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Coupling of the Thrombin Receptor to G_{12} May Account for Selective Effects of Thrombin on Gene Expression and DNA Synthesis in 1321N1 Astrocytoma Cells

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In 1321N1 astrocytoma cells, thrombin, but not carbachol, induces AP-1-mediated gene expression and DNA synthesis. To understand the divergent effects of these G protein-coupled receptor agonists on cellular responses, we examined G_q-dependent signaling events induced by thrombin receptor and muscarinic acetylcholine receptor stimulation. Thrombin and carbachol induce comparable changes in phosphoinositide and phosphatidylcholine hydrolysis, mobilization of intracellular Ca^{2+}, diglyceride generation, and redistribution of protein kinase C; thus, activation of these G_q-signaling pathways appears to be insufficient for gene expression and mitogenesis. Thrombin increases Ras and mitogen-activated protein kinase activation to a greater extent than carbachol in 1321N1 cells. The effects of thrombin are not mediated through G_i, since ribosylation of G_i/G_o proteins by pertussis toxin does not prevent thrombin-induced gene expression or thrombin-stimulated DNA synthesis. We recently reported that the pertussis toxin-insensitive G_{12} protein is required for thrombin-induced DNA synthesis. We demonstrate here, using transfection of receptors and G proteins in COS-7 cells, that $G\alpha_{12}$ selectively couples the thrombin receptor to AP-1-mediated gene expression. This does not appear to result from increased mitogen-activated protein kinase activity but may reflect activation of a tyrosine kinase pathway. We suggest that preferential coupling of the thrombin receptor to G_{12} accounts for the selective ability of thrombin to stimulate Ras, mitogen-activated protein kinase, gene expression, and mitogenesis in 1321N1 cells.

INTRODUCTION

It has recently become clear that activation of certain G protein-coupled receptors can induce downstream responses previously associated with stimulation of growth factor receptors, including activation of Ras, Raf-1, mitogen-activated protein kinase (MAPK) and cell proliferation (Jackson *et al.*, 1988; Kelvin *et al.*, 1989; Pages *et al.*, 1993; Simonson *et al.*, 1993; van Corven *et al.*, 1993; Winitz *et al.*, 1993 and see Malarkey *et al.*, 1995 and Post and Brown, 1996 for reviews). The thrombin receptor is among the best studied G protein-coupled receptors that induce cell proliferation. Thrombin, acting through proteolytic cleavage of the N terminus of its seven transmembrane domain receptor (Vu *et al.*, 1991), is a potent mitogen for fibroblasts, smooth muscle cells, and astrocytes (Obberghen-Schilling *et al.*, 1985; Cavanaugh *et al.*, 1990; Hung *et al.*, 1992; Weiss *et al.*, 1992; LaMorte *et al.*, 1993a,b and see Grand *et al.*, 1996 for review). Intracellular effectors regulated by thrombin include adenylyl cyclase and phosphoinositide-specific phospholipase C (PLC) (Grandt *et al.*, 1986; Banga *et al.*, 1988; Jones *et al.*, 1989;

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Hung *et al.*, 1992b). The thrombin receptor was also among the first G protein-coupled receptors shown to activate Ras (LaMorte *et al.*, 1993b; van Corven *et al.*, 1993).

Previous work has demonstrated divergence in the responses induced by activation of the thrombin receptor and those induced by activation of the G protein-coupled muscarinic receptors in CCL39 fibroblasts (Seuwen *et al.*, 1990; Kahan *et al.*, 1992) and 1321N1 cells (Trejo *et al.*, 1992). In CCL39 cells, thrombin induces DNA synthesis but muscarinic receptor agonists do not (Seuwen *et al.*, 1990; Kahan *et al.*, 1992). We have shown that in 1321N1 astroglial cells, thrombin but not muscarinic receptor activation leads to a sustained increase in c-*jun* mRNA, an associated increase in AP-1 DNA binding activity, and a marked increase in AP-1-mediated gene expression (Trejo *et al.*, 1992). The reason for the observed differences in the response to thrombin and muscarinic receptor activation is unclear, however, because both receptors couple to the heterotrimeric GTP-binding protein G_q (Berstein *et al.*, 1992; LaMorte *et al.*, 1993a) and, as shown here, can signal cellular responses through the activation of PLC and protein kinase C (PKC).

Experiments using pertussis toxin (PTX) to inactivate G proteins of the G_i/G_o family and studies using microinjected antibodies to these proteins suggest that the mitogenic effects of thrombin (Chambard *et al.*, 1987; LaMorte *et al.*, 1993a), like those of bombesin (Letterio *et al.*, 1986) and lysophosphatidic acid (LPA) (van Corven *et al.*, 1993), are mediated through the G_i or G_o protein(s) in various fibroblast cell lines. Activation of Ras and MAPK in response to thrombin and LPA also occur through a PTX-sensitive pathway in these cells (L'Allemain *et al.*, 1991; Howe and Marshal, 1993; van Corven *et al.*, 1993; Hordijk *et al.*, 1994). Furthermore, in related studies, expression of the constitutively activated α-subunit of G_i has been demonstrated to induce altered growth properties (Hermouet *et al.*, 1991; Pace *et al.*, 1991; Gupta *et al.*, 1992). Additionally, there is considerable evidence that release of $\beta\gamma$-subunits from pertussis toxin-sensitive G proteins accounts for the ability of some G protein-linked receptors to activate Ras and mitogen-activated protein kinase (MAPK) cascades (Crespo *et al.*, 1994; Faure *et al.*, 1994; Koch *et al.*, 1994a; Ito *et al.*, 1995). In contrast, the mechanism by which receptors activating pertussis toxin-insensitive G proteins regulate MAP kinase cascades and/or cell growth is less clear.

The G proteins G_{12} and G_{13} comprise a family of pertussis toxin-insensitive G proteins which is distinct from the G_q/G_{11} family (Strathmann *et al.*, 1993). G_{12} and G_{13} have been shown to interact with the activated thrombin receptor in platelets (Offermanns *et al.*, 1994), and our recent studies have demonstrated that $G\alpha_{12}$ is required for thrombin-induced mitogenesis in 1321N1 cells (Aragay *et al.*, 1995). Although the role of these G proteins in growth regulation has been recognized (Chan *et al.*, 1993; Xu *et al.*, 1993; Vara Prasad *et al.*, 1994; Voyno-Yasenetskaya *et al.*, 1994b; Aragay *et al.*, 1995), their direct effectors are not known. Constitutively activated GTPase-deficient mutants of $G\alpha_{12}$ and $G\alpha_{13}$ have been shown to activate the Na^+/H^+ exchanger (Dhanasekaran *et al.*, 1994; Voyno-Yasenetskaya *et al.*, 1994a) and constitutively active $G\alpha_{12}$ has been shown to increase serum-stimulated phospholipase A_2 activity (Xu *et al.*, 1993). Furthermore, EGF-stimulated MAPK activity is enhanced in fibroblasts that express activated $G\alpha_{12}$ or $G\alpha_{13}$ (Voyno-Yasenetskaya *et al.*, 1994b). More recently, $G\alpha_{12}/G\alpha_{13}$ have been shown to activate Ras and cJun N-terminal kinase (JNK) in a Ras-dependent manner (Prasad *et al.*, 1995; Collins *et al.*, 1996). The small G proteins Rac, Rho, and Cdc42 have also been implicated as mediators of $G\alpha_{12}$-induced responses, including JNK activation (Buhl *et al.*, 1995; Prasad *et al.*, 1995; Collins *et al.*, 1996; Hooley *et al.*, 1996).

