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TGF-β Signaling in A549 Lung Carcinoma Cells: Lipid Second Messengers

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Abstract Transforming growth factor-β (TGF-β) is a potent inducer of numerous extracellular matrix components, largely through a transcriptional mechanism. To define the postreceptor signaling pathways used by TGF-β in the induction of extracellular matrix gene expression, we have utilized the human lung carcinoma cell line, A549, in transfection experiments with the TGF-β inducible reporter construct, p3TP-Lux. Previous work from this laboratory using pharmacologic agents suggested that a phosphatidylcholine-specific phospholipase C and protein kinase C may be involved in early aspects of TGF-β signaling. Here we provide evidence that TGF-β induces a rapid and transient increase in diacylglycerol (DAG) production. When cells transfected with the p3TP-Lux reporter plasmid are simultaneously treated with TGF-β and a DAG kinase inhibitor, we observed a higher level of luciferase than with TGF-β alone. We also find elevated levels of phosphocholine in cells following TGF-β treatment. Further, exogenously added bacterial phosphatidylcholine phospholipase C (PC-PLC) is capable of inducing expression of the p3TP-Lux reporter to the same extent as TGF-β indicating that the bacterial PC-PLC can mimic the TGF-β effect. In contrast, neither hexanoyl sphingosine (a ceramide analogue) nor arachadonic acid induce expression of the p3TP-Lux reporter. Measurements with the fluorescent, calcium-sensitive dye, FURA2, indicated that there was no change in intracellular calcium in response to TGF-β. Furthermore, buffering intracellular calcium with the calcium chelating agent BAPTA/AM failed to block TGF-β induction of the p3TP-Lux reporter. Thus the TGF-β signaling pathway appears to involve the production of diacylglycerol but is independent of calcium. J. Cell. Biochem. 78:588–594, 2000.

Key words: transforming growth factor-β; phosphatidylcholine phospholipase C; diacylglycerol

Transforming growth factor-β (TGF-β) is the prototype for a family of polypeptide factors that induce a wide variety of responses in target cells and tissues. Prominent among these responses is induction of extracellular matrix gene expression, growth inhibition, alteration in cellular differentiation and phenotypic expression, as well as morphogenesis of developing embryos [Massague', 1996]. With regard to extracellular matrix gene expression, TGF-β has been implicated in the induction of fibrotic diseases of kidney, liver, and lung, as well as elevated extracellular matrix deposition in scarring [Border and Noble, 1994]. A wide variety of epithelially derived cell types and some hematopoietic cells are growth inhibited by TGF-β while mesenchymally derived cells show little effects on growth.

Numerous pieces of the postreceptor TGF-β signaling puzzle have been identified. TGF-β signaling is initiated upon ligand binding and activation of heterodimeric receptors possessing intrinsic serine–threonine kinase activity [Wranna et al., 1992]. Reports on postreceptor signaling mechanisms from several laboratories have demonstrated a role for several members of the mitogen-activated protein (MAP) kinase family [Hartsough and Mulder, 1995; Yamaguchi et al., 1995; Shibuya et al., 1996], protein kinase C (PKC) [Halstead et al., 1995; Weiss et al., 1995], novel protein kinases [Atfi et al., 1995], ras and rho G-proteins [Mulder and Morris, 1992; Hartsough et al., 1996; Atfi et al., 1997], and most recently members of the Smad family of proteins [Massague', 1996; Liu et al., 1997; Whitman, 1998, Zawel et al., 1998]. Interestingly, expression of dominant interfering forms of several of these signaling

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intermediates appear to inhibit TGF-β responsiveness both in terms of growth effects and as changes in gene expression [Atfi et al., 1997]. We have specifically targeted extracellular matrix gene expression by using the p3TP-Lux reporter plasmid in an attempt to define a specific signaling pathway for TGF-β [Halstead et al., 1995]. Here we show that TGF-β produces a transient increase in diacylglycerol (DAG) release from phospholipase C-catalyzed hydrolysis of phosphatidylcholine, but has no effect on intracellular calcium. Treatment of A549 cells with the phosphatidylcholine phospholipase C (PC-PLC) inhibitor, D609, blocks TGF-β signaling [Halstead et al., 1995] whereas exogenously added bacterial PC-PLC can mimic TGF-β induction of the p3TP-Lux reporter. Together with previously published evidence, the current data suggest that TGF-β may signal by activation of an isoform of PKC independent of calcium.

