers of cellular activation can be flexibly regulated by multiple pathways of protein phosphorylation. Based on these findings, this study explores the regulatory complexity of PI-specific PLC signalling as a basis for its transduction into TNF's effects on osteoblast function and the remodeling of mineralized tissue.

METHODS

Materials

Human recombinant TNF-alpha was a gift from Genentech (San Francisco, CA). Alpha-MEM (Modified Eagle's Medium), NCS (newborn calf serum and FBS (fetal bovine serum) were obtained from Gibco Laboratories (Grand Island, NY). H-7 ([1-(5-isoquinolinesulfonyl)-2-methylpiperazine]), HA1004 ([N-(2-guanidinoethyl)-5-isoquinoline-sulfonamide]) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). ET-18-OMe (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine. $3H_2O$ was purchased from Novabiochem (Switzerland). Pertussis toxin, PMA (phorbol 12-myristate, 3-acetate, 4-O-methyl ether), TPA (12-O-tetradecanoyl-phorbol-13-acetate), bovine serum albumin (BSA; Fraction V; essentially fatty acid-free), aluminum chloride, sodium fluoride, and lithium chloride were purchased from Sigma

Chemical Co. (St. Louis, MO). Scinti-Verse E scintillation fluid, ammonium phosphate, and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Springfield, NJ).

Tissue culture flasks (75 cm^2), six-well (9.4 cm^2/well) and 24-well (2.0 cm^2/well) tissue culture plates were obtained from Laboratory Disposable Products (North Haledon, NJ). Flow Scint IV scintillation fluid for flow scintography was purchased from Radiomatic Instruments and Chemical Co. (Meriden, CT). [^{3}H]Arachidonic acid, [^{3}H]I-1-P (inositol-1-phosphate), [^{3}H]I-1,4-P (inositol-1,4-phosphate), [^{3}H]I-4-P (inositol-4-phosphate) and [^{3}H]I-1,4,5-P (inositol-1,4,5-phosphate) were supplied by New England Nuclear (Division of Dupont Co., Wilmington, DE). [^{3}H]Inositol was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Cell Culture

An osteoblast cell line cloned from mouse calvaria, MC3T3-E1, was kindly provided by Dr. M. Kumegawa, Department of Oral Anatomy, Josai Dental University. Cells were cultured in 75 cm^2 tissue culture flasks containing alpha-MEM/5% NCS/5% FBS/0.01% streptomycin/100 U/ml. penicillin G. Flasks were kept in a 5% CO_2/95% air incubator at 37°C.

Measurements of Inositol Phosphates

Confluent cells in six-well plates (only a 5% well-to-well variation in cell number was found in each culture) were labeled with [^{3}H]inositol (10 uCi/well) for 24 h in culture medium. Since cellular phosphoinositide turnover times range from several hours to minutes (polyphosphoinositides) in medium containing serum (lack of G_o-synchrony), this period is sufficient to achieve isotopic equilibrium. Cells labeled for 48 h showed the same levels of the three major classes of soluble [^{3}H]inositol phosphates (see below) liberated by activators of PI-specific PLC (data not shown). After the labeling period, cells were washed 1× with alpha-MEM and incubated for 2 h in [^{3}H]inositol-free alpha-MEM containing culture medium and 20 mM $LiCl_3$ (to inhibit inositol phosphate phosphatase). Modulators were then added for various periods of time. Forskolin and 8-BrcAMP were employed at concentrations (10 uM; 0.1–1.0 mM, respectively) that have been generally used to activate cAMP-dependent protein kinase (PKA); these concentrations have been found to be required in order to achieve at least a half-maximal activation of PKA in MC3T3-E1 osteoblasts using the phosphorylation of a specific PKA substrate Kemptide [Glass et al., 1989] to measure activity [manuscript in preparation]. HA-1004 was used to inhibit PKA (Ki = 2.3 uM) [Hikada et al., 1984]; H-7 (Ki = 6.0 uM) [Hikada et al., 1984; Kawamoto et al., 1984; Inagaki et al., 1984] was used to inhibit PKA and PKC; and Et-18-OMe (Ki = 12 uM); a specific inhibitor of protein kinase C with no activity toward cAMP or cGMP-dependent protein kinases; [Helfman et al., 1983; Parker et al., 1987] was employed as a specific inhibitor of PKC. These compounds were added at concentrations that have been demonstrated in vitro to elicit a 90% or greater decrease in the activity of their respective target enzymes. The phorbol ester tumor promoters TPA and PMA were used at concentrations that were generally higher than those demonstrated to activate PKC in other cells systems. However, these concentrations were found to be required in order to induce a half-maximal or greater stimulation in membrane-associated PKC activity in MC3T3-E1 osteoblasts using the phosphorylation of N-acetylated myelin basic protein, a specific PKC substrate, to measure activity [Yasuda et al., 1990]. At the end of the stimulation period, the media was removed and [^{3}H]inositol phosphates were extracted as previously described [Hakeda et al., 1987]. [^{3}H]I-1-P, [^{3}H]I-4-P, [^{3}H]I-1,4-P, and [^{3}H]I-1,4,5-P were separated on HPLC (with retention times of 17, 18.5, 29, and 37 min, respectively) as previously described [Morgan et al., 1987] using a 220 × 4.6 mm SAX-224 Spheri-10 anion exchange column (10 micron pore size; Brownlee Labs, Santa Clara, CA) and identified by coelution with authentic radioactive standards. The radioactivities of separated inositol phosphates were measured by flow scintography using a Trace II Radioactivity Flow Monitor (Packard Instruments Co.; Downer's Grove, IL) and Flow-Scint IV scintillation fluid (1:8; v/v).

