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A G Protein $\beta\gamma$ Dimer-Mediated Pathway Contributes to Mitogen-Activated Protein Kinase Activation by Thyrotropin-Releasing Hormone Receptors in Transfected COS-7 Cells

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ABSTRACT

Activation of mitogen-activated protein kinase (MAPK) is induced by adding thyrotropin-releasing hormone (TRH) to COS-7 cells cotransfected with TRH receptors and an epitope-tagged MAPK. Long term treatment of the cells with pertussis toxin has no effect on TRH-induced MAPK activation. Incubation of the cells with the protein kinase C (PKC) inhibitor GF109203X causes an almost complete inhibition of MAPK activation by the PKC activator phorbol-12-myristate-13-acetate. In contrast, only ~50% of the TRH-induced MAPK activity is inhibited by GF109203X, indicating that activation of MAPK by TRH is only partially dependent on PKC. The inhibitory effect of GF109203X is additive with that of $p21^{N17ras}$, a dominant negative mutant of $p21^{ras}$ that exerts little effect on PKC-dependent MAPK activation by phorbol-12-myristate-13-acetate. The TRH-induced activation of MAPK also is inhibited partially by overexpression of transducin α subunits (αt), an agent known to sequester free G protein $\beta\gamma$ dimers. However, the inhibitory potency of αt on TRH-induced activation is about half of that obtained in cells transfected with m2 muscarinic receptors, which activate MAPK exclusively through $\beta\gamma$ dimers. The effect of αt is also additive with that of GF109203X but not with that of $p21^{N17ras}$. MAPK activation is not induced by the constitutively active form of $G_{\alpha q}$ due to an inhibitory effect of its expression at a step downstream of that at which PKC-dependent and -independent routes to MAPK converge. Our results demonstrate that TRH receptors activate MAPK by a pathway only partially dependent on PKC activity. Furthermore, they indicate that $\beta\gamma$ dimers of a pertussis and cholera toxin-insensitive G protein are involved in the PKC-independent fraction of the dual signaling route to MAPK initiated in the TRH receptor.

The GH_3 rat anterior pituitary cell line often is used as a model for lactotrophs because it produces prolactin and possesses functional receptors for TRH and other regulatory neuropeptides (e.g., vasoactive intestinal peptide or bombesin) that act to increase prolactin secretion [see Ben-Jonathan *et al*. (1989) and Corette *et al*. (1995) for reviews]. Both early releases of preformed prolactin and sustained secretion of newly synthesized hormone contribute to TRH effects (Gershengorn, 1986). Activation of the GH_3 cell TRH-R promotes its interaction with one or more G proteins with subsequent regulation of several effectors, including PLC and different ionic channels (Corette *et al*., 1995; Gershengorn and Osman, 1996). As for many cell types and agonists, phosphorylation of proteins found in GH_3 cell cytosolic, microsomal, and nuclear fractions has been repeatedly reported for TRH (Drust *et al*., 1982; Drust and Martin, 1982; Sobel and Tashjian, 1983; Drust and Martin, 1984; Jefferson *et al*., 1991), and phosphorylation-dephosphorylation cycle or cycles have been implicated in regulation of GH_3 cell electrical activity by modulation of specific ionic currents (Barros *et al*., 1992, 1993; Delgado *et al*., 1992). Among other protein kinases (e.g., PKC and Ca^{2+}/calmodulin kinase; Drust and Martin, 1984; Martin *et al*., 1990; Jefferson *et al*., 1991), MAPK has been shown recently to be stimulated by TRH in GH_3 cells (Ohmichi *et al*., 1994). Although partially dependent on PKC activity, a PKC-independent pathway also was found to couple the TRH-R to MAPK activation; however, the

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ABBREVIATIONS: TRH, thyrotropin-releasing hormone; MAPK, mitogen-activated protein kinase; Met-TRH, methyl-thyrotropin-releasing hormone; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; TRH-R, thyrotropin-releasing hormone receptor; PLC, phosphoinositide-specific phospholipase C; PTX, pertussis toxin; m1-R, m1 acetylcholine muscarinic receptor; CTX, cholera toxin; MBP, myelin basic protein; EGF, epidermal growth factor; m2-R, m2 acetylcholine muscarinic receptor; αt, G protein α subunit of retinal transducin; PIP_2, phosphatidylinositol bisphosphate; α-QL, mutationally activated G protein α subunit; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

molecular entities involved in such a coupling remain obscure. The physiological role of this TRH effect is not known, but early tyrosine phosphorylation leading to MAPK activation has been suggested to be involved in TRH-induced prolactin secretion (Kanda *et al.*, 1994; Ohmichi *et al.*, 1994). Furthermore, the known regulatory role of the enzyme on gene transcription makes it a candidate to participate in prolactin synthesis or proliferative and neuronal trophic responses also reported for TRH (Metcalf and Jackson, 1989).

Cloning of the GH_3 cell TRH-R allowed the deduction of its amino acid sequence and its inclusion in the superfamily of seven transmembrane-spanning G protein-coupled receptors (Gershengorn and Osman, 1996). Activation of the ubiquitous MAPK by growth factor receptors that possess intrinsic tyrosine kinase activity has been largely recognized. Mitogenic signals from these receptors involve a series of SH_2- and SH_3-dependent protein/protein interactions resulting in $p21^{ras}$-dependent MAPK activation [see Malarkey *et al.* (1995) and Marshall (1995) for reviews]. Recently, a number of receptors that couple to heterotrimeric G proteins have been shown to stimulate MAPK activity (Crespo *et al.*, 1994a; Faure *et al.*, 1994; Russell *et al.*, 1994; Hawes *et al.*, 1995; Malarkey *et al.*, 1995; Van Biesen *et al.*, 1995; Luttrell *et al.*, 1996), including both receptors that couple to G_q and G_i. It has been suggested that MAPK activation in COS cells via receptors coupled to members of the $G_{q/11}$ family largely occurs through a PKC-dependent pathway. In this case, the GTP-bound α subunit of $G_{q/11}$ would activate PLC, which also would activate MAPK via a mechanism not completely understood (Faure *et al.*, 1994; Hawes *et al.*, 1995; Luttrell *et al.*, 1996). In contrast, MAPK activation by receptors coupled to PTX-sensitive G proteins (e.g., G_i or G_o) occurs via a pathway PKC independent, $p21^{ras}$ mediated, and dissociable from PLC activation. $\beta\gamma$ dimers released from the PTX-sensitive G protein and one or more tyrosine phosphorylations are important components of the coupling cascade to MAPK initiated in this type of receptors (Crespo *et al.*, 1994a; Faure *et al.*, 1994; Koch *et al.*, 1994; Hawes *et al.*, 1995; Van Biesen *et al.*, 1995; Luttrell *et al.*, 1996). However, it has been reported that stimulation of MAPK activity by G_q-coupled m1-R is partially independent of PKC activity (Crespo *et al.*, 1994b), and some inhibitory effect of overexpression of transducin α subunit on m1-R-induced MAPK activation also has been obtained (Crespo *et al.*, 1994a). This opens the possibility that a PKC-independent $\beta\gamma$ dimer-mediated route also is involved, at least in part, in MAPK activation via receptors coupled to PTX-insensitive G proteins.

In this report, the transduction pathways linking TRH-R stimulation to MAPK activation are explored using COS-7 cells transfected with the TRH-R and an epitope-tagged MAPK. Our results indicate that as in pituitary cells, TRH-Rs activate MAPK via a pathway only partially dependent on PKC activity. Furthermore, they show that $\beta\gamma$ dimers of a PTX- and CTX-insensitive G protein are involved in the PKC-independent fraction of the dual signaling route to MAPK initiated in the TRH receptor.

Materials and Methods

Chemicals. TRH, carbachol, PMA, MBP, genistein, PTX, CTX, and culture medium were purchased from Sigma Chemie (Deisenhofen, Germany). Serum was from Biochrom (Berlin, Germany). Anti-hemagglutinin monoclonal antibody 12CA5 and human EGF was from Boehringer-Mannheim Biochemica (Mannheim, Germany). [γ-^{32}P] ATP (7000 Ci/mmol, 160 mCi/ml) and *myo*-[^{3}H]inositol (17.6 Ci/mmol, 1 Ci/ml) were obtained from Amersham International (Buckinghamshire, UK). [^{3}H]Met-TRH (pGlu-3-methyl-His-ProNH_2; 82.5 Ci/mmol, 1 mCi/ml) was from New England Nuclear (Boston, MA). GF109203X was from Calbiochem (San Diego, CA). DEAE-dextran and Gamma-Bind Sepharose beads were from Pharmacia Biotech (Uppsala, Sweden) (St. Quentin, France). All other reagents were purchased from Sigma and were the highest quality available.

