MECHANISMS OF THYROTROPIN-RELEASING HORMONE-INDUCED ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASES AND PROLACTIN GENE TRANSCRIPTION

by

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ABSTRACT

Thyrotropin-releasing hormone (TRH) stimulates prolactin secretion and gene transcription. The specific signaling pathways that mediate TRH signaling to prolactin gene transcription have not been clearly defined. In GH_3, rat pituitary tumor cells, TRH has been shown to activate the mitogen-activated protein kinases (MAPKs). Several studies have reported that activation of the MAPK pathway is sufficient to activate prolactin gene transcription. These results suggest that MAPK could be a good candidate for mediating TRH signaling to the prolactin gene. The goal of this thesis is to investigate the role for MAPK in TRH-induced prolactin transcription and to further elucidate the signaling pathways that mediate TRH effect on MAPKs.

TRH was found to induce a sustained activation of MAPK in GH3 cells, supporting a possible role in transcription regulation. Treatment with a selective MAPK kinase (MEK) inhibitor, PD98059, completely blocked TRH-induced Erk2 activation. Expression of a kinase-defective MEK mutant or treatment with PD98059 substantially reduced TRH induction of both prolactin gene transcription and GAL4-Elk dependent reporter gene activity, suggesting that MAPK activation is necessary for TRH-induced prolactin transcription. Previous studies have found that the prolactin promoter contains several binding sites for members of the Ets family of transcription factors (Ets sites), which are important for mediating the MAPK response of the prolactin promoter. Mutations of the Ets

sites within the prolactin promoter impaired the response to both TRH and MAPK, demonstrating that the response elements in the prolactin promoter for TRH and MAPK are colocalized. This finding further supports a role for MAPK in mediating TRH-induced prolactin transcription.

Previous studies have provided evidence that TRH effects on MAP kinases may be mediated by tyrosine kinases and the adaptor proteins, Grb2 and Shc. We found that TRH stimulates tyrosine phosphorylation of the EGF receptor. Treatment with a specific EGF receptor inhibitor, tyrphostin AG1478, or expression of a kinase-defective EGF receptor mutant provided evidence that TRH-stimulated tyrosine phosphorylation of the receptor is due to activation of the receptor's intrinsic tyrosine kinase activity. Furthermore, the intrinsic kinase activity is required for TRH-induced activation of MAP kinases, Grb2 association, tyrosine phosphorylation of HER2 and Shc and full activation of prolactin gene expression. TRH-induced tyrosine phosphorylation of the EGF receptor appears to be sensitive to change in extracellular Ca^{2+} concentration and requires PKC activation, as determined by pharmacological agents. These results are consistent with a model in which the activated EGF receptor and HER2 heterodimers serve as a scaffold for downstream adaptor proteins that mediate TRH signaling to MAP kinase activation and gene transcription.

CHAPTER I

INTRODUCTION

Physiological Functions of Thyrotropin-Releasing Hormone

Thyrotropin-releasing hormone (TRH, pyroglutamine-histidine-proline-amide) is a peptide hormone synthesized in the hypothalamus, stored in the median eminence and reaches target cells in the anterior pituitary via the hypothalamic-hypophyseal portal system. Like many other hormones, TRH is derived from post-translational cleavage of a large precursor molecule [1]. The cDNA sequence of the rat TRH precursor encodes a protein with a molecular size of 29.2 kDa containing an N-terminal signal peptide, five repeating sequences of Lys-Arg-Gln-His-Pro-Gly-Lys/Arg-Arg, separated by four intervening peptide sequences of varying lengths and followed by a C-terminal trailer sequence [61] (Fig.1). The signal peptide is cleaved upon entering endoplasmic reticulum [92]. During the formation of secretory vesicles at the trans-Golgi network, the rat pro-TRH is probably cleaved by an endopeptidase, which cleaves the prohormone on the carboxyl side of Arg/Lys-Arg sites, and a carboxypeptidase-B-like enzyme, which cleaves C-terminal Arg and Lys, to a

family of peptides including the TRH precursor, flanking and intervening sequences [1, 92]. The TRH precursor is then amidated and cyclized to TRH.