The aim of the present study was to identify early signaling events that would distinguish between the pathways used by thrombin and carbachol in 1321N1 cells and to determine whether coupling to G_q, G_i/G_o, or G_{12} mediates thrombin's unique effects on AP-1-regulated gene expression and DNA synthesis. In this report, we show that thrombin and muscarinic receptors have comparable effects on $G\alpha_q$-mediated signaling pathways but are distinguished by their effects on Ras and MAPK. We also demonstrate that the effects of thrombin on gene expression and DNA synthesis are not mediated though pertussis toxin-sensitive G_i or G_o proteins. By coexpressing receptor and G protein cDNA in COS-7 cells, we further show that the thrombin receptor preferentially couples to AP-1-mediated gene expression through the pertussis toxin-insensitive $G\alpha_{12}$. We suggest that the thrombin receptor interacts with G_{12} to activate as yet unidentified effector pathways which require tyrosine kinase activity and lead to growth responses.

MATERIALS AND METHODS

Cell Culture

Human 1321N1 astrocytoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). In most experiments, 1321N1 cells were plated at 1.2×10^6 cells/100-mm culture plate and used 4 days after plating, following 18 h of serum deprivation in 0.1% bovine serum albumin/DMEM. COS-7 cells were cultured in DMEM/10% FCS plus antibiotics. For transfection experiments, cells were plated at 2.5×10^5 cells/60-mm dish 1 day before transfection.

DNA Synthesis

Cells were plated at $1.5–2 \times 10^4$ cells/12-mm coverslip (70% confluency) and grown in DMEM/5% FCS overnight. Following 18 h of serum deprivation, cells were treated with agonist and labeled with

bromodeoxyuridine (BrdUrd, Amersham, Durham, NC) for 24 h. Cells were fixed in 95% ethanol:5% glacial acetic acid and immunostained with a mouse monoclonal antibody to BrdUrd (Amersham) and a secondary rhodamine-conjugated anti-mouse antibody (Cappel). The fraction of cells synthesizing DNA was quantitated using a Zeiss Axiophot photomicroscope and a 40× Neofluar objective. The percentage of labeled nuclei was determined in fields of 100–300 cells using two coverslips per experimental condition. For [^{3}H]thymidine incorporation, 1321N1 cells were plated on 24-well dishes at 5×10^4 cells/well, grown in DMEM/5% FCS overnight, washed, and then maintained in 0.1% bovine serum albumin/serum-free media for 48 h. Cells were treated with agonist in the same media for an additional 24 h with 1–2 μCi/ml [^{3}H]thymidine included during the last 6 h of incubation. Cells were fixed with methanol, washed with 10% and 0.5% trichloroacetic acid, and dissolved in 1 N sodium hydroxide. Radioactivity was quantified using liquid scintillation counting.

Phosphoinositide Hydrolysis

For examination of inositol trisphosphate ($InsP_3$) formation in 1321N1 cells, phosphoinositides were labeled by incubating cells for 18 h with 10 μCi/ml [^{3}H]inositol in DMEM. Cells were washed twice before the addition of the agonist. Incubations were terminated with 10% trichloroacetic acid and [^{3}H]$InsP_3$ was separated on Dowex AG 1X-8 as described (Masters *et al.*, 1984). For studies in COS-7 cells, transfected cells were labeled overnight with 2–3 μCi/ml [^{3}H]inositol, washed, and treated with agonist in the presence of 10 mM LiCl. Cell lysates were prepared and [^{3}H]inositol monophosphate accumulation was quantitated as described (Masters *et al.*, 1984).

Phospholipase D Activation (Phosphatidylethanol Formation)

Astrocytoma cells grown in 35-mm plates were labeled with 3 μCi/ml [^{3}H]myristic acid for 18 h and then treated with agonist or vehicle in the presence of 1% ethanol. Reactions were terminated by replacing medium with 1 ml of ice-cold methanol. Lipids were extracted according to Bligh and Dyer (1959) and separated by TLC, and [^{3}H]phosphatidylethanol and [^{3}H]phosphatidic acid were identified using cold standards, scraped, and quantified by liquid scintillation counting as described (Nieto *et al.*, 1994).

Intracellular Calcium

Intracellular calcium concentrations were determined by the use of the fluorescent Ca^{2+} indicator fura-2 as described (McDonough *et al.*, 1988). Briefly, cells were loaded with 1 μM fura-2AM for 15 to 20 min at 37°C and then resuspended in fura-free buffer. Cells were stimulated with agonists and fluorescence was monitored with a SPEX Fluorolog spectrofluorometer with excitation and emission set at 340 and 510 nm, respectively.

Diglyceride Formation

Unlabeled 1321N1 cells were treated in serum-free medium with vehicle or agonist for 5 min. The reaction was stopped with methanol and cell lipids were extracted using the method of Bligh and Dyer (1959). The method of Preiss *et al.* (1986) was used to quantitatively measure diacylglycerol in a 700-μl aliquot of the organic phase as described (Trilivas and Brown, 1989).

PKC Redistribution

Changes in the subcellular distribution of PKC were detected by immunoblotting with isoform-specific PKC antibodies as described (Trilivas *et al.*, 1991). Briefly, membrane and cytosolic fractions were prepared from cells treated for various times with thrombin (0.5 U/ml) or carbachol (500 μM), added to SDS sample buffer (Laemmli, 1970) and aliquots of 60 μg of protein were resolved by SDS-PAGE.

Immunoblotting

Samples separated by SDS-PAGE were electrophoretically transferred to Immobilon membranes (Millipore, Bedford, MA). PKC-α was detected by blotting with an isozyme-specific monoclonal antibody (1:100; Amersham) and PKC-ϵ, was detected with an isozyme-specific polyclonal anti-PKC antibody (1:500, a gift from Dr. B. Strulovici), followed by [^{125}I]protein A as previously described (Trejo *et al.*, 1992). Autoradiographs were scanned with an LKB UltroScan XL densitometer to quantify PKC immunoreactivity. For detection of immunoreactive G protein α-subunits, membrane fractions (100 μg) were resolved by 12.5% SDS-PAGE, transferred to an Immobilon membrane, and incubated with polyclonal antisera specific for $G\alpha_i$ (LaMorte *et al.*, 1993a), $G\alpha_o$, $G\alpha_{12}$, or $G\alpha_{q/11}$ (provided by Dr. M. Simon), followed by ^{125}I-protein A and subjected to autoradiography.