Expression plasmids. The expression plasmid pcDNA3-HA-MAPK (provided by Dr. J. S. Gutkind, National Institute of Dental Research, National Institutes of Health, Bethesda, MD) contains an amino-terminal hemagglutinin-tagged murine p42-MAPK cDNA. Its protein product (referred to here as HA-MAPK) can be recognized efficiently by the murine monoclonal antibody 12CA5. The previously described cDNAs for rat TRH receptor isoforms (de la Peña *et al.*, 1992) were cloned in pcDNA3 (InVitrogen, San Diego, CA) as *Hin*dIII/*Eco*RI fragments. m1-R and m2-R cDNAs were subcloned in pcDNA3 as *Bam*HI/*Xba*I and *Xho*I/*Xba*I fragments, respectively, transferred from constructs in pBluescript KS^+ (Stratagene, La Jolla, CA). $G_{\alpha t}$ was cloned in pcDNA3 as an *Eco*RI/*Xho*I fragment transferred from pcDNA I. The plasmid constructs of wild-type or mutationally active G protein α subunits cloned in pcDNA I and that of $p21^{N17ras}$ cloned in pcDNA 3 also were generously provided by Dr. J. S. Gutkind.

Transient expression in COS-7 cells. COS-7 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1 mixture) supplemented with 0.13% $NaHCO_3$, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum. Plasmid DNA transfection of subconfluent COS-7 was performed by the DEAE-dextran technique as described previously (del Camino *et al.*, 1997).

MAPK assay. MAPK activity in COS-7 cells transfected with HA-MAPK was determined as described previously (Crespo *et al.*, 1994a). COS-7 cells were cotransfected with the different DNA constructs (1–2 μg/plate) and the expression plasmid pcDNA 3-HA-MAPK (1–2 μg/plate). The total amount of plasmid DNA was equalized in all 60-mm plates with vector DNA (pcDNA3) when necessary. Two days after transfection, cells were serum-fasted overnight and then stimulated with the different agents. After the indicated periods of time, cells were washed with cold phosphate-buffered saline and lysed in a buffer containing 10 mM EGTA, 40 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 20 mM HEPES, pH 7.5. After centrifugation, clarified supernatants were immunoprecipitated with an anti-hemagglutinin monoclonal antibody 12CA5 for 1 hr at 4°. Immunocomplexes were recovered with the use of Gamma-Bind Sepharose beads. Pellets of individual plates were washed three times with phosphate-buffered saline solution supplemented with 1% Triton X-100; once with 0.5 M LiCl in 100 mM Tris, pH 7.5; and once with kinase reaction buffer [12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate, 12.5 mM MOPS, pH 7.5]. Reactions were performed at 30° for 30 min by the addition to pellets of a 30-μl volume of kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP, 20 μM unlabeled ATP, and 1.5 mg/ml MBP. Reactions were terminated by the addition of 5× Laemmli's buffer, boiled, and electrophoresed on 15% polyacrylamide gels. Phosphorylated MBP was visualized by autoradiography and quantified with the aid of an InstantImager (Packard, Meriden, CT).

Measurement of [^{3}H]Met-TRH binding and phosphatidylinositol hydrolysis in transfected cells. Binding assays to COS-7 cells were performed as described previously (del Camino *et al.*, 1997). For assays of inositol phosphate accumulation, cells were incubated 48 hr after transfection with 1 μCi/ml *myo*-[^{3}H]inositol for 24 hr at 37° in inositol-free medium supplemented with 5% fetal bovine serum. Cells were incubated for an additional 6 hr in serum-free conditions and stimulated with experimental agents for 45 min

in the presence of 10 mM LiCl. Inositol phosphates were extracted subsequently and analyzed by ion exchange chromatography using AG1-X8 resin (BioRad, Hercules, CA).

Statistical analysis. Unless otherwise indicated, data are expressed in the histograms as mean ± standard error for the number of experiments indicated, which were performed with duplicate or triplicate samples. Due to significant day-to-day differences in MAPK activation levels, only data obtained on the same day with different treatments were used for comparison. Fold stimulation in the graphs corresponds to MAPK activity levels compared with those obtained in untreated cells. Basal activity without any treatment has not been subtracted in any case. Thus, 1-fold activation in the presence of an agonist plus an inhibitor would correspond to a 100% inhibition of the agonist-induced activation.

Results

The addition of TRH to COS-7 cells cotransfected with rat GH_3 cells TRH-R and an epitope-tagged MAPK caused a time- and concentration-dependent activation of the enzyme (Fig. 1). The increase in activity measured 5 min after the start of 1 μM TRH treatment averaged 7.2 ± 0.6-fold (47 experiments) of control values without hormone. Although less marked than the activation reached by stimulation of COS-7 EGF endogenous receptors with EGF (22.3 ± 3.8-fold, 21 experiments; see Fig. 1A), these results demonstrate that the TRH-R is able to activate MAPK in transfected cells, as demonstrated previously in GH_3 anterior pituitary cells (Ohmichi *et al*., 1994). Both isoforms of the receptor (TRH-R_{412} and TRH-R_{387}; de la Peña *et al*., 1992) induced equal activations of the kinase (Fig. 1A) and showed a similar dependence on hormone concentration (not shown). Thus, subsequent experiments were performed routinely with the TRH-R_{412} long isoform. The MAPK activation in response to TRH was rapid, reaching a maximum after 5 min of exposure to the neuropeptide that slightly declined thereafter up to 30 min of treatment (Fig. 1, B and D). The activation level at 5 min after stimulation depended on the concentration of TRH (Fig. 1, C and D). The EC_{50} value was obtained at 1–10 nM, a value similar to the K_d value for hormone-receptor interactions in GH_3 cells (Hinkle, 1989), and maximal stimulation levels were obtained at ~100 nM TRH.

It has been shown previously that both G_i- and $G_{q/11}$-coupled receptors are able to activate MAPK in COS cells (Crespo *et al*., 1994a; Faure *et al*., 1994; Russell *et al*., 1994; Hawes *et al*., 1995; Van Biesen *et al*., 1995; Luttrell *et al*., 1996). It also is known that TRH-Rs are coupled to PTX- and CTX-insensitive $G_{q/11}$ for activation of PLC in GH_3 cells and to other G proteins for other effects (Gershengorn and Osman, 1996). As an initial approach to determine the coupling specificity of TRH-R to MAPK activation, we used bacterial toxins for covalent modification of COS-7 G proteins. As shown in Fig. 2, the response to TRH was not altered by pretreatment of the cells with PTX or CTX. Failure to detect toxin effects was not due to lack of modifications of cellular G proteins by the toxins, because MAPK activation mediated by m2-R was 83% inhibited by PTX pretreatment, as reported previously (Crespo *et al*., 1994a).

TRH-R stimulation in anterior pituitary cells causes $G_{q/11}$-mediated PIP_2 hydrolysis and PKC activation (Gershengorn and Osman, 1996). However, both PKC-dependent and -independent pathways are involved in GH_3 cell MAPK activation (Ohmichi *et al*., 1994). Previous work in COS-7 cells has demonstrated two main pathways for G protein-mediated MAPK activation. G_i-coupled receptor-mediated MAPK activation is $G_{\beta\gamma}$ subunit mediated, $p21^{ras}$ dependent, PKC independent, and dissociable from PLC activation (Crespo *et al*., 1994a; Faure *et al*., 1994; Koch *et al*., 1994; Hawes *et al*., 1995; Van Biesen *et al*., 1995; Luttrell *et al*., 1996). The