MATERIALS AND METHODS

The human lung carcinoma cell line, A549, was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cultures were grown at 37°C in humidified 5% CO_2. Diacylglycerol kinase inhibitor II, wortmannin, AACOCF3, BAPTA/AM, and FURA2/AM were purchased from Calbiochem, Corp., San Diego, CA. *Bacillus cereus* PC-PLC and 4-bromophenacyl bromide were from Sigma Chemical Corp, St. Louis, MO. [methyl-^{14}C]-choline chloride (40–60 mCi/mmol) was obtained from New England Nuclear, Boston, MA and luciferase reagents were purchased from Promega Corp., Madison, WI. DAG kinase assay kit was from Amersham Corp., Arlington Heights, IL. Porcine TGF-β1 and epidermal growth factor (EGF) were from R & D Systems, Minneapolis, MN. Transient transfections with the p3TP-Lux reporter plasmid and assay of luciferase activity were as described previously [Halstead et al., 1995]. For DAG analysis, cells were grown to near confluency in 100-mm glass tissue culture dishes. Culture medium was changed to serum-free DMEM for 60 min, followed by treatment with either TGF-β, EGF, or PC-PLC. Cultures were then extracted on ice with chloroform-methanol and processed for determination of DAG content using the DAG kinase assay kit following manufacturer's protocol.

For intracellular calcium analysis, cells were grown on microscope slide coverslips at low density for maximum cell spreading. Cells were loaded with FURA2/AM by incubation for 3 h in serum-free medium containing 5 μM FURA2/AM. Probenecid (100 μM), an inhibitor of the multidrug resistance transporter, was included to inhibit efflux of the dye. After washing cells several times in phosphate-buffered saline, coverslips were mounted in a microscope slide chamber that permits addition of test agents to living cells while viewing and monitoring. Intracellular calcium was determined by fluorescent digital imaging microscopy (PTI Imagemaster, Pistcataway, NJ). Briefly, images were obtained alternately with excitation at 340 nm and 380 nm and emission at >510 nm. Random cells within the field were selected, and changes in fluorescence were monitored after the addition of TGF-β or other agents. Intracellular calcium was calculated from the ratio of fluorescence determined at 340 and 380 nm according to the method described by Grynkiewicz et al. [1985]. To assess the effect of chelating intracellular calcium on TGF-β responsiveness, A549 cells were transfected with the p3TP-Lux plasmid followed by loading of the cells with the calcium chelating agent, BAPTA/AM at 5 μM for 3 h. Cultures were then treated with TGF-β and luciferase activity was determined.

To measure hydrolysis of phosphatidylcholine in response to TGF-β, cells were metabolically labeled with ^{14}C-choline (50 μCi/dish) for 48 h following the protocol of Larrodera et al. [1990]. Following treatment with TGF-β, chloroform-methanol extracts were prepared and ^{14}C-phosphocholine was released to the aqueous phase determined by thin layer chromatography and scintillation counting [Bligh and Dyer, 1959].

RESULTS AND DISCUSSION

TGF-β Stimulates DAG Release

A549 cells were grown to near confluency followed by transfer to serum-free medium for 1 h. Cultures in duplicate were treated with either TGF-β, EGF, or bacterial PC-PLC for the indicated times. Chloroform-methanol extracts were prepared and DAG content was measured as described above. The results

