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Transforming growth factor beta stimulates phosphoinositol metabolism and translocation of protein kinase C in cultured astrocytes

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Transforming growth factor beta (TGF-β) is a regulatory peptide found in many normal and neoplastic tissues, including brain, with a diverse range of cellular effects. The transmembrane biochemical signals by which TGF-β exerts these effects and the second messenger systems that may amplify them are unknown. We investigated the effects of TGF-β upon membrane phosphoinositol metabolism and protein kinase C activity in cultured astrocytes. We found that exposure of astrocyte enriched cultures to TGF-β resulted in the stimulation of phosphoinositol lipid turnover to inositol phosphates and in the apparent redistribution of protein kinase C from cytosol to membrane.

Transforming growth factor beta (TGF-β) is a 25 kDa peptide growth factor found in almost all tissues, both normal and neoplastic, which interacts with a wide variety of cells through specific cellular receptors [22, 30]. It belongs to a family of transforming growth factors that are operationally defined by their ability to reversibly promote a transformed phenotype with anchorage-independent growth in non-tumorigenic fibroblastic cells [15]. It can also exert growth inhibitory as well as stimulatory effects, or can promote differentiated functions, depending on the cell type and assay system [15, 22, 23]. Initially, TGF-β was thought to be related to the induction of malignant cell growth in vivo, but its presence in so many different normal tissues and its capacity to stimulate numerous cell types in various ways suggest that it plays important roles in the physiologic regulation of cell growth and differentiation. Little is known, however, about the biochemical second messenger mechanisms by which TGF-β exerts its cellular effects.

Most cells have at least two classes of receptors that are linked either to the adenyl

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cyclase second messenger pathway, or to the system in which membrane phospholipids are hydrolyzed by phospholipase C, leading to the formation of diacylglycerol, which activates protein kinase C, and inositol 1,4,5-triphosphate which in turn mobilizes intracellular calcium [3, 19].

TGF-β is found in normal brain [22]. The brain contains the substrates and enzymes involved in membrane phosphoinositol (PI) lipid metabolism [11, 29]. A number of receptors associated with PI lipid metabolism are present in brain and brain derived tissue, including cells of glial origin [16, 20, 21, 24]. To determine whether the interaction of TGF-β with its receptors is linked to this second messenger system in glial cells, we investigated the effects of TGF-β upon phosphoinositol metabolism and protein kinase C activity in these cells. We found that exposure of astrocytic cultures prepared from neonatal rat brain cortex to TGF-β resulted in the stimulation of PI lipid turnover to inositol phosphates, and in the apparent redistribution of protein kinase C activity from cytosol to membrane.

Astrocyte cultures were prepared from neonatal rat brain according to a modification of the method of Frangakis as previously described [12, 25]. For PI lipid turnover determination, confluent first passage astrocyte cultures in 6 well multiplates (Costar) were prelabeled for 48 hours with 10 μCi *myo*-[2-^{3}H]inositol (15 Ci/mM, ARC, St. Louis, MO.) in 1 ml of inositol-free, serum-free Dulbecco's modified Eagle's medium (DMEM). Inositol phosphate formation was examined by modifications of previously described methods [1, 4]. After incubation with various concentrations of TGF-β (R&D Systems, Minneapolis, MN) in DMEM containing 5 mM LiCl at 37°C, the supernatant of a 5% trichloroacetic acid (TCA) deproteinized cell extract of each well was washed 6 times each with 3 ml of water saturated diethyl ether and neutralized with 1 M $KHCO_3$. Inositol phosphates in the supernatant were bound to 1 ml of a 50% (w/v) slurry of Dowex AG 1–8X resin (formate form, BioRad, Richmond, CA), washed free of labeled inositol with 5 mM myoinositol (6 × 5 ml), and eluted with 1 ml of 1 M ammonium formate/0.1 M formic acid. The radioactivity in a 0.8 ml aliquot of the eluate was determined by liquid scintillation counting.

Inositol lipids in the pellet of the deproteinized cells were extracted with 0.5 ml H_2O/1.5 ml chloroform/methanol (1:2 v/v), and then with another 0.5 ml H_2O/1 ml chloroform. The radioactivity in 0.2 ml of the organic layer was quantitated by liquid scintillation counting.

To prepare cells for protein kinase C investigations, confluent astrocyte cultures rinsed twice with serum-free medium, were incubated with TGF-β in serum-free medium at 37°C. The incubation was terminated by rinsing twice with ice-cold calcium-free phosphate-buffered saline (PBS), and scraping the cells in homogenization medium containing 20 mM HEPES, pH 7.5, 2 mM EDTA, 5 mM EGTA, 0.25 M sucrose and 10 mM β-mercaptoethanol. The suspension was sonicated for 15 s and centrifuged at 40,000 *g* for 1 h at 4°C. These supernatants and pellets were used for all protein kinase C assays.