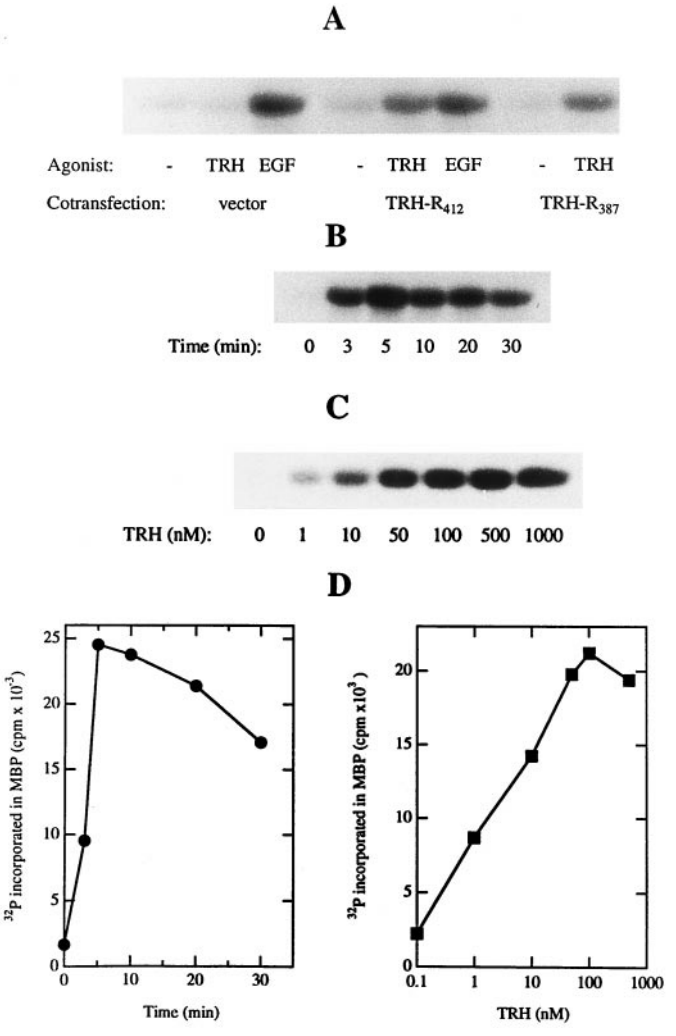

Fig. 1. Stimulation of MAPK in COS-7 cells after stimulation of transfected TRH-R. A, TRH increases the enzymatic activity of an epitope-tagged MAPK when cotransfected with both isoforms of the TRH-R. COS-7 cells were cotransfected with pcDNA 3 plasmid DNA (*vector*) or with the same plasmid containing the sequences encoding the long (*TRH-R_{412}*) or the short (*TRH-R_{387}*) isoforms (de la Peña *et al*., 1992) of the TRH-R and HA-MAPK as described in Materials and Methods. MAPK activity was determined in serum-fasted cells after a 30-min treatment with (*TRH*) or without (−) 1 μM TRH. MAPK activity in response to stimulation of COS-7 cell endogenous EGF receptors with 100 ng/ml EGF is shown for comparison. Radioactivity incorporated in MBP as a result of the enzymatic activity of immunoprecipitated HA-MAPK was visualized by autoradiography. B, Time course of MAPK activation by TRH in TRH-R-transfected COS-7 cells. Incorporation of radioactivity in MBP was measured in reactions performed as in A after stimulation of cells cotransfected with TRH-R_{412} and HA-MAPK. Cells were stimulated with 1 μM TRH for the indicated periods of time. A representation of the amount of radioactivity incorporated in MBP as a function of the duration of TRH treatment is shown in D (*left*). C, Dependence of MAPK activation on TRH concentration. MAPK activity was measured in HA-MAPK immunoprecipitates of COS-7 cells expressing TRH-R and stimulated with the indicated TRH concentrations for 5 min. The representation of the amount of radioactivity incorporated in MBP as a function of the TRH concentration is shown in D (*right*). A–C, Major bands correspond to the position of MBP in the autoradiograms.

mechanism or mechanisms leading to MAPK activation by G_q-coupled receptors, such as m1-R and α_{1B}-adrenergic receptor, remain controversial. Thus, a $p21^{ras}$-independent, PLC- and PKC-mediated MAPK activation pathway insensitive to $G_{\beta\gamma}$ subunit sequestrants (e.g., overexpression of transducin α subunits or a peptide derived from the carboxyl terminus of the β-adrenergic receptor kinase) has been involved in such a response (Faure *et al.*, 1994; Koch *et al.*, 1994; Hawes *et al.*, 1995). However, stimulation of MAPK activity by m1-R only partially dependent on PKC and partial inhibitions of m1-R induced activations by transducin α subunits also have been reported (Crespo *et al.*, 1994a, 1994b). To determine whether a PKC-independent pathway might be involved in TRH-induced activation of MAPK, we initially depleted COS-7 cells of PKC by a 18-hr treatment with 1 μM PMA. This prevented subsequent activation of MAPK by short term treatment with PMA and TRH. However, a several-fold MAPK activation was detected at the end of the prolonged PMA treatment, even without MAPK stimulants (data not shown). This might confound experimental interpretations. As an alternative approach, we used the nontoxic PKC-specific inhibitor bisindolylmaleimide GF109203X, previously shown to act as a potent *in vivo* PKC inhibitor in COS-7 cells (Crespo *et al.*, 1994b; Coso *et al.*, 1995). As shown in Fig. 3, treatment of the cells with 1 μM GF109203X almost completely abolished the PMA-stimulated MAPK activity. Furthermore, no detectable effect of the inhibitor was obtained on the PKC-independent activation of the enzyme by EGF. Finally, only a partial inhibition of ~50% of the TRH-induced activation of MAPK was obtained in the presence of GF109203X. This suggests that as in pituitary cells, MAPK activation in transfected COS-7 cells is only partially dependent on PKC.

As stated, PKC-dependent activation of MAPK in COS-7 cells involves a pathway independent of $p21^{ras}$ (Crespo *et al.*, 1994a, 1994b; Russell *et al.*, 1994; Hawes *et al.*, 1995; Luttrell *et al.*, 1996). Further support for the existence of a PKC-independent fraction of TRH-induced MAPK activation was obtained in experiments in which $p21^{ras}$ function is impaired by expression of the dominant negative mutant $p21^{N17ras}$. As shown in Fig. 4A, little effect of $p21^{N17ras}$ expression is de-

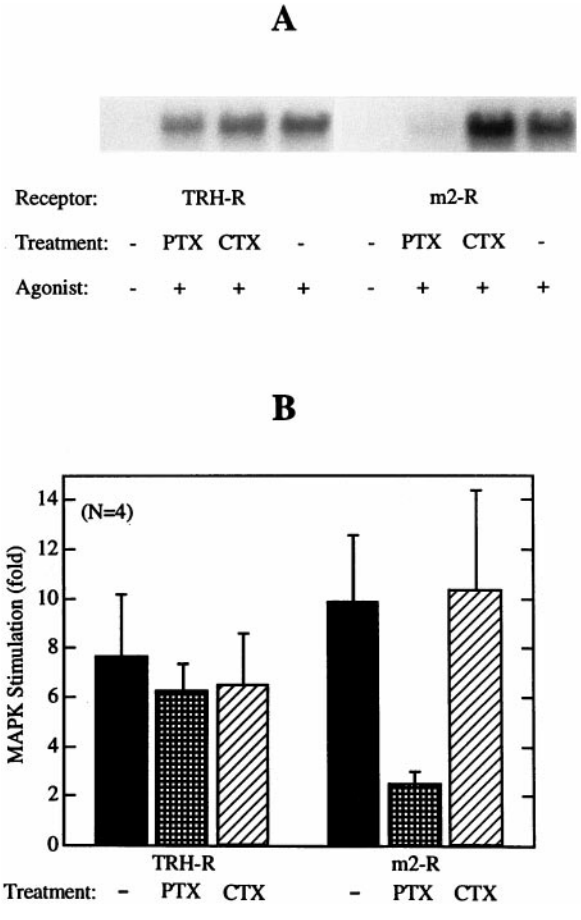

Fig. 2. Effect of PTX and CTX treatment on MAPK activation caused by stimulation of TRH-R and m2-R in receptor-transfected COS-7 cells. COS-7 cells were transiently transfected with DNA plasmids encoding HA-MAPK and TRH-R or m2-R as described in Materials and Methods. When indicated, cells were preincubated overnight with PTX (100 ng/ml) or CTX (200 ng/ml) before stimulation with 1 μM TRH (*TRH-R*) or 1 mM carbachol (*m2-R*) for 5 min. A, Autoradiogram showing the radioactivity incorporated in MBP. Only the representative result of a single reaction from triplicate samples is shown for every individual treatment. B, Averaged results of four experiments performed in triplicate. *Bars*, averaged MAPK activity expressed as fold stimulation respective to that obtained without TRH or carbachol for TRH-R- and m2-R-transfected cells, respectively. Significantly lower MAPK activation values were obtained after the addition of carbachol to cells expressing m2-Rs and incubated with PTX ($p < 0.05$ versus untreated cells, paired t test).