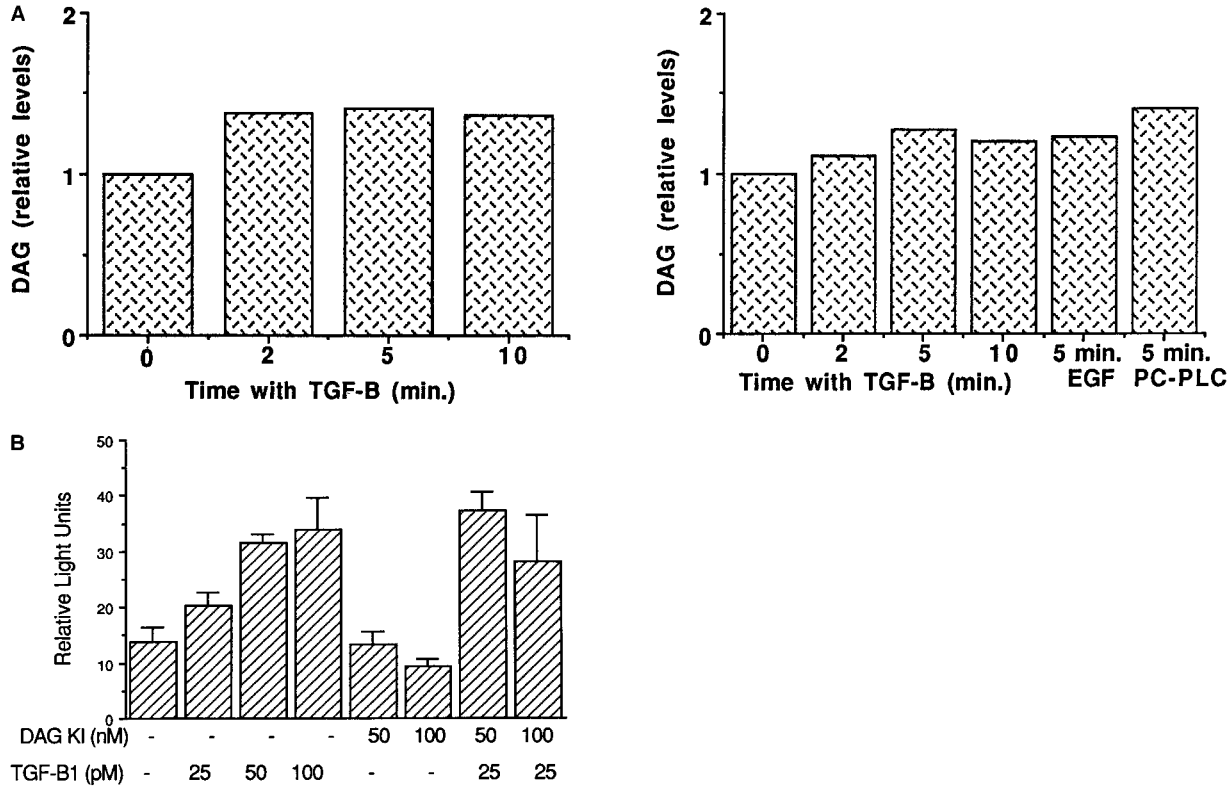

Fig. 1. **A:** Transforming growth factor (TGF)-β induces a transient increase in diacylglycerol (DAG) production. A549 cells were grown for 24 h in 100-mm glass petri dishes. For treatments, the growth medium was replaced with serum-free Dulbecco's modified Eagle's medium supplemented with either 200 pM TGF-β1 or 200 ng/ml epidermal growth factor (EGF) for the indicated times. Monolayers were then extracted in ice with chloroform-methanol as described in Materials and Methods. DAG content was analyzed using the DAG kinase assay kit from Amersham, Corp. Thin-layer chromatography plates were quantitated using a Molecular Dynamics Storm 840 Scanner. Two separate experiments are shown with the determinations preformed on duplicate cultures. The data were normalized to untreated, control cultures and represent the averages of determinations performed on duplicate cultures. The duplicate values varied by less than 10%. **B:** Inhibition of DAG kinase enhances TGF-β induction of p3TP-Lux. A549 cells were transiently transfected with the p3TP-Lux plasmid as described. The following day, cultures were treated in serum-free medium with 25, 50, or 100 pM TGF-β1 alone or with the indicated concentrations of DAG kinase inhibitor II (Calbiochem) alone or with 25 pM TGF-β. Results presented represent the averages of determinations done on triplicate cultures and corrected for transfection efficiency using a β-galactosidase control plasmid.