RESULTS

We have previously found that the treatment of MC3T3-E1 osteoblasts with TNF-alpha activates PI-specific PLC, as demonstrated by the specific breakdown of PI, elevation in the mass of diacylglycerol and increased turnover of [^{32}P]orthophosphoric acid and [^{3}H]arachidonic acid in PI [Rapuano, 1991]. Table I shows that

TNF-alpha induces a 36% increase in the release of I-1-P in cells prelabeled with [^{3}H]inositol within 5 min. of incubation. The stimulated release of I-1-P parallels the changes in the mass of PI (decrease) and diacylglycerol (increase) that we have previously demonstrated within the same period of incubation of MC3T3-E1 cells with TNF-alpha [Rapuano, 1991]. In the current study, we have also observed a statistically significant increase in the release of [^{3}H]inositol phosphates from prelabeled MC3T3-E1 osteoblasts from 5252 ± 246 (n = 3) to 8212 ± 406 (n = 3) cpm/sample ($P < 0.05$) within 2 min of exposure to TNF-alpha. Importantly, the metabolism of polyphosphoinsitides by PLC in the same incubates was demonstrated by a simultaneous 4–5-fold increase in the formation of their main direct hydrolysis products, I-1, 4-P and I-1,4,5-P (Table I). In other experiments, TNF-alpha also induced a 3–4-fold increase in the total production of [^{3}H]inositol phosphates in serum-free media from 7814 ± 740 (n = 6) to 27,826 ± 3036 (n = 6) cpm/sample, although osteoblasts were otherwise routinely incubated with TNF-alpha in serum-containing medium (see Methods). We have also demonstrated in an earlier study that $A1F_4^-$, which has been shown to activate a G protein coupled to a PI-specific PLC in fibroblasts [Paris, 1987], also stimulates the production of [^{3}H]inositol phosphates in MC3T3-E1 osteoblasts [Rapuano, 1994]. We showed that, in the presence of $LiCl_3$, [^{3}H]inositol phosphates released from prelabeled osteoblasts accumulate with maximal production observed between 30–60 min (see Table II) [Rapuano, 1994]. Accordingly, in this study, in experiments in which osteoblasts were pretreated with inhibitors of PI-specific PLC, cells were subsequently incubated with stimulators for 30–60 min to attain peak levels of liberated [^{3}H]inositol phosphates in order to accurately test effects of inhibitors. Under these conditions, as shown in Figure 1 and Table II, the main metabolite of PI-specific PLC action on polyphosphoinositides (30 min of TNF incubation) was I-4-P, a demonstrated breakdown product of I-1,4-P and I-1,4,5-P [Morgan et al., 1987]. Table II also reveals that pertussis toxin, which inhibits some G proteins by promoting the ADP-ribosylation of a cysteine residue [Casey et al., 1990; Pang, 1990], blocked 53% of the TNF-stimulated formation of total inositol phosphates. The effects of protein kinase activity on the responsiveness of PI-specific PLC to stimulation by TNF-alpha or $A1F_4^-$ was investigated with activators and inhibitors of protein kinase A and C. Table III demonstrates that the incubation of osteoblasts with phorbol esters, including TPA (1 uM) or PMA (20 uM), which activate cellular protein kinase C [for review see Nishizuka, 1986; Kikkawa, 1986], had no significant effect on basal formation of [^{3}H]inositol phosphates. Despite the lack of effect of phorbol esters on the activity of PI-specific PLC, we have found that PMA (20 uM) caused a substantial breakdown of phospholipids and the release of [^{3}H]arachidonic from prelabeled MC3T3-E1 phospholipids [Rapuano and Bockman, 1996]. In fact, 88% of the arachidonic acid mobilized by phorbol esters was derived from the breakdown of PE by PLC and PLA_2, clearly indicating that other phospholipase pathways distinct from PI-specific PLC are responsive to stimulation by a phorbol ester in osteoblasts [Rapuano and Bockman, 1996]. Importantly, in