Protein kinase C activity was determined by measuring the incorporation of radiolabel from [^{32}P]ATP into exogenous lysine-rich histones by modification of the method described by Takai et al. [27]. In a total volume of 250 μl, the standard reaction

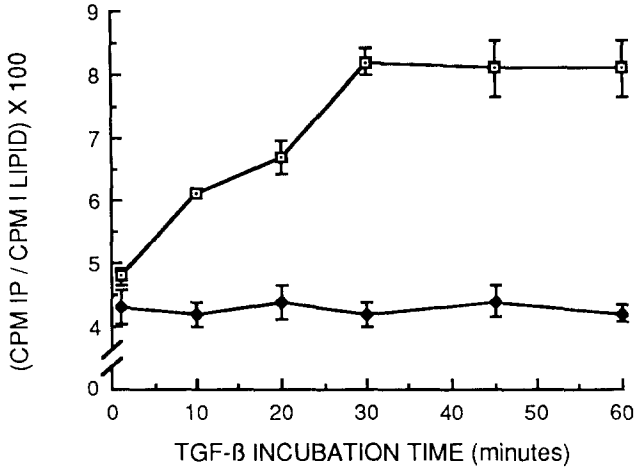

Fig. 1. Time course of TGF-β-induced accumulation of [^{3}H]inositol phosphates. Astrocyte-enriched cultures were incubated for the periods initiated with 50 pM TGF-β (□) or no addition (◆). The ^{3}H recovered in the inositol phosphates are corrected to a standard incorporation into lipids. The values are the means of 3 determinations ±S.E.M. Similar results were obtained in 3 independent experiments.

mixture contained 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 μM $CaCl_2$, 20 μg/ml phosphatidylserine, 20 μg/ml 1,2-diolein (or an equivalent volume of deionized H_2O), 80 μg/ml histones, 0.2 nmol ATP, and 2 μCi [^{32}P]ATP (4000 Ci/mmol, ICN, Irvine, CA). The reaction was initiated by addition of 5–15 μg cytosolic or membrane protein and was carried out in 1.5 ml microfuge tubes at 30°C. After incubation for 5 min, the reaction was terminated by adding 75 μl of 12 N glacial acetic acid and collected on Whatman P81 phosphocellulose papers. The filters were washed once in 30% acetic acid, twice in 15% acetic acid, and finally in acetone. The radioactivity was quantitated by Cerenkov counting and proteins determined by the method of Bradford [6]. Protein kinase C activity was calculated as the difference in activity in the presence or absence of diolein.

The time course of TGF-β stimulated [^{3}H]inositol phosphate accumulation in astrocyte enriched cultures is shown in Fig. 1. The radioactivity recovered in the [^{3}H]phosphates was corrected to a standard incorporation of ^{3}H into lipid as suggested by Bone, et al. [5]. Basal [^{3}H]inositol phosphate production did not change during the course of the incubation (1–60 min). Fifty pM TGF-β caused linear increase in [^{3}H]inositol phosphate production between 1 and 30 min. The ability of TGF-β to stimulate the accumulation of [^{3}H]inositol phosphates was concentration dependent (Fig. 2). Stimulation to 20% above control occurred at 0.05 pM TGF-β and was maximal at 70 pM where 80% above basal levels were observed. From the dose–response curve, the EC_{50} was 6.3 pM.

Basal cytosolic protein kinase C activity in these cultures averaged 8.7 ± 0.6 pmol/mg/min. Exposure of the astrocytes to TGF-β at concentrations between 0.1 and 100 pM for 45 min resulted in complete apparent redistribution of virtually all of this activity from the cytosol to the membrane. This translocation of activity was tested

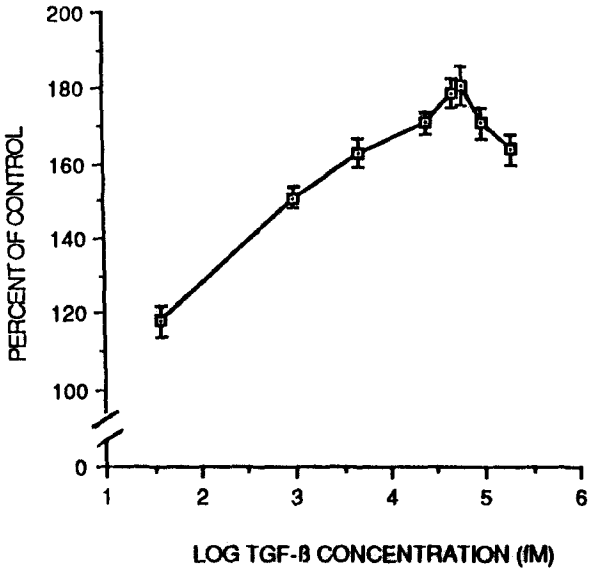

Fig. 2. Concentration dependence of TGF-β-induced [^{3}H]inositol phosphate accumulation. Astrocyte cultures were incubated with various concentrations of TGF-β for 60 minutes. The ^{3}H recovered in inositol phosphates was corrected to a standard incorporation into lipids and expressed as the percent stimulation of a control run in parallel. Values represent means $\pm$S.E.M. of 3 determinations. Essentially identical results were obtained in 3 independent experiments.

at 10 pM TGF-β, for varying incubation times from 5 to 60 min (Fig. 3). Protein kinase C activity virtually completely disappeared from the cytosol and reappeared in the membrane after 5 min of exposure to TGF-β.