shown in Fig. 1A indicate that TGF-β induces a rapid but transient increase in DAG release comparable to that observed with EGF. Treatment of cultures with bacterial PC-PLC induced a slightly higher elevation in DAG release. The results from two separate determinations, each performed in duplicate, are shown in Fig. 1A. Although DAG can bind to and activate several PKC isoforms, DAG is not known to accumulate in cells. Rather, it is rapidly converted to phosphatidic acid by the action of DAG kinase for reutilization in lipid synthesis. Blocking the conversion of DAG to phosphatidic acid via DAG kinase inhibitors has been used to enhance cellular responses to vasopressin and thrombin [de Courcelles et al., 1985; de Courcelles et al., 1989], thus implying that DAG is an important second messenger for these factors. To further emphasize a role for DAG in TGF-β signaling, A549 cells were transfected with the p3TP-Lux reporter plasmid followed by treatment with a suboptimal concentration of TGF-β (25 pM TGF-β), alone or in the presence of the DAG kinase inhibitor II. Results shown in Fig. 1B demonstrate that by inhibiting the conversion of DAG to phosphatidic acid, thus maintaining elevated DAG levels, the TGF-β response is enhanced, yielding a response comparable to that observed with 100 pM TGF-β.

TGF-β Signaling is Calcium Independent

The classic means of activation of PKC is via hydrolysis of phosphatidylinositol with con-