TABLE I. Effects of TNF-Alpha on PI Breakdown and Formation of Inositol Phosphates and Diglycerides in MC3T3-E1 Cells*

	% control ng/10^6 cells		% control cpm/sample		
	PI	DG	I-1-P	I-1,4-P	I-1,4,5-P
TNF[a]	83 ± 10 (7)	460 ± 90 (3)[1]	136 ± 7 (4)[2]	524 ± 206 (3)	369 ± 67 (4)[2]

*MC3T3-E1 osteoblasts were incubated with TNF-alpha (10 nM) for 5 min, lipids were extracted and the mass of PI and DG was determined by analysis of inorganic phosphorus and HPLC/UV spectroscopy, respectively. The data, expressed as % control (absence of TNF-alpha) PI or DG mass as shown above, has been previously reported (control levels of PI and DG were 211 ± 19 ng inorganic phosphorus/10^6 cells and 30 ± 5 ng/10^6 cells, respectively) [Rapuano, 1991]. In other experiments, cells were prelabeled with [^{3}H]inositol for 24 h, washed, and incubated with medium alone or medium + TNF-alpha (10 nM) and [^{3}H]-labeled I-1-P, I-1,4-P and I-1,4,5-P were extracted 5 min later, separated, and counted following HPLC as described in Methods. The data is expressed as % control cpm/sample. Values are presented as means ± SE (n = number of independent experiments).

[1]Significantly > control ($P < 0.01$).

[2]>Control ($P < 0.05$).

TABLE II. Effects of Pertussis Toxin on TNF-Alpha-Induced Formation of [^{3}H]Inositol Phosphates*

	cpm/sample		
	I-1-P	I-4-P	I-1,4-P
Control	6302 ± 1232 (6)	8188 ± 1163 (6)	1652 ± 200 (9)
TNF (1 nM)	6658 ± 612 (6)	9208 ± 905 (6)	2810 ± 506 (6)[5]
TNF (10 nM)	14,582 ± 1861 (9)[1]	22,812 ± 2602 (6)[3]	3222 ± 379 (9)[6]
+PT (1 ng/ml)	14,116 ± 1786 (8)	19,424 ± 4114 (5)	2106 ± 203 (7)[7]
+PT (10 ng/ml)	9758 ± 832 (8)[2]	15,565 ± 1367 (6)[4]	1870 ± 259 (9)[8]

*Cells were prelabeled with [^{3}H]inositol for 24 h, washed, and incubated with or without pertussis toxin (PT) at the indicated concentrations for 2 h. TNF-alpha was added at the indicated concentrations and [^{3}H]inositol phosphates were extracted 30 min later and analyzed as described in Table I. Pertussis toxin alone (10 ng/ml) did not diminish the basal release of [^{3}H]inositol phosphates (data not shown). I-4-P is a demonstrated breakdown product of I-1,4-P and I-1,4,5-P [Morgan, 1987]. Levels of inositol-1,4,5-phosphate in this experiment were too low to include in Table. Values are presented as means ± SE (n = number of independent experiments). Individual measurements represent CPM per sample = 1 well (of 2.5 million cells per well; well-to-well variation in cell number was only 5% at confluency—see Methods).