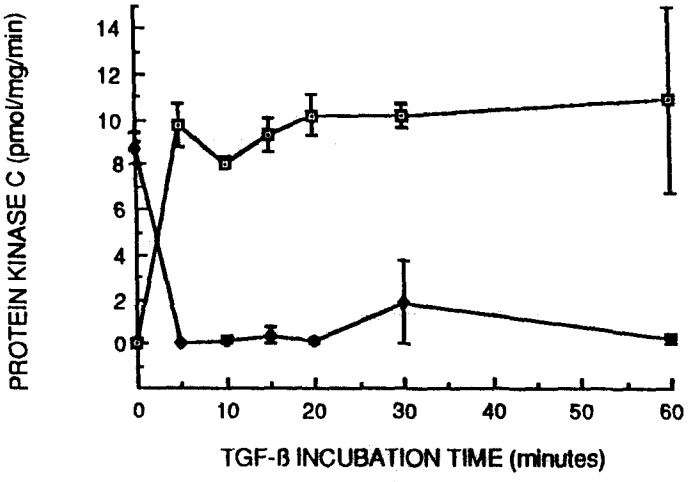

Fig. 3. Time course of TGF-β-induced redistribution of protein kinase C activity. Astrocyte cultures were incubated for designated times with 10 pM TGF-β at 37°C. Protein kinase C activity was assayed in the cytosolic (◆) and membrane (□) fractions. Values represent means $\pm$S.E.M. of 3 determinations. Essentially identical results were obtained in 3 independent experiments.

The intracellular responses produced by β adrenergic, adenosine and vasointestinal peptide receptor stimulation are among those mediated by cAMP in cultured astrocytes [10, 14]. Although TGF-β has been observed to modulate the cAMP levels stimulated by other receptor–ligand interactions, there are no reports of direct TGF-β receptor linkage to adenyl cyclase in any cell type [2].

Recent studies indicate that rat brain astrocyte cultures also have muscarinic, α_1-adrenergic and glutamatergic receptors linked to membrane phospholipid metabolism [20, 21, 24]. The coupling of receptors for peptide growth factors to PI lipid metabolism in astrocytes has not previously been reported, but such an association is known for several of these factors in other cell types. Platelet-derived growth factor stimulates PI turnover in fibroblasts, and nerve growth factor activates protein kinase C and stimulates PI lipid metabolism in PC 12 cells [8, 9, 13]. Thus, the cellular substrates and enzymes for membrane PI lipid turnover are present in astrocytes, and a precedent for the linkage of peptide growth factors to PI metabolism does exist in a number of other cells.

The magnitude of the PI lipid turnover stimulated by TGF-β in our astrocyte cultures is similar to that observed in other cell types and tissues on stimulation by a number of different agonists. Serotonin and histamine produce from 20 to 110% stimulation of inositol phosphates above basal levels in rat cerebral cortical slices [7]. Neurotensin stimulation of receptors on mouse neuroblastoma cells produces a 50% increase over basal inositol phosphates [26]. These responses are of the same order of magnitude as the 180% of control PI turnover we found in astrocyte cultures. Larger increases in the production of inositol phosphates do occur on stimulation by other agonists in some cells and tissues [4]. Such variations seem likely to reflect differences in cellular receptor characteristics or differences in the pool size of inositol lipids available for hydrolysis in response to agonist stimulation of specific receptors. The biological significance of PI turnover induced by TGF-β in these astrocyte cultures is supported by the accompanying redistribution of protein kinase C activity from cytosol to membrane, at the same concentrations and with a similar time course. The occupancy of receptors linked to the PI second messenger system activates a bifurcated biochemical pathway in which membrane lipid hydrolysis results in the generation of diacylglycerol along with inositol phosphates [3]. Upon formation of diacylglycerol, protein kinase C, which in most cells is cytosolic in its basal state, becomes membrane-bound, gains access to membrane phospholipids, and becomes activated [18]. In several cell types, the redistribution of protein kinase C results in increased phosphorylation of specific membrane proteins [17]. The ability of TGF-β to cause the redistribution of protein kinase C may be a significant step in initiating the cellular responses elicited by this growth factor.

TGF-β has diverse biological effects, and can stimulate or inhibit growth, or promote differentiation, depending on the type of cell being studied [15, 23]. These effects occur at the same concentrations of TGF-β that we found stimulated PI turnover and protein kinase C translocation in astrocytes. It seems likely that TGF-β will also have regulatory influences on the growth or differentiation of astrocytes. Whether such influences are mediated through the PI second messenger pathway remains to

be determined. Despite the variability in cellular response to TGF-β, its receptors have very similar binding characteristics across numerous cell lines and species [30]. It is possible, therefore, that the divergent cellular behaviors seen in response to TGF-β, are related to the generation of different intracellular biochemical signals. Investigation of which cells responsive to TGF-β, have receptors linked to PI lipid metabolism, and which are coupled to other pathways, should help to clarify the molecular basis of these various biological responses.

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