ADP Ribosylation

Quiescent cells were treated with 100 ng/ml pertussis toxin (PTX; List Biological Laboratories, Campbell, CA) in DMEM containing 5% FCS for various time periods. Plates were washed twice in ice-cold PBS and scraped into homogenization buffer (10 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM EGTA, and aprotinin, pH 7.5) and homogenized with a Tissuemizer (Tekmar, Cincinnati, OH) for 30 s on ice. Membranes were collected by spinning at $43{,}000 \times g$ for 60 min at 4°C and resuspended in homogenization buffer. Membrane fractions (12 μg) were incubated for 1 h at 30°C in a reaction mixture containing 3 μg of preactivated PTX and 0.25 μCi of [^{32}P]NAD. Reactions were stopped with SDS sample buffer, boiled, and proteins were analyzed by SDS-PAGE and autoradiography.

Transfection

Human 1321N1 astrocytoma cells plated at 0.6×10^6 cells/60-mm plate were transiently transfected with 2× TRE (12-*O*-tetradecanoylphorbol 13-acetate responsive element) luciferase reporter by calcium phosphate coprecipitation as described previously (Trejo *et al.*, 1992). For PTX studies, the cells were washed and then incubated with 100 ng/ml PTX for 9 h before the addition of 0.5 U/ml thrombin for an additional 24 h. Cells were then lysed in 0.1 M KPO_4 buffer (pH 7.9) containing 1% Triton X-100 and 1 mM dithiothreitol, and luciferase activity was measured by luminescence as described previously (Trejo *et al.*, 1992). For studies using COS-7 cells, cells were plated at $1–2 \times 10^5$ cells/35-mm plate and were transfected with 0.2 μg of the cDNA encoding the human thrombin receptor (Ishii *et al.*, 1995), 0.4 μg of the rat M_3 muscarinic receptor in pCD (Blin *et al.*, 1995), or backbone vector alone with or without 3.2 μg of wild-type $G\alpha_q$ or $G\alpha_{12}$ in pCIS. COS-7 cells were treated with agonist for the times indicated and luciferase expression was examined 48 h following transfection as described above.

Ras Activation

1321N1 astrocytoma cells were assayed for levels of Ras-GTP complexes as described (LaMorte *et al.*, 1993b). Briefly, cells were labeled with 0.5 mCi of [^{32}P]orthophosphate/ml in phosphate-free DMEM for 3 h. Following addition of agonist, Ras was immunoprecipitated from cell lysates using Y13-259 (provided by Dr. Y. Kaziro or purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were washed, and nucleotides were eluted and separated on Bakerflex polyethyleneimine cellulose plates. GTP and GDP were identified using cold standards and quantitated by autoradiography and densitometry.

MAPK Activation

Cells were incubated with agonist for 5 min, washed with ice-cold PBS, and lysed in 1% Triton-X buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM Na_3VO_4, and 1 mM $Na_4P_2O_7$. Insoluble material was removed by centrifugation, and cell lysates were subjected to immunoprecipitation with agarose-conjugated antibody to MAPK (Erk-1; Santa Cruz Biotechnology). Immune complexes were washed and myelin basic protein (MBP) kinase activity was assayed by resuspending the final pellet in 30 μl of kinase buffer containing MBP (50 μg/ml) and [^{32}P]ATP (4 μCi). After incubation at 30°C for 10 min, assays were terminated by the addition of SDS sample buffer. Samples were boiled, separated by SDS-PAGE, and phosphate incorporation quantitated by radioanalytical scanning (AMBIS). Activity of the HA-tagged MAP kinase and JNK were assayed in COS-7 cells transfected as described above with cDNA for HA-ERK2 or HA-JNK1 along with other plasmids of interest. At 48 h after transfection, agonists were added for 5 min (ERK) or 20 min (JNK), and cells were lysed and assayed for kinase activity as described (Collins *et al.*, 1996).

Quantitation of Expressed Receptors

COS-7 cells were transfected with various amounts of the thrombin receptor cDNA containing an epitope for the M1 monoclonal antibody (FLAG tag; Ishii *et al.*, 1995). Receptor expression was measured with a colorimetric cell surface enzyme-linked immunosorbent assay (Ishii *et al.*, 1995), and the cDNA concentration yielding the greatest expression was used in subsequent experiments. Receptor expression in cells transfected with the M_3 muscarinic receptor was directly measured by radioligand binding using [^{3}H]*N*-methyl quinuclidinyl benzilate (DuPont, Wilmington, DE).

RESULTS

The mitogenic effects of thrombin and carbachol were examined in serum-deprived 1321N1 cells using the nuclear incorporation of BrdUrd as an index of DNA synthesis. Figure 1A compares DNA synthesis in cells treated for 24 h with vehicle (a), with the stable acetylcholine analogue carbachol (b), or with thrombin (c). In three experiments, nuclear BrdUrd staining was seen in 4–8% of control (serum-deprived) cells and in 2–7% of cells treated with carbachol. In contrast, 40–55% of thrombin-treated cells incorporated BrdUrd into DNA. Responses to carbachol and thrombin were also compared using [^{3}H]thymidine incorporation, since it has been suggested that carbachol induces mitogenesis in 1321N1 cells based on this assay (Ashkenazi *et al.*, 1989; Giuzzetti *et al.*, 1996). Carbachol elicited a modest increase in [^{3}H]thymidine incorporation, but this increase was <15% of the response to thrombin (Figure 1B). Thus, in 1321N1 astroglial cells thrombin is an effective mitogen whereas carbachol is not.

To determine whether the differential effects of thrombin and carbachol on gene expression (Trejo *et al.*, 1992) and cell proliferation result from differences in their ability to regulate known phospholipid-signaling pathways, we examined agonist-induced inositol phosphate and phosphatidylethanol formation as indices of phospholipase C (PLC) and phospholipase D (PLD) activation, respectively. Activation of phospholipase-signaling pathways is accompanied by Ca^{2+} mobilization and diglyceride generation, which were also quantitated in thrombin- and carbachol-treated cells. Phospholipid-derived second messengers were examined using maximally effective concentrations of carbachol (500 μM) or thrombin (0.5 U/ml) for 15 s to 5 min (Table 1). Carbachol and thrombin did not differentially affect PLC or PLD activity as indicated by the magnitude of the peak increases in their metabolites. No differences in thrombin- and carbachol-induced phospholipase activity were observed at times up to 30 min.

The divergent effects of thrombin and carbachol on gene expression and mitogenesis could also reflect differences in the kinetics or isoform specificity of protein kinase C activation. As determined by immunoblotting, 1321N1 cells express Ca^{2+}-sensitive PKC-α (Trilivas *et al.*, 1991), Ca^{2+}-independent PKC-ε, (Nieto *et al.*, 1994), and the atypical PKC-ζ (our unpublished observation). The β, γ, or δ isoforms of PKC are not detectable (Trilivas *et al.*, 1991). The kinetics of activation of PKC isozymes by carbachol and thrombin was assessed by measuring increases in membrane-associated PKC. Since PKC-ζ was not translocated to the membrane in response to either carbachol or thrombin, its activation was not examined further. As shown in Figure 2, a transient redistribution of PKC-α was induced by carbachol and thrombin with no apparent difference in the effects of the two agonists. PKC-ε remained associated with the membrane for a longer time but was similarly affected by thrombin and carbachol. Since the kinetics and magnitude of PKC-α and PKC-ε redistribution were comparable for thrombin and carbachol, it is unlikely that PKC activation is an early signal that differentiates mitogenic and nonmitogenic receptor responses.