[1]Significantly > control ($P < 0.01$).

[2,4,7]<TNF ($P < 0.05$).

[3]>Control ($P < 0.001$).

[5]>Control ($P < 0.05$).

[6]>Control ($P < 0.005$).

[8]<TNF ($P < 0.01$).

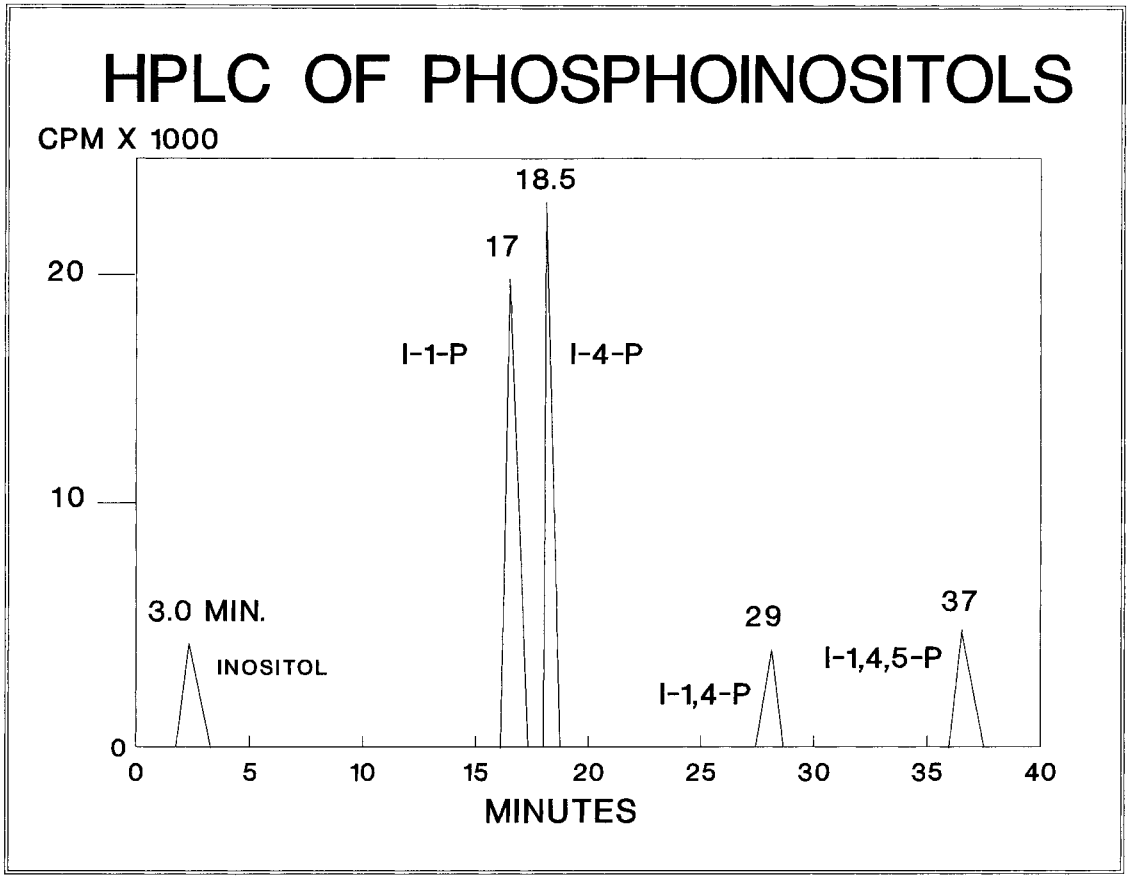

Fig. 1. HPLC profile of [^{3}H]inositol phosphates after stimulation of MC3T3-E1 cells with TNF-alpha (10 nM) for 30 min (see Results).