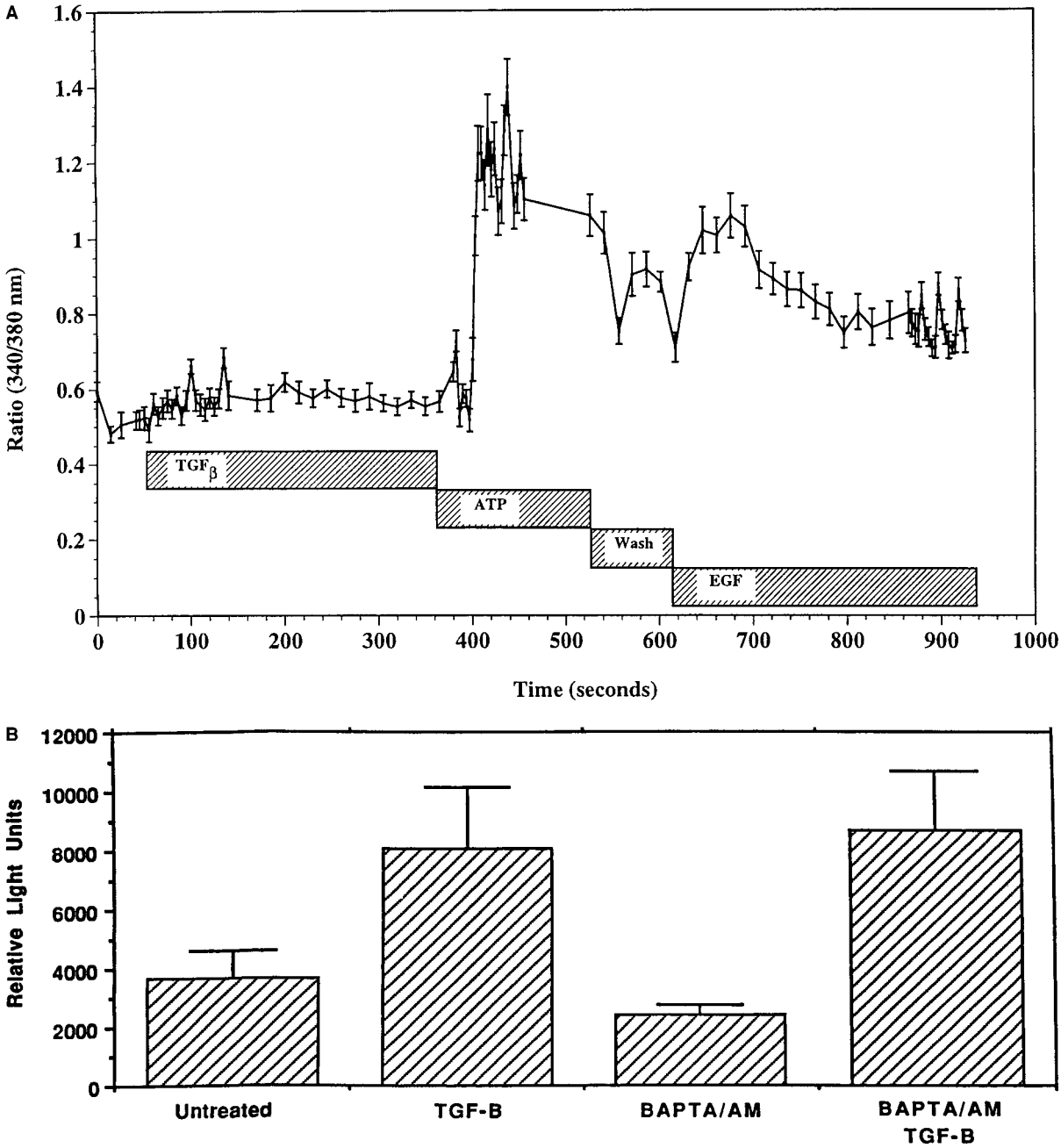

Fig. 2. Calcium-independence of transforming growth factor (TGF)-β signaling. **A:** Changes in intracellular calcium concentration were monitored by loading A549 cells with FURA2/AM and changes in fluorescence monitored in response to TGF-β, ATP, or epidermal growth factor (EGF) as described in Materials and Methods. The tracing shown represents the averages for eight cells monitored simultaneously. Essentially identical results were observed in four experiments. **B:** Chelating intracellular calcium with BAPTA/AM does not prevent TGF-β responsiveness. A549 cells were transfected with the p3TP-Lux plasmid as described. The following day, cells were loaded with BAPTA/AM (5 μM for 3 h) followed by treatment with or without 100 pM TGF-β1 for an additional 4 h. The results shown are the averages of determinations on triplicate cultures.

comitant release of DAG and IP3 followed by an increase in cytosolic calcium from the released stores. To assess whether TGF-β stimulated the release of intracellular calcium, A549 cells were grown on microscope coverslips, and changes in calcium were determined with FURA2. Fig. 2A presents the effects of sequential exposure to TGF-β, ATP, or EGF. As shown in Fig. 2A, there is no detectable change in intracellular calcium levels in re-