Another possible explanation for the observed differences in the genetic and mitogenic potential of these agonists could be that carbachol activates signaling events which inhibit gene expression and DNA synthesis. For example, agents that increase intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) inhibit growth factor-stimulated mitogenic pathways by delaying or preventing Ras-dependent activation of Raf (Cook and McCormick, 1993; Wu *et al.*, 1993; Russell *et al.*, 1994; McKenzie and Pouyssegur, 1996) and we find that increasing cAMP inhibits thrombin-induced DNA synthesis in 1321N1 cells. Since carbachol induces about a threefold increase in intracellular cAMP in 1321N1 cells in the absence of phosphodiesterase inhibitors, we considered the possibility that carbachol inhibits growth-signaling pathways. Cells were treated with thrombin or other mitogens in the presence or absence of carbachol (500 μM), and BrdUrd incorporation was examined. As shown in Figure 3,

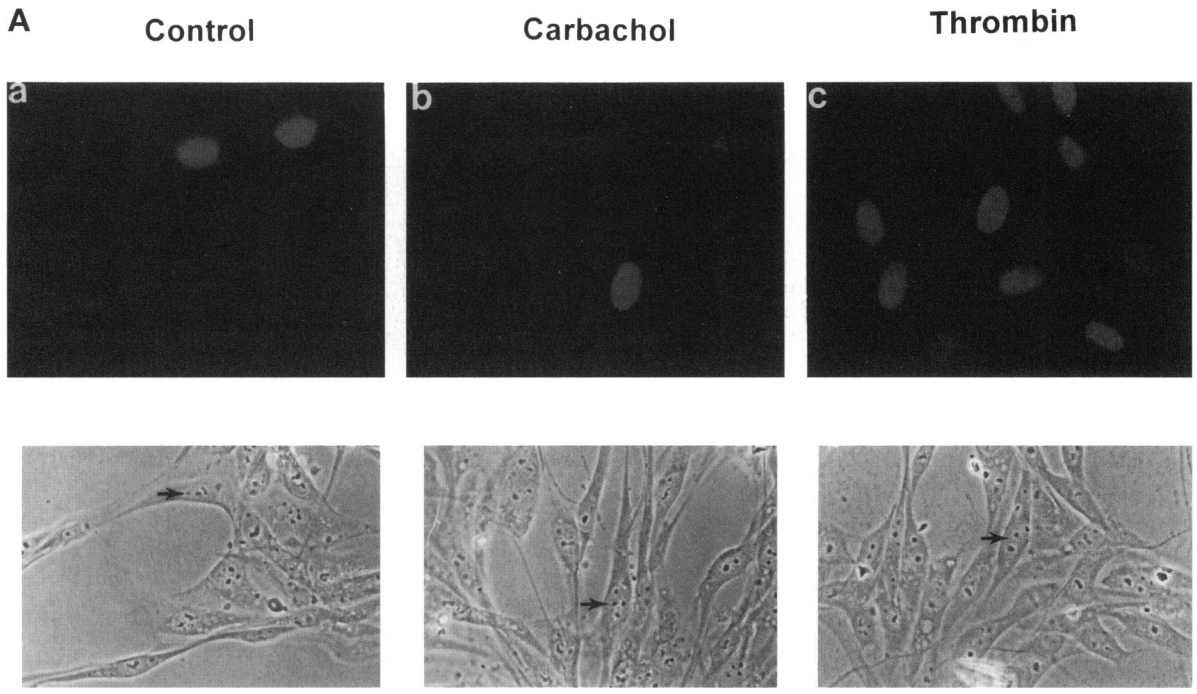

Figure 1. DNA synthesis is stimulated by thrombin but not by carbachol. (A) Serum-deprived astrocytoma cells were treated with vehicle (a), 500 μM carbachol (b), or 0.5 U/ml thrombin (c) and immunostained for nuclear incorporation of BrdUrd 24 h later as described in MATERIALS AND METHODS. The lower panels are phase-contrast images of the field of cells shown above. Each arrowhead identifies the nucleus of a cell that has incorporated BrdUrd into DNA. Cells were analyzed using a Zeiss Axiophot fluorescent microscope with a 40× Neofluar objective. Similar results were obtained in three independent experiments. (B) Serum-deprived 1321N1 cells maintained in 0.1% bovine serum albumin in serum-free media (Ctrl) or treated with carbachol (Carb) or thrombin (Thr) for 24 h, with 1–2 μCi/ml [^{3}H]thymidine included for the last 6 h. [^{3}H]Thymidine incorporation was quantified as described in MATERIALS AND METHODS. Data are means ± SEM from a single experiment performed in quadruplicate and are representative of two experiments.

carbachol did not inhibit DNA synthesis in response to either thrombin or tyrosine kinase growth factors. It is also notable that thrombin was a more effective mitogen in 1321N1 cells than any of the peptide growth factors examined. These data indicate that the differential effects of thrombin and carbachol would appear not to result from a blockade of effector pathways by carbachol, but rather from recruitment of unique effectors by thrombin.

We have previously shown that thrombin activates Ras in 1321N1 cells (LaMorte *et al.*, 1993b). We compared the effects of thrombin and carbachol on Ras activation in intact 1321N1 cells in four experiments. In a representative experiment (Figure 4), thrombin induced a 2.4-fold increase in the percentage of Ras in the GTP-bound state, whereas carbachol did not significantly increase GTP-bound Ras. Since MAPK can be activated through Ras-dependent pathways and has been implicated as a control signal for cell growth, we compared the effects of carbachol and thrombin on activation of MAPK. As shown in Figure 5, thrombin causes a significantly greater increase in MAPK activity, as assessed by the phosphorylation of MBP.