TABLE III. Effects of Protein Kinase C Agonists and Antagonists on Basal and (TNF-Alpha and ALF_4^-)-Stimulated Formation, Respectively, of [^{3}H]Inositol Phosphates*

	cpm/sample			
Condition	I-1-P	I-4-P	I-1,4-P	I-1,4,5-P
Control[a]	5984 ± 1328 (5)	7056 ± 1391 (6)	1384 ± 347 (6)	476 ± 74 (6)
TPA	8006 ± 2086 (3)	9386 ± 1258 (3)	844 ± 350 (3)	
Et-18-OMe	4487 ± 594 (6)	5624 ± 668 (6)	754 ± 90 (4)	535 ± 76 (6)
PMA	8742 ± 2316 (2)	9930 ± 1356 (2)	1692 ± 864 (2)	—
TNF	12,582 ± 1888 (3)	16,516 ± 1888 (6)[4]	2574 ± 274 (6)[8]	1070 ± 176 (6)[11]
+Et-18-OMe	5980 ± 1577 (3)[1]	8500 ± 886 (3)[5]	1824 ± 264 (2)	516 ± 264 (3)
Control[b]	5513 ± 940 (8)	6364 ± 965 (9)	1392 ± 256 (9)	542 ± 83 (9)
AlF_4^- [b]	17,576 ± 345 (3)[2]	24,072 ± 245 (3)[6]	3872 ± 511 (3)[9]	744 ± 240 (3)
+Et-18-OMe	4204 ± 1218 (3)[3]	5296 ± 1412 (3)[7]	476 ± 160 (3)[10]	376 ± 79 (3)

*Cells were prelabeled with [^{3}H]inositol and washed as described in Table I and incubated with TPA (1 uM), PMA (20 uM), TNF-alpha (10 nM) for 30 min or NaF (10 mM) + $AlCl_3^-$ (10 uM), which forms AlF_4^- as the major species [Godstein, 1964], for 60 min and [^{3}H]inositol phosphates were extracted and analyzed as described in Table I. In some samples, cells were preincubated with an inhibitor of protein kinase C, Et-18-OMe (50 uM) for 45 min before the addition of TNF-alpha or NaF/$AlCl_3^-$. Values are presented as means ± S.E. (n = number of independent experiments).
[a]Control values for experiments with TPA, PMA, or TNF-alpha.
[b]Controls for cells incubated with NaF/$AlCl_3^-$ ± protein kinase inhibitor.
[1,5]Significantly < TNF ($P < 0.05$).
[2,6,9]Significantly > control ($P < 0.001$).
[8]>Control ($P < 0.05$).
[3,7]<AlF_4^- ($P < 0.001$).
[4]Significantly > control ($P < 0.005$).
[10]Significantly < AlF_4^- ($P < 0.01$).
[11]>Control ($P < 0.05$).

cells that were treated with TNF-alpha, the preincubation of osteoblasts with TPA (1 uM) for 30 min increased TNF-evoked stimulation in the production of total [^{3}H]inositol phosphates by 34 ± 7% (n = 3; significantly greater than TNF alone; $P <$ 0.05). In other experiments, cells were pretreated with the specific PKC inhibitor compound ET-18-OMe (see Methods) 45 min before the addition of TNF-alpha or AlF_4^-. As shown in Table III, ET-18-OMe inhibited 96 and 100% of the formation of [^{3}H]inositol phosphates stimulated by TNF and AlF_4, respectively.

Table IV reveals that the the incubation of osteoblasts with the protein kinase A activator forskolin (Table IV) raised the basal levels of mono or polyphosphoinositols 2–3-fold; whereas forskolin and other protein kinase A agonists

TABLE IV. Effects of PKA Agonists on TNF-Alpha-Induced Formation of [^{3}H]Inositol Phosphates*

	cpm/sample			
	I-1-P	I-4-P	I-1,4-P	I-1,4,5-P
Control	6083 ± 588 (12)	7068 ± 579 (12)	1308 ± 86 (11)	940 ± 196 (12)
TNF	19,114 ± 972 (12)[1]	27,430 ± 2227 (12)[2]	3598 ± 511 (12)[3]	1634 ± 211 (15)[4]
Forskolin	12,136 ± 525 (3)[5]	21,312 ± 2156 (3)[6]	2328 ± 240 (3)[7]	1268 ± 109 (3)
TNF + Forskolin	35,712 ± 2907 (6)[8]	50,014 ± 4132 (6)[9]	6082 ± 900 (6)[10]	2938 ± 104 (6)[11]