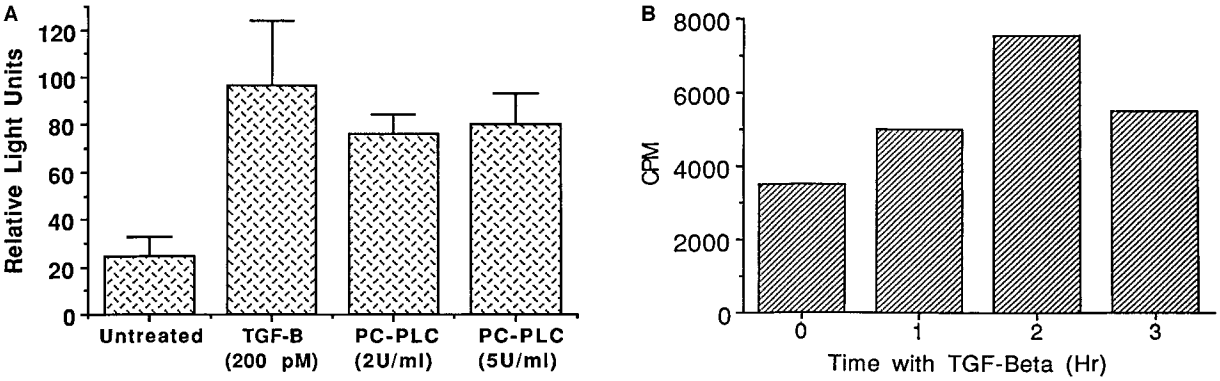

Fig. 3. Phosphatidylcholine phospholipase C (PC-PLC) as a mediator of transforming growth factor (TGF)-β responses. **A:** Bacterial PC-PLC can induce expression of the p3TP-Lux plasmid and mimic TGF-β action. A549 cells transiently transfected with the p3TP-Lux plasmid were treated with either 200 pM TGF-β1 or 2 or 5 U/ml bacterial PC-PLC for 3 h. Extracts were then prepared and assayed for luciferase activity as before. Results shown represent the averages of triplicate determinations. **B:** ^{14}-C-phosphocholine release is elevated in response to TGF-β. A549 cells were grown for 48 h in the presence of ^{14}-C-choline as described. Following experimental treatments, cultures were extracted in ice with methanol. Methanol extracts were transferred to glass tubes, chloroform added and samples vortexed vigorously. Distilled H_2O was added and samples centrifuged at 2,000*g* to separate the organic and aqueous phases. The aqueous phase was collected, dried and subjected to thin-layer chromatography (TLC) as described [Bligh and Dyer, 1959]. TLC plates were subjected to autoradiography followed by excising the spots corresponding to phosphocholine and radioactivity determined by scintillation counting. The results represent the averages of duplicate determinations.

sponse to TGF-β. In contrast, exposure to ATP or EGF induces a rapid increase in cytosolic calcium levels. Similar results were reported by Zheng et al., [1994] in examining TGF-β effects on human lung fibroblasts. It is possible that the magnitude of calcium release in response to TGF-β is below the level of delectability using this approach, therefore a second analysis of the role of calcium in TGF-β signaling was undertaken. For this analysis, A549 cells were transfected with the p3TP-Lux reporter plasmid. The following day, cultures were preloaded with BAPTA/AM, an intracellular calcium chelating agent. Following the preloading period, cultures were treated with 200 pM TGF-β for 3 h, harvested, extracted, and luciferase activities determined. As shown in Fig. 2B, TGF-β induced a 2.5-fold increase in luciferase activity. The presence of BAPTA/AM had no effect on expression of basal luciferase activity, and when cells preloaded with BAPTA/AM were treated with TGF-β they retained complete responsiveness, suggesting that calcium release was not involved in this aspect of TGF-β signaling.

Phosphatidylcholine Hydrolysis May Mediate TGF-β Signaling

Data previously obtained from this laboratory using pharmacologic agents that block hydrolysis of phospholipids suggest that phosphatidylcholine may be the source of a lipid second messenger in a TGF-β signaling pathway. Thus, D609, an inhibitor of phosphatidylcholine phospholipase C, can block TGF-β signaling whereas U73122, an inhibitor of phosphatidylinositol phospholipase C, is ineffective [Halstead et al., 1995]. To date, the mammalian form of PC-PLC has not been purified, however, the bacterial isoform of this enzyme is commercially available. A549 cells that had been previously transfected with the p3TP-Lux reporter and subsequently treated with bacterial PC-PLC demonstrates that this bacterial enzyme can mimic the effect of TGF-β on induction of the reporter gene as shown in Fig. 3A.

To demonstrate that phosphatidylcholine hydrolysis occurs in A549 cells following TGF-β treatment, A549 cells were metabolically labeled with ^{14}C-choline for 48 h. The labeled cultures were treated with either TGF-β or bacterial PC-PLC for varying times followed by extraction with chloroform-methanol. The aqueous phase was collected and subjected to thin-layer chromatography and autoradiography. Fig. 3B shows that ^{14}C-phosphocholine is released following treatment of prelabeled A549 cells. DAG is not the only lipid second messenger capable of acti-