In fibroblasts thrombin-induced Ras activation, MAPK activation, and mitogenesis occur through a G_i/G_o-dependent pathway (Chambard *et al.*, 1987; Kelvin *et al.*, 1989; LaMorte *et al.*, 1993a; van Corven *et al.*, 1993). The M_3 muscarinic receptor in 1321N1 cells does not interact with G_i/G_o (Hughes *et al.*, 1984; Masters *et al.*, 1985a); therefore, we hypothesized that selective coupling of the thrombin receptor to G_i/G_o might account for the differential effects of thrombin and carbachol on nuclear responses. Western blot analysis (Figure 6A) using antisera specific for G protein α-subunits demonstrated that 1321N1 cells express pertussis toxin-sensitive $G\alpha_i$ (39 kDa) and $G\alpha_o$ (40 kDa) as well as the PTX-insensitive G proteins $G\alpha_{q/11}$ (42 kDa) and $G\alpha_{12}$ (43 kDa). Pertussis toxin

Table 1. Carbachol and thrombin stimulate intracellular $InsP_3$, PEth, Ca^{2+}, and DAG generation

	$InsP_3$ (cpm)	PEth (cpm)	$[Ca^{2+}]_i$ (nM)	DAG (pmol)
Basal	64 ± 5	389 ± 10	100	173 ± 16
500 μM carbachol	400 ± 26	711 ± 21	820	287 ± 20
0.5 U/ml thrombin	428 ± 40	689 ± 27	800	271 ± 49

Phospholipid-derived second messengers were measured as described in MATERIALS AND METHODS. Values indicate peak responses to maximal concentrations of agonists and were measured at the following times: $InsP_3$ at 2.5 min; phosphatidylethanol (PEth) at 2.5 min; $[Ca^{2+}]_i$ at 15 s, and diacylglycerol (DAG) at 5 min.

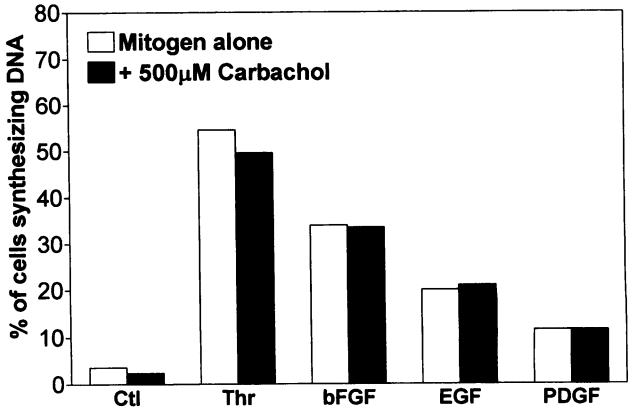

Figure 3. Carbachol does not inhibit mitogen-stimulated DNA synthesis. Quiescent astrocytoma cells were treated with either thrombin (Thr; 0.5 U/ml), basic fibroblast growth factor (bFGF; 40 ng/ml), epidermal growth factor (EGF; 1 μg/ml), or platelet-derived growth factor (PDGF; 50 ng/ml), either alone or with 500 μM carbachol, for 24 h and analyzed for DNA synthesis by BrdUrd incorporation as described. The percentage of cells undergoing DNA synthesis was quantitated for 100 to 300 cells on duplicate coverslips in a single representative experiment.

treatment was then used to assess the involvement of G_i/G_o in thrombin-induced mitogenic signaling in 1321N1 astroglial cells. The time-dependent decrease in ribosylation of PTX-sensitive G proteins indicates that the toxin is effective at ribosylating its substrates in intact 1321N1 cells within 6 h (Figure 6B). To prove that G_i was functionally inactivated by pertussis toxin treatment, we demonstrated that inhibition of isoproterenol-stimulated cAMP by the adenosine analogue phenylisopropyl adenosine was prevented by pretreatment of cells with PTX (our unpublished observation).

The effect of PTX on thrombin-mediated gene expression and DNA synthesis was then examined. Thrombin-induced AP-1-mediated gene expression was assessed by examining the ability of thrombin to transactivate an AP-1-sensitive luciferase reporter gene, as described previously (Trejo *et al.*, 1992). Under conditions that lead to maximal ADP ribosylation of pertussis toxin-sensitive G proteins, the ability of

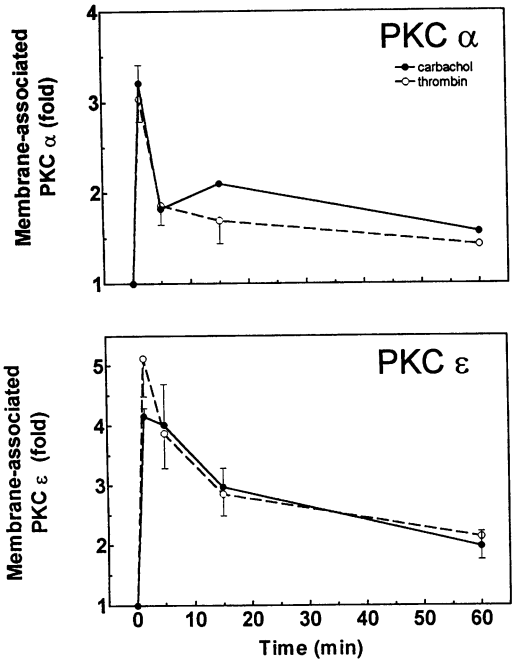

Figure 2. Carbachol and thrombin induce similar increases in membrane-associated PKC-α and PKC-ε. Cells were treated with carbachol (●) or thrombin (○) for various time periods, and membrane and cytosolic fractions were prepared and analyzed by SDS-PAGE as described in MATERIALS AND METHODS. The proteins were transferred to Immobilon membranes and probed with antibodies specific for the α and ε isoforms of PKC. Immunoreactive PKC was quantitated by laser densitometry of autoradiographs, and results are expressed as fold induction relative to unstimulated controls. Data shown are means ± SEM of one to five experiments.

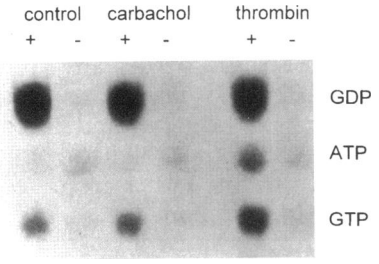

Figure 4. Ras-bound GTP is increased by thrombin but not by carbachol. Quiescent cells were labeled for 3 h with [^{32}P]orthophosphate, washed, and then treated with vehicle (control), 500 μM carbachol, or 0.5 U/ml thrombin for 10 min. Cell lysates were prepared and subjected to immunoprecipitation with (+) or without (−) Y13-259 and nucleotides separated as described in MATERIALS AND METHODS. Levels of GDP and GTP were quantitated by autoradiography and densitometry and the ratio of GTP to total (GDP plus GTP) nucleotide was calculated. Results shown are representative of four independent experiments.

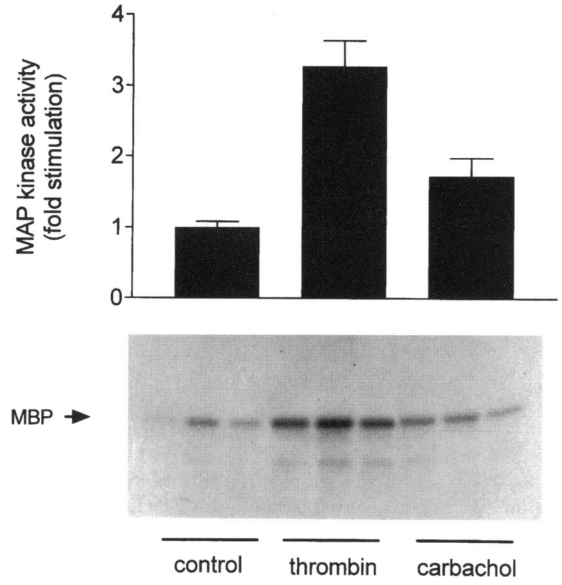

Figure 5. MAP kinase activity is differentially increased by thrombin versus carbachol. Cells were treated with vehicle (control), thrombin (0.5 U/ml) or carbachol (500 μM) for 5 min. Cell lysates were prepared and MAPK was immunoprecipitated and assayed as described in MATERIALS AND METHODS. [^{32}P] incorporation into MBP was quantitated by radioanalytic scanning. Data shown are means ± SEM of a single experiment performed in triplicate and are representative of four experiments.