*Cells were prelabeled with [^{3}H]inositol and washed as described in Methods and incubated with TNF-alpha (10 nM). In some samples, forskolin (50 uM) or 8-BrcAMP (1 mM) was added 20–45 min before the time TNF-alpha was added or before the start of the control (no TNF) incubation period. [^{3}H]inositol phosphates were extracted 30 min after the addition of TNF-alpha and analyzed as described in Table I. Values are presented as means ± S.E. (n = number of independent experiments).
[1–3,5,6]Significantly > control ($P < 0.001$).
[4]>Control ($P < 0.05$).
[7]>Control ($P < 0.05$).
[8]>TNF, forskolin or control ($P < 0.001$).
[9]>Control or TNF ($P < 0.001$) and forskolin ($P < 0.005$).
[10]>TNF or forskolin ($P < 0.05$) and control ($P < 0.001$).
[11]>TNF ($P < 0.005$), forskolin or control ($P < 0.001$).

increased AlF_4^--induced [^{3}H]inositol phosphate formation to the same degree [Rapuano and Bockman, 1996a]. In addition, the combined effects of TNF-alpha and forskolin in cotreated cells were additive for each class of [^{3}H]inositol phosphates (Table IV). Moreover, preincubation with the protein kinase A inhibitor HA1004 blocked 77 and 91%, respectively, of the release of [^{3}H]phosphoinositols stimulated by TNF-alpha or the combination of TNF-alpha (10 nM) + forskolin (50 uM) (Table V). In contrast, HA1004 inhibited only 30% of AlF_4^--promoted [^{3}H]inositol phosphate production (Table V).

DISCUSSION

Although we have previously shown that TNF induces the breakdown of PI in MC3T3-E1 osteoblasts, the present study is the first to demonstrate that the monokine can induce hydrolysis of the polyphosphoinositides to release I-1,4-P and I-1,4,5-P. However, TNF-induced hydrolysis of polyphosphoinositides is most dramatically shown by the formation of I-4-P (a breakdown product of I-1,4-P and I-1,4,5-P) [see Morgan et al., 1987]. That the principal targets of TNF or AlF_4^--stimulated PLC are PIP and PIP_2 and not PI is supported by the findings that I-1,4,5-P and I-1,4,5-P are the major phosphoinositide breakdown products released after 5 min and I-4-P is the major product after 30 min (Tables II–V). I-4-P was also found to be the major phosphoinositol formed in anterior pituitary cells in response to gonadotropin-releasing hormone [Morgan et al., 1987]. The formation of this particular intermediate of polyphosphoinositol metabolism may account for the apparent lack of PI-specific PLC activation (measured strictly by the appearance of I-1,4,5-P) by TNF-alpha, despite a G protein dependency for other actions, observed in one study [Yanaga et al., 1992]. It is not surprising that this report perhaps along with other simi-

TABLE V. Effects of a PKA Inhibitor on the Formation of [^{3}H]Inositol Phosphates Stimulated by AlF_4^- or TNF*

	cpm/sample			
	I-1-P	I-4-P	I-1,4-P	I-1,4,5-P
Control[a]	5824 ± 1016 (6)	6390 ± 1090 (6)	1056 ± 298 (5)	892 ± 308 (6)
HA-1004	7792 ± 634 (11)	8360 ± 599 (11)	970 ± 96 (11)	818 ± 103 (10)
TNF	22,354 ± 1852 (4)[1]	29,724 ± 4294 (5)[5]	2140 ± 214 (6)[11]	1176 ± 250 (6)
TNF + HA-1004	8416 ± 2702 (5)[2]	12,900 ± 3786 (5)[6]	1480 ± 176 (6)[12]	856 ± 156 (6)
TNF + Forsk.	31,806 ± 2818 (9)	46,144 ± 3305 (9)[7]	5136 ± 611 (12)[13]	2246 ± 287 (9)[16]
TNF + Forsk./HA-1004	8012 ± 1502 (3)[3]	11,184 ± 1001 (3)[8]	1060 ± 210 (3)[14]	472 ± 186 (3)[17]
Control[b]	4848 ± 1368 (3)	4880 ± 1548 (3)	732 ± 104 (3)	672 ± 226 (3)
AlF_4^-	16,140 ± 272 (3)[4]	22,890 ± 968 (3)[9]	2250 ± 346 (4)[15]	1134 ± 226 (4)
AlF_4^- + HA-1004	13,842 ± 1500 (3)	16,016 ± 1450 (3)[10]	1962 ± 566 (3)	760 ± 194 (3)