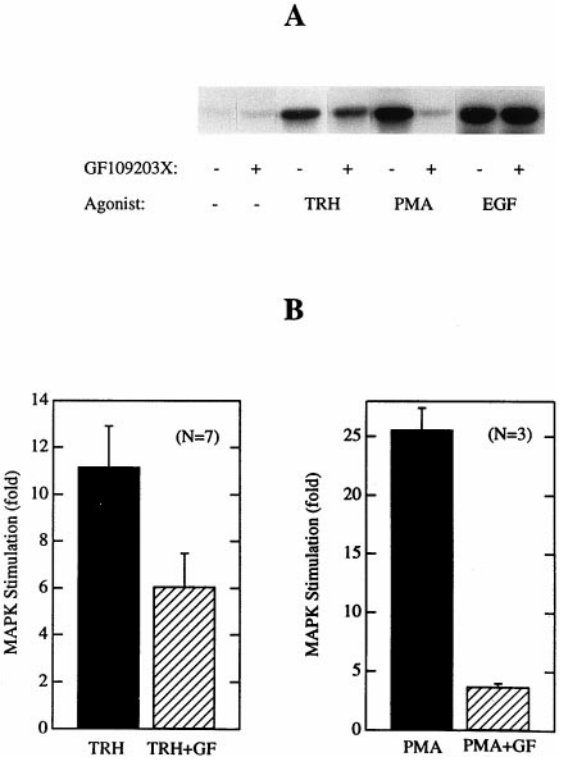

Fig. 3. Effect of the PKC inhibitor GF109203X on TRH-, PMA-, and EGF-mediated MAPK activation. COS-7 cells were cotransfected with plasmid DNA encoding HA-MAPK and stimulated for 5 min with 1 μM TRH or 50 ng/ml PMA or for 3 min with 100 ng/ml EGF as indicated. Plasmid-encoding TRH-R was included for transfection of TRH-stimulated cells. Before stimulation, cells were preincubated with 3.5 μM GF109203X for 60 min. The inhibitor was present during treatment with different agonists. A, Autoradiogram of the radioactivity incorporated in MBP. B, Averaged results for the number of experiments indicated (*N*) and performed in triplicate for TRH-treated (*left*) or PMA-treated (*right*) cells. *GF*, presence of GF109203X. MAPK activity is expressed as fold stimulation respective to that obtained without agonists. Treatment with GF109203X significantly reduced MAPK activation by TRH or PMA ($p < 0.01$ versus untreated cells, paired *t test).*

tected on PKC-dependent MAPK activation by PMA. However, a 51% inhibition of TRH-induced MAPK activity is exerted by introduction of $p21^{N17ras}$ into the cells. It is interesting that as for the GF109203X effect and consistent with the coexistence of PKC-dependent and -independent pathways going to MAPK from TRH-R, only a partial inhibition of the TRH-induced enzyme activation was obtained in the presence of $p21^{N17ras}$. This partial reduction of TRH-induced activation was not due to a lowered potency of $p21^{N17ras}$ to inhibit Ras signaling. Thus, a similar 60% inhibition of the TRH-evoked response was obtained in a second set of experiments in which the Ras-dependent m2-R response (Crespo *et al*., 1994a; Russell *et al*., 1994) was almost abolished (87% inhibition) by coexpression of $p21^{N17ras}$ (Fig. 4B). Finally, the inhibitory effect of $p21^{N17ras}$ was not related to an indirect effect on TRH-R expression, because similar amounts of [^{3}H]Met-TRH binding sites were obtained in cells transfected with TRH-R alone (20×10^3 receptors/cell, mean of three determinations) or with both TRH-R and $p21^{N17ras}$ (25×10^3 receptors/cell), as measured by [^{3}H]Met-TRH saturation binding. These results demonstrate that $p21^{N17ras}$ constitutes a useful tool to specifically distinguish between pure PKC-dependent responses, such as that promoted by PMA, and PKC-independent Ras-mediated activations, such as that induced by m2-R. Furthermore, they indicate that a PKC-dependent route coexists with a Ras-dependent pathway leading to MAPK activation in response to TRH.

The demonstration of both a PKC-dependent and -independent way to activate MAPK suggests the existence of a dual pathway initiated in the TRH-R. However, only partial inhibitions are induced by agents presumably acting on only one of both routes. This does not exclude that the action of these agents is exerted at different levels of a single route. To demonstrate that the TRH effect is indeed due to operation of two independent pathways converging at a point downstream of the step inhibited by GF109203X and $p21^{N17ras}$, additivity of inhibitions caused by these agents was studied. As shown in Fig. 5, both GF109203X and $p21^{N17ras}$ caused a partial inhibition of MAPK activation by TRH, which amounted to 67% and 75%, respectively, in this set of experiments. Furthermore, the TRH-induced activity was completely abolished (inhibition level of 97%) when both agents

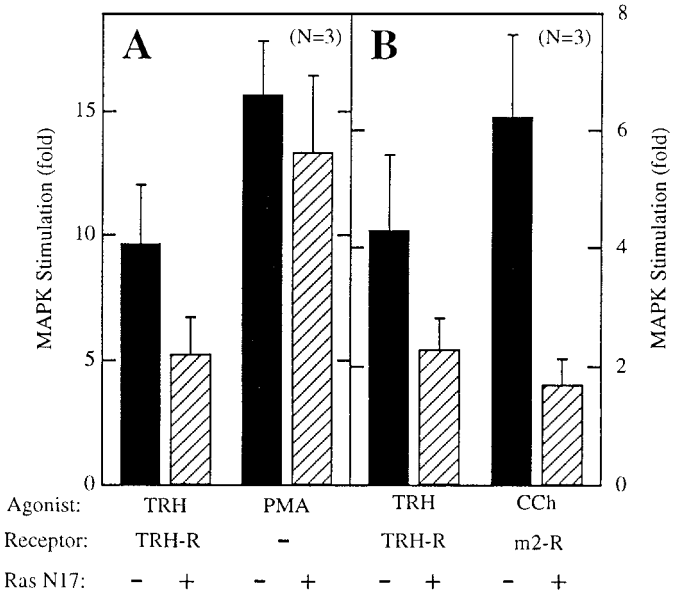

Fig. 4. Effect of expression of the dominant negative form of $p21^{ras}$ ($p21^{N17ras}$) on MAPK activation. A, Expression of $p21^{N17ras}$ partially inhibits TRH-mediated, but not PMA-mediated, MAPK activation. COS-7 cells were cotransfected with DNA plasmids encoding HA-MAPK and TRH-R plus either vector DNA alone (*filled bars*) or plasmid DNA encoding $p21^{N17ras}$ (*striped bars*). Plasmid DNA containing TRH-R sequence was not used for PMA-treated cells. Cells were stimulated with 1 μM TRH or 50 ng/ml PMA for 5 min. B, Expression of $p21^{N17ras}$ inhibits TRH- and carbachol-mediated MAPK activation differently in cells expressing TRH-R and m2-R, respectively. COS-7 cells were cotransfected with DNA plasmids encoding HA-MAPK and TRH-R or m2-R plus either vector DNA alone (*filled bars*) or plasmid DNA encoding $p21^{N17ras}$ (*striped bars*). Cells were stimulated with 1 μM TRH (*TRH-R*) or 1 mM carbachol (*m2-R*) for 5 min. MAPK activity is expressed as fold stimulation respective to that obtained without agonists. Values shown represent mean ± standard error from three separate experiments performed in triplicate. Different batches of cells were used to obtain the data. Except for PMA-induced activations, significantly lower MAPK activation values were obtained in cells cotransfected with $p21^{N17ras}$ ($p < 0.05$ versus non-$p21^{N17ras}$-containing cells, paired t test).

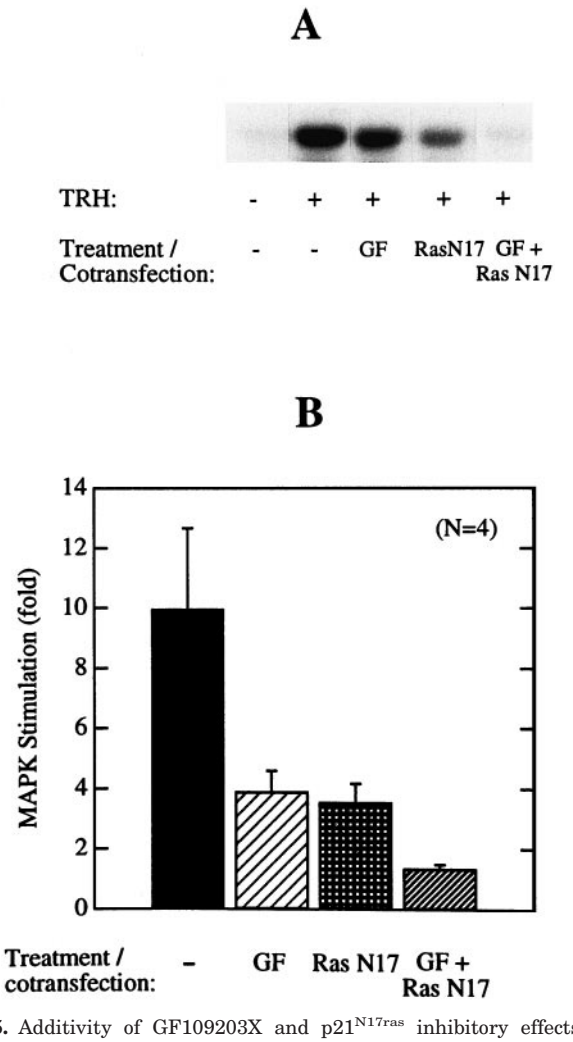

Fig. 5. Additivity of GF109203X and $p21^{N17ras}$ inhibitory effects on TRH-induced MAPK activation. COS-7 cells were cotransfected with DNA plasmids encoding HA-MAPK and TRH-R. When indicated, plasmid encoding $p21^{N17ras}$ was included for transfection (*RasN17*). Performance of a 60-min preincubation with 3.5 μM GF109203X before 5-min stimulation of the cells with 1 μM TRH is indicated. In this case, the PKC inhibitor was maintained during the TRH treatment. A, Autoradiogram showing the radioactivity incorporated into MBP. B, Averaged results of four experiments performed with triplicate samples. MAPK activity is expressed as fold stimulation respective to that obtained in the absence of TRH. MAPK activation values were significantly lower in the presence of GF109203X, $p21^{N17ras}$, or both ($p < 0.05$ versus control, paired t test).

were used at the same time. This indicates that two routes couple TRH-R to MAPK activation: a PKC-dependent pathway, probably involving a $G_{q/11}$ protein coupled to phospholipase C activation and generation of diacylglycerol, and a second route that involves $p21^{ras}$ and activates MAPK independent of PKC activity increases.