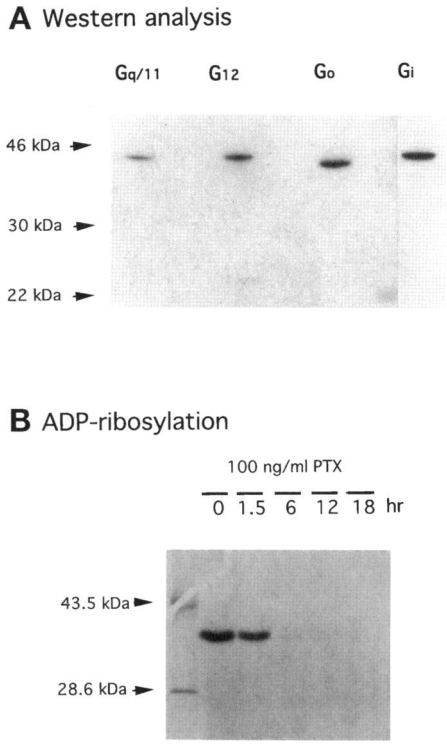

Figure 6. 1321N1 cells express multiple G α proteins and pertussis toxin-sensitive substrate(s). (A) 1321N1 membrane proteins were separated on SDS-PAGE and blotted onto Immobilon membranes and probed with antisera against $G\alpha_{q/11}$, $G\alpha_{12}$, $G\alpha_o$, or $G\alpha_i$. Antibodies were visualized with [^{125}I]-protein A. (B) Cells were treated with 100 ng/ml of PTX for 1.5 to 18 h. Membrane fractions (12 μg protein) were prepared and incubated for 1 h at 30°C with 3 mg of preactivated PTX and 0.25 μCi of [^{32}P]NAD. The samples were then analyzed by SDS-PAGE and autoradiography. The numbers to the left indicate approximate molecular mass (kDa) of marker proteins.

thrombin to stimulate luciferase expression was unchanged (Figure 7A). In addition, thrombin-induced DNA synthesis, assessed by BrdUrd incorporation into DNA, was unaffected by pertussis toxin treatment (Figure 7B). These data indicate that in 1321N1 cells, unlike most cell systems, thrombin does not induce AP-1-mediated gene expression or stimulate DNA synthesis through G_i/G_o.

These results suggested that thrombin-induced gene expression and DNA synthesis were not mediated by G_i/G_o and that activation of G_q-signaling pathways was not sufficient for these growth responses. Since our recent studies using microinjection of antibodies to $G\alpha_{12}$ demonstrated that G_{12} is required for thrombin-induced DNA synthesis (Aragay *et al.*, 1995), we hypothesized that the thrombin receptor preferentially coupled to $G\alpha_{12}$.

To determine whether the M_3mAChR and thrombin receptor show differential receptor coupling to G proteins, we transiently transfected COS-7 cells with the thrombin receptor or M_3 muscarinic receptor alone or with expression vectors encoding wild-type $G\alpha_q$ or $G\alpha_{12}$. We first established that the thrombin and M_3 muscarinic receptors were expressed in transfected COS-7 cells and that coexpressed G proteins did not alter the level of receptor expression. In COS-7 cells transfected with 0.4 μg of the M_3 muscarinic receptor cDNA, the number of M_3 muscarinic receptors was approximately 300 fmol/mg protein as assessed by radioligand-binding assays. The number of thrombin receptors expressed in COS-7 cells could not be quantitatively determined since radioligands for competitive binding studies are not available. However, increases in the expression of the epitope-tagged thrombin receptor were demonstrated using the enzyme-linked immunosorbent assay (our unpublished observation). We used Western blot analysis to verify expression of $G\alpha_q$ and $G\alpha_{12}$ in COS-7 cells. In COS-7 cells transfected with backbone vector, endogenous $G\alpha_q$ was readily detectable whereas only low levels of $G\alpha_{12}$ were seen. The expression of both $G\alpha_q$ and $G\alpha_{12}$ was markedly increased in cells transfected with the corresponding G protein α-subunit cDNA.

The ability of $G\alpha_q$ and $G\alpha_{12}$ to couple the thrombin or M_3 muscarinic receptor to AP-1 mediated gene expression was assessed by cotransfecting receptor and G protein along with the AP-1-regulated 2× TRE-luciferase reporter gene. Neither carbachol nor throm-

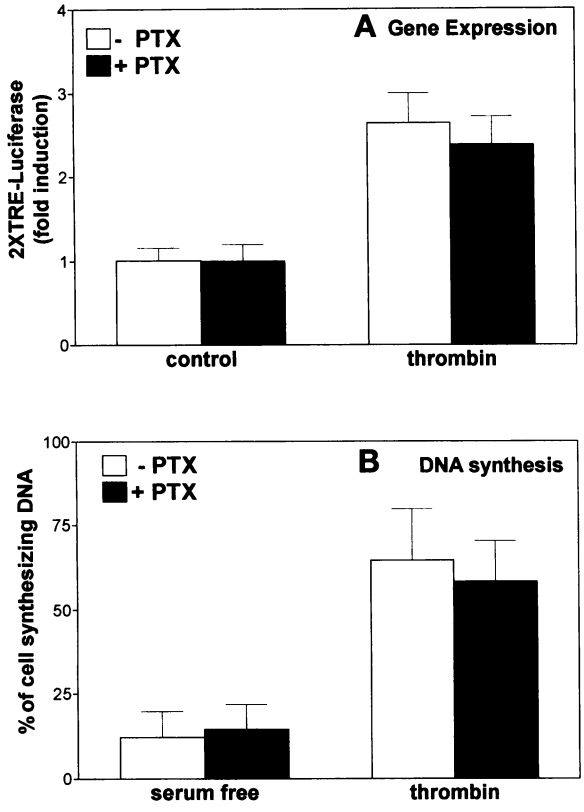

Figure 7. Pertussis toxin does not inhibit thrombin-induced activation of AP-1-mediated gene expression or DNA synthesis. (A) 1321N1 cells were transiently transfected with a 2× TRE-luciferase reporter plasmid by the calcium phosphate method as described in MATERIALS AND METHODS. Transfected cells were washed and left untreated (□) or incubated with 100 ng/ml PTX (■) for 9 h. Cells were then stimulated with thrombin (0.5 U/ml) for 24 h and assayed for luciferase activity. The fold induction in thrombin-treated cells is calculated by comparison to untreated cells following normalization for total protein. Each bar represents the mean ± SEM of four separate experiments, each containing two to three replicates. (B) Quiescent cells were left untreated (□) or treated with 100 ng/ml of PTX (■) for 12 h before the addition of thrombin and BrdUrd to the incubation medium for an additional 24 h. The percentage of cells undergoing DNA synthesis was quantitated following immunostaining for BrdUrd as described in MATERIALS AND METHODS. Results shown are means ± SEM of four separate experiments.