*Cells were prelabeled with [^{3}H]inositol and washed as described in Methods, incubated with TNF-alpha (10 nM) or NaF (10 mM) + $AlCl_3^-$ (10 uM) for 30 min and [^{3}H]inositol phosphates were extracted and analyzed as described in Table I. In some samples, HA-1004 was added 45 min before the addition of TNF or NaF + $AlCl_3^-$. HA1004 had no effects on basal production of [^{3}H]phosphoinositols (data not shown). Values are presented as means ± S.E. (n = number of independent experiments).

[a]Control group for experiments with TNF.

[b]Control group for experiments with AlF_4^- (see Methods).

[1,5,9]>Control ($P < 0.001$).

[2]<TNF ($P < 0.001$).

[3]<TNF + forskolin ($P < 0.001$).

[4]>Control ($P < 0.005$).

[6]<TNF ($P < 0.005$).

[7]>TNF ($P < 0.025$).

[8]<TNF + forskolin ($P < 0.001$).

[10]<AlF_4^- ($P < 0.05$).

[11]>Control ($P < 0.05$).

[12]Significantly < TNF ($P < 0.05$).

[13]>TNF ($P < 0.01$).

[14]<TNF + forskolin ($P < 0.01$).

[15]>Control ($P < 0.01$).

[16]>TNF ($P < 0.025$).

[17]<TNF + forskolin ($P < 0.01$).

lar studies failed to observe this signalling pathway for TNF since the (RIA, ELISA, or HPLC-based) methodologies selected for analysis did not measure I-4-P. In such studies, an observed longterm (over a time course of 10–120 min) activation of other phospholipase pathways, such as phospholipase A_2 (PLA_2), by TNF-alpha, may occur secondarily in response to an earlier PLC-mediated release of second messengers from PI (polyphosphoinositols and diacylglycerol) that activate PLA_2 (via increased intracellular calcium flux and membrane translocation of protein kinase C). Importantly, our earlier study demonstrated a TNF-induced rapid (5 min) decrease in PI mass concomitantly with diacylglycerol formation at a time when the breakdown of other major phospholipids (by any phospholipase pathway) had not been observed [Rapuano, 1991]. The latter finding argues against a rapid cytotoxic effect of TNF that would increase membrane permeability to calcium thereby promoting a nonselective activation of multiple phospholipase pathways. In view of the considerations and findings discussed above, the evidence presented here supports at least a specific activation of PI-specific PLC by TNF in osteoblasts if not a broader role for the enzyme in the diverse cellular effects of TNF. It is also suggested that activation occurs via a G-protein-dependent receptor-mediated mechanism, since such a mechanism preferentially stimulates the PLC-mediated hydrolysis of polyphosphoinositides over phosphatidylinositol [Majerus, 1986].