Previous work in GH_3 cells indicated the possible participation of tyrosine phosphorylation or phosphorylations in the PKC-independent activation of MAPK by TRH (Ohmichi *et al.*, 1994). However, the mechanism or mechanisms leading to tyrosine kinase activity increases, or the nature of the tyrosine kinase or kinases implicated in the effect of the neuropeptide is not known. In COS-7 cells, the $p21^{ras}$-mediated and PKC-independent effects of G_i protein-coupled receptors on MAPK are exerted by liberation of $\beta\gamma$ dimers and subsequent stimulation of $p21^{ras}$, with participation of one or more tyrosine kinases (Hawes *et al.*, 1995; Van Biesen *et al.*, 1995; Luttrell *et al.*, 1996). To determine the role of free G protein $\beta\gamma$ dimers in MAPK activation by TRH, we used the overexpression of αt to sequester $\beta\gamma$ subunits after they are released from G proteins by receptor stimulation (Crespo *et al.*, 1994a; Faure *et al.*, 1994). The presence of αt did not modify significantly the amount of expressed TRH-Rs, as measured by [^{3}H]Met-TRH saturation binding (40×10^3 receptors/cell in the absence versus 33×10^3 receptors/cell in the presence of αt, mean of three determinations). As shown in Fig. 6, expression of αt did not affect the activation of MAPK by PMA but inhibited 77% of its activation when mediated by m2 muscarinic receptors. This result parallels those previously obtained by others, demonstrating that the activation of MAPK by m2-R is mediated predominantly via $\beta\gamma$ dimers released from G_i (Crespo *et al.*, 1994a; Faure *et al.*, 1994). Furthermore, TRH-induced activation of the enzyme via TRH-R was inhibited in the presence of αt. However, consistent with coexistence of a PKC-dependent and a $\beta\gamma$ subunit-mediated activation initiated in the TRH-R, the inhibition of the TRH response by αt was reduced to a 52%. It is important to note that this effect of αt overexpression is specifically exerted on $\beta\gamma$ subunit-mediated signals and is not due to a nonspecific impairment of G protein function by chronically sequestering $\beta\gamma$ dimers. Thus, the inositol phosphates produced in response to TRH were 1225 ± 360 cpm/mg of protein in COS-7 cells transfected with the TRH-R and 1056 ± 125 cpm/mg of protein when the same batch of cells was cotransfected with the receptor and αt. This demonstrates that the αq/11-mediated stimulation of PIP_2 hydrolysis in response to TRH remained unaffected in the presence of αt.

The existence of two independent routes to MAPK from the TRH-R also would predict the combined effect of a PKC inhibitor (GF109203X) and a $\beta\gamma$ subunit scavenger (α_t) to be additive. On the other hand, it could be expected that blockade of the Ras-dependent pathway by $p21^{N17ras}$ is not enhanced in the presence of the $\beta\gamma$ sequestrant αt, which presumably acts at a step of the same route closer to the TRH-R. Fig. 7 shows that both predictions are indeed correct. As shown in Fig. 7A, the inhibition of MAPK activation by TRH was 41% and 44% in cells cotransfected with αt or incubated with GF109203X, respectively. Such an inhibition was increased to 81% when the two treatments were performed simultaneously with the same batch of cells. In contrast, although 35% and 65% inhibition was obtained in the presence of αt and $p21^{N17ras}$, respectively, no further increase respect to the inhibition caused by $p21^{N17ras}$ was observed in the presence of both $p21^{N17ras}$ and αt (Fig. 7B). These data confirm the hypothesis of a dual pathway, which is consistent with a PLC- and PKC-dependent route plus a PKC-independent route mediated by $\beta\gamma$ dimers leading to Ras activation.

The $\beta\gamma$ dimer-mediated activation of MAPK by different receptors in COS cells has been always associated with liberation of these subunits from PTX-sensitive receptor-coupled G proteins. In addition, expression of specific sets of $\beta\gamma$ dimers has been shown repeatedly to induce MAPK activation in these cells (Crespo *et al.*, 1994a; Faure *et al.*, 1994; Hawes *et al.*, 1995; Van Biesen *et al.*, 1995). However, our results demonstrate that the TRH-induced activation is com-

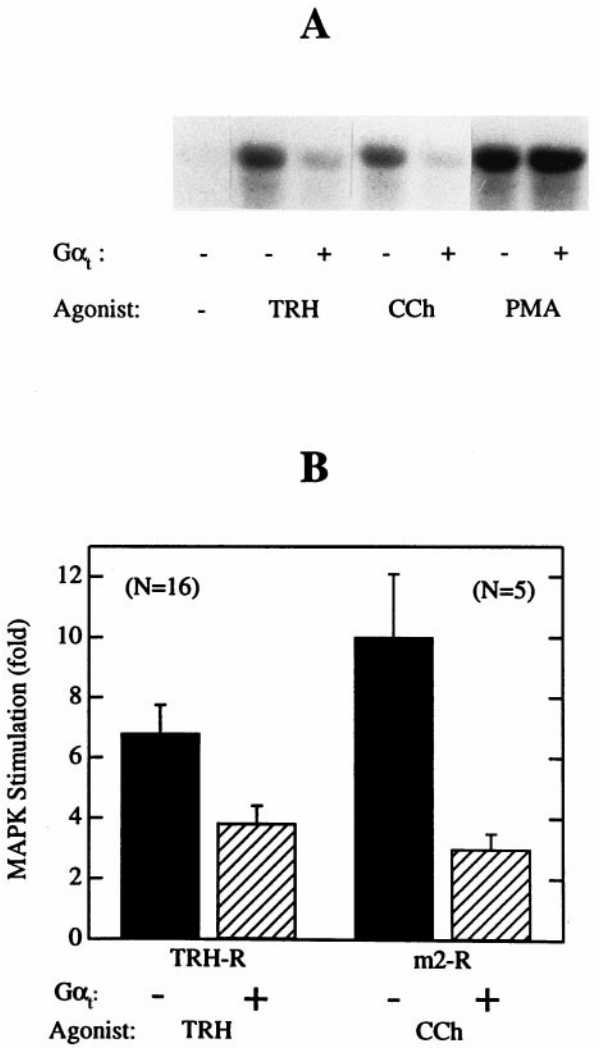

Fig. 6. Effect of expression of αt on MAPK activation induced by stimulation of TRH-R and m2-R. COS-7 cells were cotransfected with DNA plasmids encoding HA-MAPK and TRH-R or m2-R as indicated, plus either vector alone (*filled bars*) or plasmid DNA encoding αt (*striped bars*). Plasmid-containing receptor sequences was not used for PMA-treated cells. Cells were stimulated for 5 min with 1 μM TRH (*TRH*; TRH-R-transfected cells), 1 mM carbachol (*CCh*; m2-R-transfected cells), or 50 ng/ml PMA. A, Autoradiogram showing the radioactivity incorporated into MBP. B, Averaged results for the number of experiments indicated (*N*) and performed in triplicate for TRH- or carbachol-stimulated cells. MAPK activity is expressed as fold stimulation respective to that obtained in the absence of agonists. Significantly lower MAPK activation values for TRH and carbachol were obtained in the presence of αt ($p < 0.01$ versus controls, paired *t* test).

pletely insensitive to treatment of the cells with bacterial toxins. To gain some insights into the nature of the G protein coupling of TRH-Rs to MAPK activation, several wild-type and α-QLs were transfected with or without the TRH-R. Initially, we expected, as for exogenously introduced $\beta\gamma$ subunits, that the presence of mutationally activated forms of the G protein or proteins coupled to the TRH receptor would mimic the TRH-induced activation.