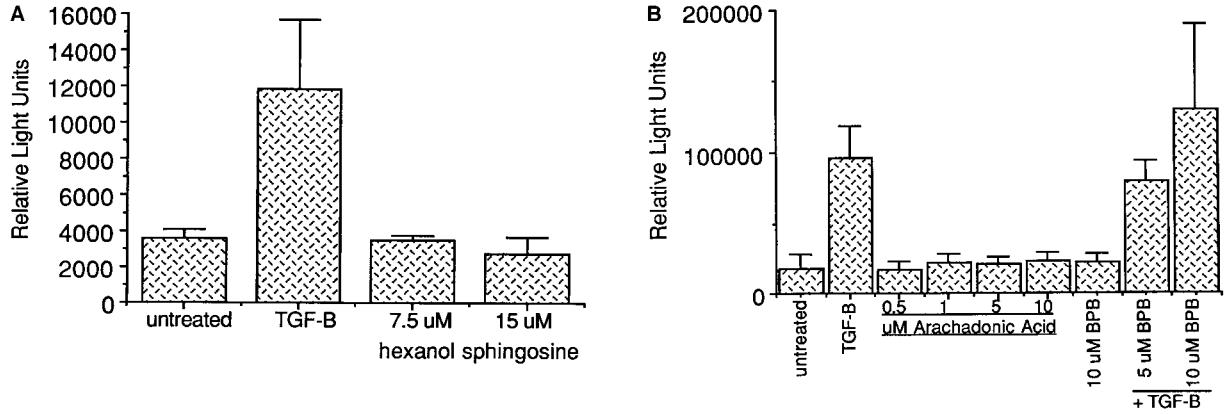

Fig. 4. Additional lipid second messengers do not induce p3TP-Lux expression. Neither ceramide nor arachadonic acid appear capable of inducing expression of the p3TP-Lux reporter. Transiently transfected A549 cells were treated with either hexanol sphingosine, a ceramide analogue (**A**), or arachadonic acid (**B**). Some cultures were also treated with a PLA_2 inhibitor, bromophenacyl bromide (BPB) along with TGF-β. Inhibition of phospholipase A_2 (PLA_2) does not block TGF-β responsiveness. The results represent the averages of determinations on triplicate cultures.

vating PKC. Arachadonic acid, ceramide, and variously phosphorylated forms of phosphatidylinositol may facilitate PKC activation [Liscovitch and Cantley, 1994; Lozano et al., 1994; Toker et al., 1994; Divecha and Irvine, 1995]. Fig. 4 shows that in A549 cells neither arachadonic acid nor a ceramide analogue, hexanoyl sphingosine, are capable of inducing expression of the p3TP-Lux reported. Also, inhibition of $cPLA_2$ (Fig. 4B) via bromophenacyl bromide [Rowles and Gallacher, 1996] or AACOCF 3 (not shown) does not block TGF-β responsiveness of the cells.

Transcriptional activation of several extracellular matrix genes comprises one of the principle actions of TGF-β. Data presented here demonstrates that TGF-β stimulation of A549 human lung carcinoma cells results in a rapid, transitory increase in DAG without detectable changes in intracellular calcium levels. We also observe release of phosphocholine and show that exogenously applied bacterial PC-PLC can mimic the TGF-β effect of induction of expression of the p3TP-Lux reporter plasmid. In contrast, neither arachadonic acid nor a ceramide analogue has this effect. Coupled with previous data from this and other laboratories implicating PKC in TGF-β signaling, the present data suggests a role for calcium-independent PKC isoforms of the novel and/or atypical classes in TGF-β signaling. Of note are the observations of Akimoto et al., [1996] and Moriya et al., [1996] that EGF and platelet-derived growth factor (PDGF) can activate aPKC-lambda and nPKC-epsilon in a wortmannin-sensitive manner. Also of note are the observations of Ahmed et al., [1998] suggesting a role for atypical PKCs in the transcriptional activation of latent TGF-β binding protein-2. We also observe changes in subcellular distribution of certain calcium-independent PKC isoforms in A549 cells in response to TGF-β (unpublished observations). Disatnik et al., [1995] have reported that TGF-β may activate nPKC-epsilon in cardiac myocytes, thus supporting our observations. Several reports indicate that TGF-β can activate the MAP kinase pathway [Hartsough and Mulder, 1995; Yamaguchi et al., 1995; Weiss et al., 1995; Shibuya et al., 1996]. Given that MAP kinase may be activated directly by PKC [Blumer et al., 1994; Berra et al., 1995], our results are not inconsistent with a role for MAP kinases in some aspects of TGF-β signaling.

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