Two more of our findings in particular support the role of a G protein in the stimulatory action of TNF-alpha on phosphoinositide metabolism in osteoblasts. The finding which most strongly supports the above hypothesis demonstrated that at least half of TNF's effects on phosphoinositol release was sensitive to inhibition by pertussis toxin, which is specific for G proteins. Secondly, we have also shown that the effects of TNF-alpha and AlF_4^-, a known activator of G proteins, are both entirely prevented by a specific protein kinase C inhibitor. This latter finding suggests that TNF-alpha and AlF_4^- share a common protein kinase C-dependent step for the activation of PI-specific PLC. This final common pathway for activation is not dependent upon a direct (protein kinase C-dependent) modulation of the PLC enzyme, since neither protein kinase C agonists or antagonists altered its basal activity. Neither is the permissive action of protein kinase C (or the potentiation by phorbol esters) on TNF's stimulation of phosphoinositide breakdown due to direct effects on the cytokine's receptor, since it has been shown to be down regulated by protein kinase C agonists, leading to a decreased responsiveness to TNF-alpha [Johnson, 1988]. Therefore, we suggest that protein kinase C regulates the activation of PI-specific PLC by phosphorylating G proteins that are required by both TNF-alpha and AlF_4^- for coupling to the phospholipase enzyme. We have shown that the incubation of MC3T3-E1 osteoblasts with TNF-alpha stimulates the cell's production of PGE_2 [Rapuano, 1991], which can activate PI-specific PLC via a G protein-dependent mechanism [Tokuda, 1991]. However, the increase in PGE_2 levels clearly lagged behind (2 h; data not shown) that of inositol phosphates (5 min; Table I), whose production, therefore, was independent of prostaglandin synthesis in TNF-alpha-treated cells. Accordingly, this study is the first to demonstrate a requirement for G proteins in the direct coupling between TNF and PI-specific PLC.

TNF-alpha and AlF_4^- have been shown to induce cAMP formation in fibroblasts [Zhang et al., 1988] and MC3T3-E1 osteoblasts [Tokuda et al., 1993], respectively. Therefore, some of their effects in this study may derive from the activation of adenylate cyclase, protein kinase A and cross-talk with PI-specific PLC coupled to other cellular receptors through multiple G proteins. We can not rule out that cAMP plays a minor role in the actions of AlF_4^-, since 30% of its stimulation of phosphoinositide breakdown was sensitive to inhibition by HA1004. However, we have shown that TNF-alpha promotes the release of [^{3}H]inositol phosphates within 2 min of exposure, before an increase in cAMP levels was measured in fibroblasts incubated with TNF-alpha [Zhang et al., 1988]. Moreover, our other findings have shown that 53% of TNF's stimulation of [^{3}H]inositol phosphate formation was blocked by pertussis toxin, whereas the activation of PI-specific PLC in MC3T3-E1 cells by bradykinin [Yanaga et al., 1991] or PGE_2 [Tokuda et al., 1991] was either insensitive or weakly sensitive (25%), respectively, to pertussis toxin. In addition, it has been shown that protein kinase A agonists had no significant effect on PGE_2-induced phosphoinositol formation [Kozawa et al., 1992]. We have also found that PGF_{2alpha}, which we have found is

the most potent activator of this phospholipase pathway in MC3T3-E1 cells, is completely resistant to the inhibitory effects of pertussis toxin [unpublished findings]. Therefore, it is unlikely that the action of TNF-alpha on PI-specific PLC results indirectly from cAMP-dependent regulation of G proteins that are coupled to other membrane receptors for prostaglandins or other hormones.

Although pertussis toxin generally inactivates G proteins that are inhibitory to the effector molecule [Simon et al., 1991], phosphoinositide breakdown can be inhibited by pertussis toxin in a number of cell types [Brandt et al., 1985; Pfeilschifter, 1986; Nakamura, 1985; Smith et al., 1985]. Our findings, suggesting that TNF-alpha activates multiple G proteins (both pertussis toxin-sensitive and insensitive), that are stimulatory to PI-specific PLC, have not been previously reported in any cell system. Alternatively, our data suggests also that TNF-alpha may activate a single pertussis toxin-sensitive G protein (which may not be completely accessible to pertussis toxin in MC3T3-E1 cells) that is phosphorylated perhaps at different sites by PKA and PKC. In summary, this study presents new evidence that G protein(s) coupled to a membrane TNF-alpha receptor may be independently regulated by different pathways of protein phosphorylation. Furthermore, although earlier studies have provided evidence that G proteins are rapidly phosphorylated [Carlson et al., 1989] in some cases via cAMP [Gunderson, 1990] or protein kinase C-mediated [Katada et al., 1985] mechanisms, no previous investigation has identified multiple protein kinase pathways regulating a single receptor/G protein-mediated signalling response.

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