In the absence of receptors, wild-type αq or α13 subunits did not induce a significant activation of MAPK (not shown). Furthermore, although a modest enhancement of MAPK activity was observed sporadically in the presence of α_q-QL, no consistent activation of the kinase was induced by transfecting the GTPase-deficient mutationally activated α subunits of G_{i2}, G_s, and G_{13} (data not shown) as well as of G_q (Fig. 8). Previous experiments with activated mutants of G protein α subunits in COS cells yielded conflicting results. Thus, although a modest enhancement of MAPK activity has been reported in response to activated αq and αs (Faure *et al*., 1994), a lack of activation on expression of αq-QL (Qian *et al*., 1993; Crespo *et al*., 1994a) or αs-QL (Crespo *et al*., 1994a, 1995) also has been shown. The lack of activation in the presence of αq-QL does not seem to be coherent with the demonstrated ability of this constitutively active subunit to persistently activate COS cell PLC, causing a sustained high steady state level of inositol phosphates (Qian *et al*., 1993, Crespo *et al*., 1994). Thus, elevated diacylglycerol levels could be expected under these conditions with a concomitant PKC-dependent elevation of MAPK activity. A possible explanation for these paradoxical results can be obtained from experiments in which MAPK activation by different agents is studied in the presence of αq-QL. As shown in Fig. 8, the TRH-induced activation of MAPK is strongly inhibited by αq-QL expression. Similar results were obtained with the activated form of α13 but not with the wild-type form of $G_{\alpha q}$ or with activated αi2-QL or αs-QL (not shown). This indicates that failure to activate MAPK by αq-QL alone is not due to lack of expression of the subunit. Furthermore, it also demonstrates that one or more steps of the cascade leading to MAPK are inhibited in the presence of the activated αq. Fig. 8 also shows that the inhibitory effect of αq-QL coexpression is not exclusive for TRH-induced MAPK activation. Thus, a similar inhibitory effect is exerted on MAPK activation induced by the addition of carbachol to cells expressing another G_q-coupled receptor, such as the m1-R. Nevertheless, the receptor-independent and PKC-mediated activation caused by PMA also was inhibited by the constitutively active form of αq. This result would be compatible with an inhibitory effect due to down-regulation of PKC after its chronic stimulation in response to sustained elevations of diacylglycerol. However, the presence of αq-QL similarly inhibited the PKC-independent activations induced by stimulation of endogenous EGF (Fig. 8) or transfected m2 muscarinic receptors (not shown). Thus, although some involvement of PKC down-regulation cannot be completely excluded for pathways that use the kinase, our results indicate that the inhibitory effect of αq-QL is exerted at a step downstream of that at which PKC-dependent and -independent routes converge.

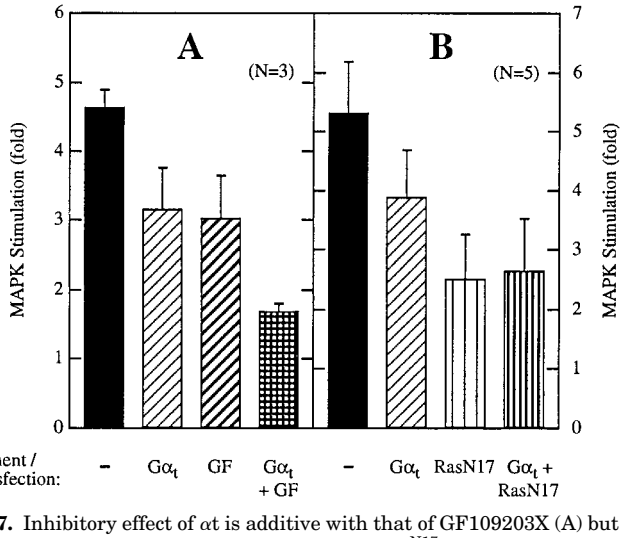

Fig. 7. Inhibitory effect of αt is additive with that of GF109203X (A) but not with inhibition induced by expression of $p21^{N17ras}$ (B). COS-7 cells were cotransfected with DNA plasmids encoding HA-MAPK and TRH-R, plus either vector alone (*filled bars* and *GF*) or plasmid DNA-encoding $G_{\alpha t}$ with or without $p21^{N17ras}$ (*RasN17*). When indicated, cells also were incubated with 3.5 μM GF109203X for 60 min before the addition of TRH (*GF*). Cells were stimulated for 5 min with 1 μM TRH. Averaged results are shown for the indicated number of experiments performed with triplicate samples. Different batches of cells were used to obtain the data. MAPK activity is expressed as fold stimulation respect to that obtained in the absence of TRH. Significantly lower MAPK activation values were obtained in the presence of GF109203X, $p21^{N17ras}$, or αt, either alone or combined ($p < 0.05$ versus control, paired t test).

Discussion

Recent work in GH_3 anterior pituitary cells demonstrated the ability of TRH-R stimulation to activate MAPK (Ohmichi

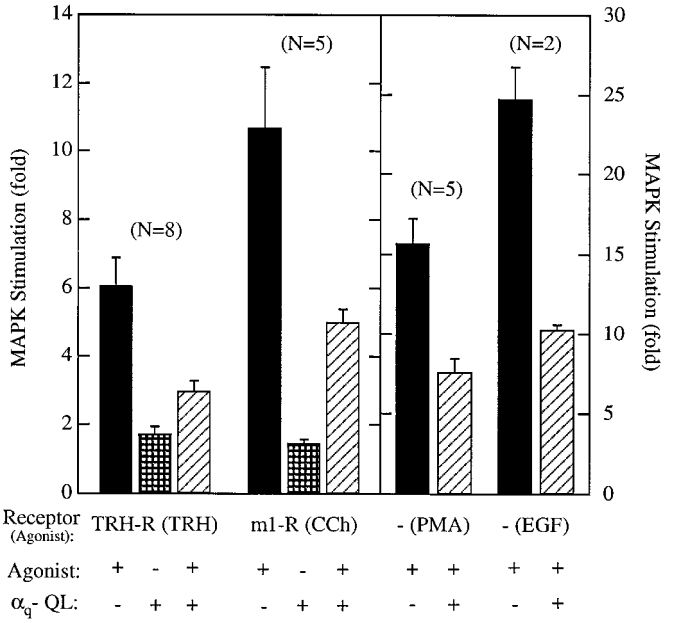

Fig. 8. Effect of expression of αq-QL on MAPK activation induced by stimulation of TRH-R, m1-R, EGF-R, or PKC. COS-7 cells were cotransfected with DNA plasmids encoding HA-MAPK and TRH-R or m1-R as indicated, plus either vector alone or plasmids encoding αq-QL. Plasmid-containing receptor sequences were not used for PMA- or EGF-treated cells. When indicated, cells were stimulated with 100 ng/ml EGF (*EGF*) for 3 min or with 1 μM TRH (*TRH*), 1 mM carbachol (*CCh*), or 50 ng/ml PMA (*PMA*) for 5 min. Averaged results are shown for the number of experiments indicated (*N*) and performed in triplicate. Note the absence of MAPK stimulation in agonist-untreated cells expressing the activated α subunit of G_q. In all cases, MAPK activation values were significantly lowered in the presence of αq-QL ($p < 0.05$ versus controls, paired t test).

et al., 1994). Coupling of TRH-R to bacterial toxin-insensitive αq/11 protein activates a signaling cascade starting in PLC-mediated hydrolysis of PIP_2 that seems to be important for TRH-induced physiological effects (Gershengorn and Osman, 1996). However, PTX-sensitive G proteins are involved in TRH regulation of ionic channels in pituitary cells (Gollasch *et al*., 1993). Furthermore, CTX-sensitive G_s-like proteins have been implicated in modifications of cell excitability by the neuropeptide (Barros *et al*., 1993, 1994) and in coupling TRH-R to PLC activation in *Xenopus laevis* oocytes [de la Peña *et al*. (1995) but see Quick *et al*. (1994) and Stehno-Bittel *et al*. (1995)]. The route or routes linking TRH-R to pituitary cells MAPK activation are not completely defined. Furthermore, the nature of the transducer or transducers implicated in this effect is not known (Ohmichi *et al*., 1994). To gain further insights into these issues, we used COS-7 cells as a model, in which both TRH-R and an epitope-tagged MAPK are exogenous elements introduced by transfection. Our results demonstrate a clear stimulation of the kinase by TRH in a time- and concentration-dependent manner. This contrasts with the lack of MAPK activation reported previously using Rat-1 cells transfected with the TRH-R (Lee *et al*., 1995). Furthermore, it allows the performance of studies of the molecular components and characteristics of the coupling cascade started in TRH-R with a cellular model widely used to define these entities.