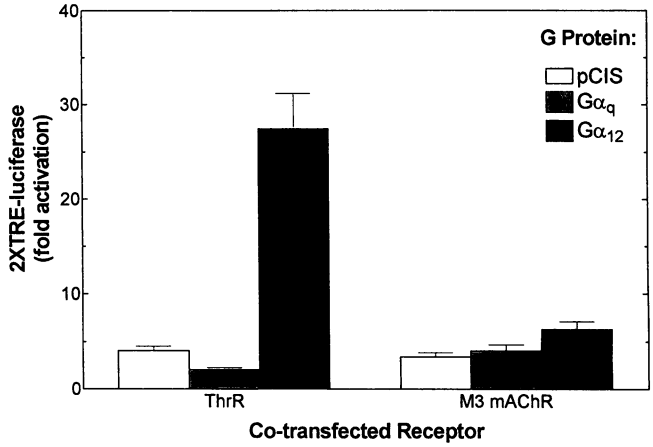

Figure 8. $G\alpha_{12}$ selectively couples the thrombin receptor to gene expression. COS-7 cells were transiently transfected with the cDNA encoding either the thrombin receptor (ThrR; 0.2 μg) or the M_3 muscarinic receptor (M_3 mAChR; 0.4 μg) with either wild-type $G\alpha_q$ (3.2 μg), $G\alpha_{12}$ (3.2 μg), or backbone vector (pCIS, 3.2 μg) along with 1.4 μg of the 2× TRE-luciferase reporter gene. Cells were treated with thrombin (0.5 U/ml) or carbachol (500 μM) for 48 h, and luciferase activity was measured in cell lysates as described in MATERIALS AND METHODS. The results are the means ± SEM of three experiments performed in triplicate.

bin induced transcriptional activation of the AP-1-sensitive reporter gene in the absence of coexpressed receptor. In cells cotransfected with the cDNA for the thrombin or the M_3 muscarinic receptor, threefold to fourfold ligand-dependent increases in 2× TRE-mediated luciferase expression were observed (Figure 8). These responses were pertussis toxin insensitive and were not increased by coexpression of $G\alpha_q$. However, in cells cotransfected with the thrombin receptor and $G\alpha_{12}$ cDNA, there was a marked synergistic increase in thrombin-stimulated gene expression (from 4- to 28-fold). In contrast, expression of $G\alpha_{12}$ along with the M_3 muscarinic receptor did not significantly increase carbachol-stimulated AP-1 reporter gene expression (Figure 8), even when muscarinic receptor expression was further increased by transfection with a sixfold higher amount of muscarinic receptor cDNA.

In a separate series of experiments, we examined coupling of these receptors and G proteins to PLC activation. COS-7 cells were transfected with receptor and G protein cDNA as described above, labeled overnight with [^{3}H]inositol, washed, and then incubated with thrombin or carbachol in the presence of LiCl. Agonist treatment increased inositol monophosphate accumulation only in cells transfected with the thrombin receptor or M_3 muscarinic receptor. Cells cotransfected with $G\alpha_q$ or $G\alpha_{12}$ along with receptor cDNA did not show greater agonist-stimulated PLC activity (Figure 9).

To further explore the mechanism for the synergistic effect of the thrombin receptor and $G\alpha_{12}$ on 2XTRE activation, we measured MAP kinase (ERK2) and Jun kinase (JNK1) activities. COS-7 cells were cotransfected with HA-tagged MAP kinase expression plasmids along with the thrombin receptor and $G\alpha_{12}$ cDNAs. Both MAP kinase and Jun kinase were activated by thrombin in cells expressing thrombin receptors. The stimulatory effect of thrombin on MAP or Jun kinase activity was not significantly greater when $G\alpha_{12}$ was coexpressed along with the receptor.

The involvement of a tyrosine kinase pathway in the observed synergy between $G\alpha_{12}$ and the thrombin receptor was assessed by the use of cell-permeable

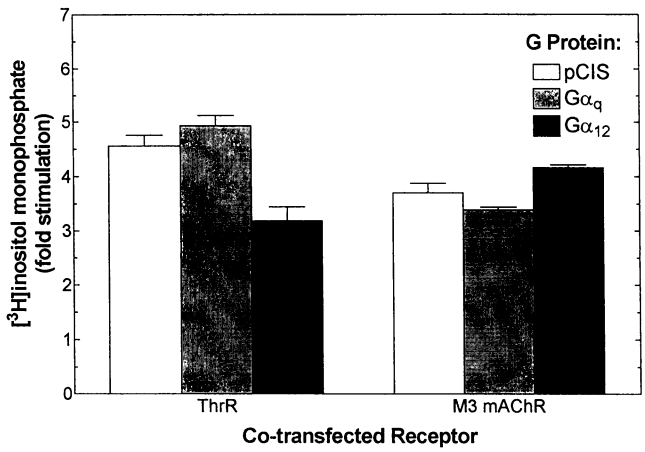

Figure 9. $G\alpha_q$ and $G\alpha_{12}$ do not affect receptor-mediated phosphoinositide hydrolysis. COS-7 cells were transiently transfected with the cDNA encoding either the thrombin receptor (ThrR; 0.2 μg) or the M_3 muscarinic receptor (M_3 mAChR; 0.4 μg) along with either wild-type $G\alpha_q$ (3.2 μg), $G\alpha_{12}$ (3.2 μg), or backbone vector (pCIS, 3.2 μg). Transfected cells were labeled overnight with 2 to 3 μCi/ml [^{3}H]inositol, washed, and treated with thrombin (0.5 U/ml) or carbachol (500 μM) for 30 min, and [^{3}H]inositol monophosphate accumulation was measured in cell lysates as described in MATERIALS AND METHODS. The results are the means ± SEM of two to three experiments performed in triplicate.

tyrosine kinase inhibitors. Both herbimycin and genistein produced a dose-dependent inhibition of the enhanced TRE-luciferase activation seen when $G\alpha_{12}$ was coexpressed with the thrombin receptor (Figure 10). These data suggest that AP-1 activity may be