The pioneering studies in pituitary cells (Ohmichi *et al*., 1994) indicated that a PKC-dependent pathway coexisted with a second route to MAPK activation independent of PKC activity, possibly involving tyrosine phosphorylation of *Shc* proteins, and in which activated Raf-1 and $p21^{ras}$ presumably played a role. That a PKC-dependent pathway also partially links TRH-R to MAPK activation in COS-7 cells is indicated by our results obtained after inhibition of PKC. Initially, we used a procedure for depletion of conventional PKC through prolonged treatment with a high concentration of PMA. Our data, although consistent with a participation of PKC in TRH effects, were complicated by possible secondary effects of this treatment that resulted in high basal MAPK activities to a varying extent even before the addition of an agonist. Thus, it is possible that alterations of cellular components during the prolonged exposure to PMA or maintenance of a small fraction of highly active PKC after the long PMA treatment contributed to the observed results. As an alternative, we used the nontoxic PKC-specific inhibitor GF109203X under conditions previously shown to effectively block phosphorylation of endogenous PKC substrates as well as biological responses induced by phorbol esters in COS-7 cells (Crespo *et al*., 1994b; Coso *et al*., 1995). GF109203X pretreatment inhibited MAPK activation without affecting basal activities. However, TRH-induced activation was only partially inhibited, indicating that the effect of the hormone involves both PKC-dependent and -independent mechanisms. It is important to note that the partial effect of GF109203X is not due to a limited effect of the inhibitor on PKC enzymatic activity, because the exclusively PKC-dependent action exerted by PMA was almost completely abolished by GF109203X.

Depending on the receptor, MAPK activation in COS-7 cells may be mediated by PTX-sensitive or -insensitive G proteins and be either $p21^{ras}$ or PKC dependent, respectively. Thus, MAPK activation via α_{2A}-adrenergic, m2 muscarinic acetylcholine, D_2 dopamine, A1 adenosine, and endogenous lysophosphatidic acid receptors is mediated by βγ subunits derived from PTX-sensitive G proteins in a $p21^{ras}$-dependent manner (Luttrell *et al*., 1996, and references therein). In contrast, PTX-insensitive signaling to MAPK by G_q-coupled α_{1B}-adrenergic and m1 muscarinic receptors has been reported to be insensitive to expression of a dominant negative mutant of $p21^{ras}$ and of a $G_{\beta\gamma}$-sequestrant β-adrenergic receptor kinase peptide (Faure *et al*., 1994; Koch *et al*., 1994; Hawes *et al*., 1995). This has been interpreted as indicating that MAPK activation by G_q-coupled receptors is G_q mediated, PKC dependent, $p21^{ras}$ independent, and not dissociable from the ability of these receptors to activate PLC (Hawes *et al*., 1995). However, only a partial dependence on PKC activity has been reported by other authors for m1-R (Crespo *et al*., 1994b), and some inhibition of m1-R-activating effects by βγ-sequestering αt subunits has been observed in COS-7-transfected cells (Crespo *et al*., 1994a). Our results indicate that although totally insensitive to PTX treatment, the TRH-induced increases in MAPK activity are in part transduced by a βγ-mediated and $p21^{ras}$-dependent pathway. Thus, (1) only a fraction of the TRH-induced activation was blocked by a PKC inhibitor that abolished PKC-dependent activations triggered by treatment with PMA; (2) activation was inhibited partially by overexpression of αt subunits, and (3) expression of the dominant negative $p21^{N17ras}$ form of $p21^{ras}$ inhibited TRH-induced activation of MAPK but not the PKC-dependent activation induced by PMA. As for the PKC inhibitor, the reduction in TRH-induced activation by $p21^{N17ras}$ was only partial in the same cells in which the totally Ras-dependent activation initiated by the m2-R was almost abolished by the Ras competitor. The additivity of $p21^{N17ras}$ and PKC inhibitor GF109203X effects indicates that the $p21^{ras}$-mediated route differs from the PKC-dependent pathway initiated in the TRH-R. This is supported further by our results showing an additive inhibition caused by the PKC inhibitor and a βγ subunit scavenger (αt) but not by $p21^{N17ras}$ and αt, which presumably act at different levels of the same Ras-dependent route. These results demonstrate that not only can receptors coupled to PTX-sensitive G_i or G_o use βγ dimers to signal for MAPK activation but also a similar pathway can be used, at least in part, for MAPK activity increases transduced exclusively by G proteins insensitive to PTX and CTX. Interestingly, a $p21^{ras}$-dependent route regulated by PTX-insensitive G protein or proteins has been reported for thrombin and angiotensin II receptors in fibroblasts and cardiac myocytes, respectively (Chen *et al*., 1996; Sadoshima *et al*., 1996).

Although insensitive to PTX and CTX, the identity of the G protein or proteins coupling TRH-R to MAPK activation is not known. Our efforts to advance in this direction were greatly hampered by the inability to mimic the TRH effects with mutationally activated forms or to potentiate the hormonal effects with wild-type forms of specific α subunits. Failure to detect any effect of βγ dimer sequestrants on G_q-mediated responses has been taken as an indication that only βγ subunits of PTX-sensitive G_i or G_o, and not those of $G_{q/11}$, are able to transduce signals to MAPK (Crespo *et al*., 1994b; Russell *et al*., 1994). However, this interpretation is challenged by our results showing that a fraction of the

toxin-insensitive effect in response to TRH is mediated by βγ subunits. This would be consistent with the reported tyrosine phosphorylation of *Shc* leading to $p21^{ras}$ and Raf-1 activation in pituitary GH_3 cells, presumably involving $G_{q/11}$, although the toxin sensitivity of this TRH-induced effect was not investigated (Ohmichi *et al*., 1994). Nevertheless, the possibility cannot be completely excluded that βγ subunits released from a TRH-R-coupled toxin-insensitive G protein other than $G_{q/11}$ also contribute to TRH effects on MAPK.

It is known that introduction in COS cells of the constitutively active from of $G_{\alpha q}$ (αq- QL) persistently activates PLC, causing a sustained high steady state level of inositol phosphates to levels similar to those reached by stimulation of G_q-coupled receptors such as the m1-R (Qian *et al*., 1993). However, a similar activation of MAPK is not observed even though clearly PKC-dependent effects are obtained in response to PMA or to stimulation of TRH-R and m1-R. A plausible hypothesis to explain this apparent paradox is that long term stimulation of one or several cell components causes their inactivation at some time after the appearance of the permanently active αq-QL. Because prolonged activation of PKC by PMA leads to down-regulation of the enzyme, it remained possible that a similar mechanism due to sustained elevation of diacylglycerol participates in prevention of MAPK activation by αq-QL. This would be compatible with the αq-QL-induced inhibition of the PKC-mediated activation promoted by PMA. However, our results provide a more straightforward interpretation of the data: the inhibitory effect of αq-QL expression is exerted at a step downstream of that at which PKC-dependent and -independent routes to MAPK converge (e.g., at the level of Raf-1 or MAPK). This is supported by the similar inhibition of the PKC-independent activations in response to stimulation of EGF or m2 muscarinic receptors. Further work is necessary to localize the specific step or steps of the cascade inhibited in the presence of αq-QL.

In summary, as shown previously in GH_3 anterior pituitary cells (Ohmichi *et al*., 1994), TRH-Rs activate MAPK in transfected COS-7 cells via a dual pathway only partially dependent on PKC activity. βγ subunits of a PTX- and CTX-insensitive G protein are involved in the PKC-independent fraction of the signaling route to MAPK initiated in the TRH-R. Thus, it is tempting to hypothesize that a similar βγ-dependent mechanism is the cause of the PKC-independent fraction of MAPK activation involving tyrosine phosphorylation in pituitary cells. The identity of the G protein or proteins coupling TRH-Rs to MAPK activation remains to be established. Finally, although the relevance of TRH-induced MAPK activation in pituitary cell physiology is not known, we verified recently that, as previously shown for other G protein-coupled receptors (Crespo *et al*., 1994a), prolonged challenge with TRH leads to the formation of proliferation foci in transfected NIH 3T3 cells (P. de la Peña and J. S. Gutkind, unpublished observations). This opens up the possibility that some of the TRH effects can be related to its action as a proliferative factor under certain physiological or pathological conditions.

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References

Barros F, Delgado LM, del Camino D, and de la Peña P (1992) Characteristics and modulation by thyrotropin-releasing hormone of an inwardly rectifying K^+ current in patch-perforated GH_3 anterior pituitary cells. *Pflueg Arch Eur J Physiol* **422:** 31–39.

Barros F, Mieskes G, del Camino D, and de la Peña P (1993) Protein phosphatase 2A reverses inhibition of inward rectifying K^+ currents by thyrotropin-releasing hormone in GH_3 pituitary cells. *FEBS Lett* **336:**433–443 .

Barros F, Villalobos C, García-Sancho J, del Camino D, and de la Peña P (1994) The role of the inwardly rectifying K^+ current in resting potential and thyrotropin-releasing hormone-induced changes in cell excitability of GH_3 rat anterior pituitary cells. *Pflueg Arch Eur J Physiol* **426:**221–230.