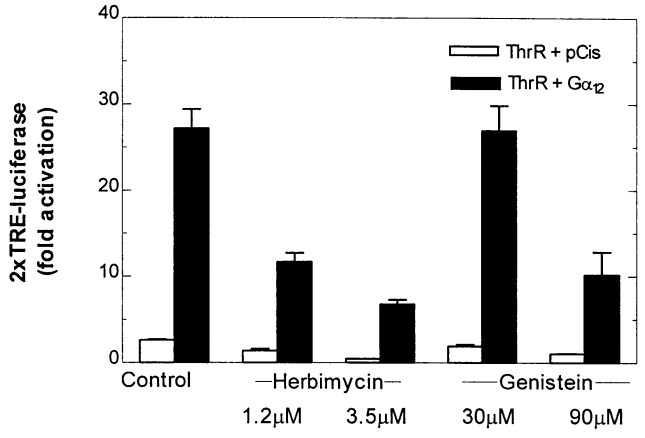

Figure 10. Tyrosine kinase inhibitors block thrombin-stimulated gene expression. COS-7 cells were transiently transfected with the cDNA encoding the thrombin receptor (ThrR; 0.2 μg), $G\alpha_{12}$ (3.2 μg), and 1.4 μg of the 2× TRE-luciferase reporter gene. Transfected cells were pretreated with the indicated concentrations of either herbimycin A or genistein for 30 min before addition of thrombin (0.5 U/ml). Luciferase activity was measured 12 h after thrombin addition. Results shown are mean ± SE of triplicate plates from one experiment representative of three.

increased through a tyrosine kinase-dependent pathway regulated by the thrombin receptor and its interaction with the $G\alpha_{12}$ protein.

DISCUSSION

1321N1 astrocytoma cells express muscarinic receptors of the M_3 subtype (Wall *et al.*, 1991) which appear to regulate a phosphatidylinositol 4,5-bisphosphate-specific PLC through G_q (Masters *et al.*, 1985a; Berstein *et al.*, 1992). The thrombin receptor in these cells likewise couples to PLC through the pertussis toxin-insensitive G protein, G_q (Jones *et al.*, 1989; LaMorte *et al.*, 1993). In this study we show that there are no apparent differences in the initial activation of PLC by muscarinic and thrombin receptor stimulation as assessed by the formation of the biologically active metabolite [^{3}H]$InsP_3$ or by the resultant increase in intracellular Ca^{2+} in response to carbachol and thrombin. The two agonists also have comparable effects on phospholipase D activation and diglyceride formation at the times examined. The divergent effects of carbachol and thrombin on gene expression and DNA synthesis are therefore unlikely to be explained by a quantitative difference in the ability of these two agonists to activate either PLC- or PLD-signaling pathways.

The activation of MAPK signaling pathways are thought to play an important role in integrating and transmitting transmembrane signals required for gene expression and cell growth. There is considerable evidence that pertussis toxin-sensitive G proteins mediate mitogenic responses and activation of MAPK cascades elicited by some G protein-coupled receptors (Chambard *et al.*, 1987; LaMorte *et al.*, 1993a; Winitz *et al.*, 1993; Crespo *et al.*, 1994; Hordijk *et al.*, 1994). It has been proposed that G_i-coupled receptors regulate Ras-dependent signaling cascades through the release of G protein $\beta\gamma$-subunits (Crespo *et al.*, 1994; Faure *et al.*, 1994; Koch *et al.*, 1994a; Touhara *et al.*, 1994; Touhara *et al.*, 1995) and that this may result from tyrosine phosphorylation of the adaptor protein Shc (Touhara *et al.*, 1995; van Biesen *et al.*, 1995). In 1321N1 cells, PTX treatment effectively inactivates G_i, as indicated by the complete ADP ribosylation of endogenous $G\alpha_i$ protein and blockade of inhibition of cAMP formation by the adenosine analogue, phenylisopropyl adenosine. However, PTX does not significantly affect the ability of thrombin to induce either AP-1-mediated gene expression or DNA synthesis (Figure 7). Therefore our results, in distinction to earlier findings in other systems (Chambard *et al.*, 1987; Kelvin *et al.*, 1989), indicate that neither α_i nor β,γ-subunits derived from G_i or other pertussis toxin-sensitive proteins are required for coupling the thrombin receptor to transcriptional or mitogenic responses in 1321N1.

Several recent reports demonstrate that agents that increase intracellular cAMP inhibit growth factor-in-

duced mitogenic signaling (Burgering *et al.*, 1993; Cook and McCormick, 1993; Wu *et al.*, 1993). The observation that stimulation of M_1 muscarinic acetylcholine receptors stably expressed in Rat-1a fibroblasts inhibited growth factor-stimulated mitogenic pathways (Russell *et al.*, 1994) suggested that the failure of the muscarinic receptor to induce gene expression and mitogenesis could result from activation of signaling pathways that block cellular growth. However, although we found that carbachol increased intracellular cAMP (threefold) in 1321N1 cells, carbachol did not inhibit mitogenesis induced by thrombin or other growth factors. The lack of a mitogenic response to carbachol is also unlikely to be explained by muscarinic receptor desensitization or depletion of carbachol from the media since phosphoinositide hydrolysis is stimulated for up to 6 h following the addition of carbachol to 1321N1 cells (Nieto *et al.*, 1994). These data indicate that carbachol does not activate pathways that inhibit cell proliferation, but rather that thrombin activates unique effectors that induce genetic and mitogenic changes.

Previous studies in 1321N1 cells demonstrate that $G\alpha_{12}$ participates in thrombin-induced gene expression and DNA synthesis (Aragay *et al.*, 1995). We have expanded on these studies by comparing the coupling of the thrombin and M_3 muscarinic receptor to $G\alpha_{12}$ by examining AP-1-mediated gene expression in COS-7 cells. We demonstrate here that there is a ligand-dependent, synergistic increase in AP-1-mediated gene expression in COS-7 cells cotransfected with the thrombin receptor and $G\alpha_{12}$ cDNA but not with the M_3 muscarinic receptor and $G\alpha_{12}$. Since activation of expressed thrombin and M_3 muscarinic receptor leads to equivalent increases in inositol phosphate production (Figure 9), it appears that expressed receptors couple equally well to $G\alpha_q$ and PLC activation. We therefore interpret the synergistic interaction of the thrombin receptor and $G\alpha_{12}$ on AP-1-mediated gene expression as reflecting a greater capacity of the thrombin receptor (versus M_3 muscarinic receptor) to interact with and signal through $G\alpha_{12}$. Since we see no synergistic effect of the receptor and $G\alpha_{12}$ on MAP kinase or Jun kinase activation, other effectors regulated through $G\alpha_{12}$ must be responsible for increasing AP-1 activity. We suggest that these effectors include tyrosine kinases since the synergy is inhibited in COS-7 cells treated with genistein or herbimycin.

We have shown that in 1321N1 cells G_i is not required and G_q is not sufficient to transduce mitogenic signals from the thrombin receptor. We further demonstrate using COS-7 cells that the thrombin receptor, but not the M_3 muscarinic receptor, couples to AP-1-mediated gene expression through the pertussis toxin-insensitive G protein $G\alpha_{12}$. We postulate that the thrombin receptor in 1321N1 cells, through its interaction with $G\alpha_{12}$, activates pathways involving tyrosine kinases which cooperate with G_q-regulated pathways to effect growth responses.

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