Ben-Jonathan N, Arbogast LA, and Hyde JF (1989) Neuroendocrine regulation of prolactin release. *Prog Neurobiol* **33:**399–447.

Chen Y-H, Grall D, Salcini AE, Pelicci PG, Pouyssegur J, and Obberghen-Schilling EV (1996) Shc adaptor proteins are key transducers of mitogenic signaling mediated by the G protein-coupled thrombin receptor. *EMBO J* **15:**1037–1044.

Corette BJ, Bauer CK, and Schwarz JR (1995) Electrophysiology of anterior pituitary cells, in *The Electrophysiology of Neuroendocrine Cells* (Scherübl H and Hescheler J, eds) pp 101–143, CRC Press, Boca Raton, FL.

Coso OA, Chiariello M, Kalinec G, Kyriakis JM, Woodgett J, and Gutkind JS (1995) Transforming G protein-coupled receptors potently activate JNK (SAPK): evidence for a divergence from the tyrosine kinase signaling pathway. *J Biol Chem* **270:** 5620–5624.

Crespo P, Cachero TG, Xu N, and Gutkind JS (1995) Dual effect of β-adrenergic receptors on mitogen-activated protein kinase: evidence for a βγ-dependent activation and a $G_{\alpha s}$-cAMP-mediated inhibition. *J Biol Chem* **270:**25259–25265.

Crespo P, Xu N, Daniotti JL, Troppmair J, Rapp UR, and Gutkind JS (1994b) Signaling through transforming G protein-coupled receptors in NIH 3T3 cells involves c-Raf activation: evidence for a protein kinase C-independent pathway. *J Biol Chem* **269:**21103–21109.

Crespo P, Xu N, Simonds WF, and Gutkind JS (1994a) Ras-dependent activation of MAP kinase pathway mediated by G-protein βγ subunits. *Nature (Lond)* **369:**418–420.

de la Peña P, del Camino D, Pardo LA, Domínguez P, and Barros F (1995) G_s couples thyrotropin-releasing hormone receptors expressed in *Xenopus* oocytes to phospholipase C. *J Biol Chem* **270:**554–559.

de la Peña P, Delgado LM, del Camino D, and Barros F (1992) Two isoforms of the thyrotropin-releasing hormone receptor generated by alternative splicing have indistinguishable functional properties. *J Biol Chem* **267:**25703–25708.

del Camino D, Barros F, Pardo LA, and de la Peña P (1997) Altered ligand dissociation rates in thyrotropin-releasing hormone receptors mutated in glutamine 105 of transmembrane helix III. *Biochemistry* **36:**3308–3318.

Delgado LM, de la Peña P, del Camino D, and Barros F (1992) Okadaic acid and calyculin A enhance the effect of thyrotropin-releasing hormone on GH_3 rat anterior pituitary cells excitability. *FEBS Lett* **311:**41–45.

Drust DS and Martin TFJ (1982) Thyrotropin-releasing hormone rapidly and transiently stimulates cytosolic calcium-dependent protein phosphorylation in GH_3 pituitary cells. *J Biol Chem* **257:**7566–7573.

Drust DS and Martin TFJ (1984) Thyrotropin-releasing hormone rapidly activates protein phosphorylation in GH_3 pituitary cells by a lipid-linked, protein kinase C-mediated pathway. *J Biol Chem* **259:**14520–14530.

Drust DS, Sutton CA, and Martin TFJ (1982) Thyrotropin-releasing hormone and cyclic AMP activate distinctive pathways of protein phosphorylation in GH_3 pituitary cells. *J Biol Chem* **257:**3306–3312.

Faure M, Voyno-Yasenetskaya TA, and Bourne HR (1994) cAMP and βγ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J Biol Chem* **269:**7851–7854.

Gershengorn MC (1986) Mechanism of thyrotropin-releasing hormone stimulation of pituitary hormone secretion. *Annu Rev Physiol* **48:**515–526.

Gershengorn MC and Osman R (1996) Molecular and cellular physiology of thyrotropin-releasing hormone receptors. *Physiol Rev* **76:**175–191.

Gollasch M, Kleuss C, Hescheler J, Wittig B, and Schultz G (1993) G_{i2} and protein kinase C are required for thyrotropin-releasing hormone-induced stimulation of voltage-dependent Ca^{2+} channels in rat pituitary GH_3 cells. *Proc Natl Acad Sci USA* **90:**6265–6269.

Hawes BE, Van Biesen T, Koch WJ, Luttrell LM, and Lefkowitz RJ (1995) Distinct pathways of G_i- and G_q-mediated mitogen-activated protein kinase activation. *J Biol Chem* **270:**17148–17153.

Hinkle PM (1989) Pituitary TRH receptors. *Ann N Y Acad Sci* **553:**176–187.

Jefferson AB, Travis SM, and Schulman H (1991) Activation of multifunctional Ca^{2+}/calmodulin-dependent protein kinase in GH_3 cells. *J Biol Chem* **266:**1484–1490.

Kanda Y, Koike K, Ohmichi M, Sawada T, Hirota K, and Miyake A (1994) A possible involvement of tyrosine kinase in TRH-induced prolactin secretion in GH_3 cells. *Biochem Biophys Res Commun* **199:**1447–1452.

Koch WJ, Hawes BE, Allen LF, and Lefkowitz RJ (1994) Direct evidence that G_i-coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G_{\beta\gamma}$ activation of $p21^{ras}$. *Proc Natl Acad Sci USA* **91:**12706–12710.

Lee TW, Anderson LA, Eidne KA, and Milligan G (1995) Comparison of the signalling properties of the long and short isoforms of the rat thyrotropin-releasing hormone receptor following expression in Rat 1 fibroblasts. *Biochem J* **310:**291–298.

Luttrell LM, Hawes BE, Van Biesen T, Luttrell DK, Lansing TJ, and Lefkowitz RJ (1996) Role of c-Src tyrosine kinase in G protein-coupled receptor- and $G_{\beta\gamma}$ subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* **271:** 19443–19450.

Malarkey K, Belham CM, Paul A, Graham A, McLees A, Scott PH, and Plevin R (1995) The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. *Biochem J* **309:**361–375.

Marshall CJ (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80:**179–185.

Martin TFJ, Hsieh K-P, and Porter BW (1990) The sustained second phase of hormone-stimulated diacylglycerol accumulation does not activate protein kinase C in GH_3 cells. *J Biol Chem* **265:**7623–7631.

Metcalf G and Jackson IMD (1989) Thyrotropin-releasing hormone: biomedical significance. *Ann N Y Acad Sci* **553:**473–475.

Ohmichi M, Sawada T, Kanda Y, Koike K, Hirota K, Miyake A, and Saltiel AR (1994) Thyrotropin-releasing hormone stimulates MAP kinase activity in GH_3 cells by divergent pathways: evidence of a role for early tyrosine phosphorylation. *J Biol Chem* **269:**3783–3788.

Qian N-X, Winitz S, and Johnson GL (1993) Epitope-tagged Gq α subunits: expression of GTPase-deficient α subunits persistently stimulates phosphatidylinositol-specific phospholipase C but not mitogen-activated protein kinase activity regulated by the M1 muscarinic acetylcholine receptor. *Proc Natl Acad Sci USA* **90:**4077–4081.

Quick MW, Simon MI, Davidson N, Lester HA, and Aragay AM (1994) Differential coupling of G protein α subunits to seven-helix receptors expressed in *Xenopus* oocytes. *J Biol Chem* **269:**30164–30172.

Russell M, Winitz S, and Johnson GL (1994) Acetylcholine muscarinic m1 receptor regulation of cyclic AMP synthesis controls growth factor stimulation of Raf activity. *Mol Cell Biol* **14:**2343–2351.

Sadoshima J-I and Izumo S (1996) The heterotrimeric G_q protein-coupled angiotensin II receptor activates $p21^{ras}$ via the tyrosine kinase-Shc-Grb2-Sos pathway in cardiac myocytes. *EMBO J* **15:**775–787.

Sobel A and Tashjian AH Jr (1983) Distinct patterns of cytoplasmic protein phosphorylation related to regulation of synthesis and release of prolactin by GH cells. J *Biol Chem* **258:**10312–10324.

Stehno-Bittel L, Krapivinsky G, Krapivinsky L, Perez-Terzic C, and Clapham DE (1995) The G protein $\beta\gamma$ subunit transduces the muscarinic receptor signal for Ca^{2+} release in *Xenopus* oocytes. *J Biol Chem* **270:**30068–30074.

Van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM, and Lefkowitz RJ (1995) Receptor-tyrosine-kinase- and G$\beta\gamma$-mediated MAP kinase activation by a common signalling pathway. *Nature (Lond)* **376:**781–784.

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