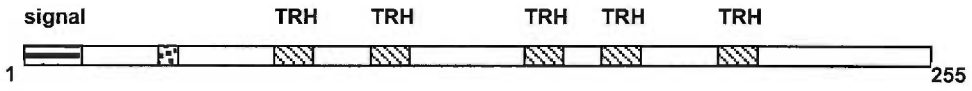


Figure 1. The structure of prepro-TRH

TRH is rapidly degraded by a pyroglutamyl aminopeptidase in tissues and serum to TRH free acid, the stable cyclized metabolite (cyclo(His-Pro)) and its constituent amino acids [92]. The activity of the anterior pituitary TRH-degrading enzyme is rapidly and potently stimulated by thyroid hormones [214]. The potency of the thyroid hormone effect on the degradation of TRH indicates that this may be an important regulatory mechanism. Several studies indicate TRH biosynthesis is regulated at the transcriptional level [215, 216]. Analysis of the structure of the prepro-TRH gene reveals a glucocorticoid receptor binding site and thyroid hormone receptor binding site in the 5'-flanking region of the gene, suggesting a possible regulatory role for glucocorticoid and thyroid hormone [217].

TRH is known for its role in maintaining normal thyroid function [2, Fig. 2]. TRH stimulates the release of thyroid-stimulating hormone (TSH) from the anterior pituitary; subsequently, TSH stimulates the secretion of thyroid hormone from the thyroid gland. Thyroid hormone regulates energy production and protein

synthesis in most tissues of the body. Elevated thyroid hormone has a negative feedback effect to suppress TSH release and to inhibit the expression of both

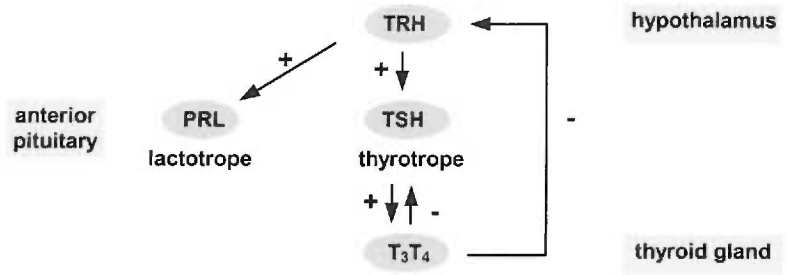


Figure 2. Feedback regulation of TRH, TSH and prolactin expression

TSH in the anterior pituitary and TRH in the hypothalamus. In addition to its role in controlling thyrotroph function, TRH acts on the secretion of other anterior pituitary hormones in a variety of physiological and pathophysiological settings. For example, in primary hypothyroidism, elevated TRH stimulates prolactin release by the pituitary [2]. Prolonged stimulation by TRH can increase the biosynthesis of prolactin and TSH in the pituitary by affecting their transcription levels [2, 3], resulting in increased prolactin and TSH secretion.

Signal Transduction of the TRH Receptor

The TRH signal transmits into its target cells in the anterior pituitary through its membrane receptor, a member of the G protein-coupled receptor (GPCR) family [4]. The TRH receptor couples to G_q to stimulate the activity of phospholipase Cβ (PLCβ) [5], leading to increased intracellular levels of diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3) [6]. DAG activates

protein kinase C (PKC), which results in redistribution of PKC from the cytosol to the membrane compartments [7, 8]. IP3 causes the release of Ca^{2+} from intracellular stores, resulting in a first phase of Ca^{2+} elevation [9]. A second phase of Ca^{2+} elevation after TRH stimulation is due to membrane depolarization, which leads to Ca^{2+} influx through L-type voltage-sensitive calcium channels (VSCCs) [10]. The precise mechanisms and transducers that activate these calcium channels during the TRH response are still unresolved. Antisense studies have provided evidence that TRH stimulation of VSCC involves $G\alpha_{i2}$, and this effect of $G\alpha_{i2}$ is dependent on simultaneous activation of protein kinase C [11]. Increases in intracellular Ca^{2+} lead to activation of a Ca^{2+}/calmodulin-dependent protein kinase in GH_3 pituitary tumor cells [12]. It has also been shown that TRH can activate the mitogen-activated protein kinase (MAPK) signaling pathway, also known as the extracellular signal-regulated kinase (ERK), in GH_3 cells [13]. Thus, multiple kinase pathways including PKC, the Ca^{2+}/calmodulin-dependent kinase and MAPK are activated by TRH.

Like other G protein-coupled receptors, the TRH receptor signaling attenuates after prolonged stimulation by TRH. The processes involve receptor desensitization, TRH-TRHR complex internalization and TRH receptor down-regulation by modulating receptor expression [14].

Regulation of Prolactin Gene Transcription

Structure of the Prolactin Gene

Prolactin is a hormone that stimulates breast development and also induces milk production after parturition. The rat prolactin gene is approximately 10kb in size and is composed of five exons [15]. The mature prolactin mRNA is about 1 kb and encodes a 227-amino acid protein that includes a 28-amino acid signal peptide, which is cleaved upon entering the endoplasmic reticulum.

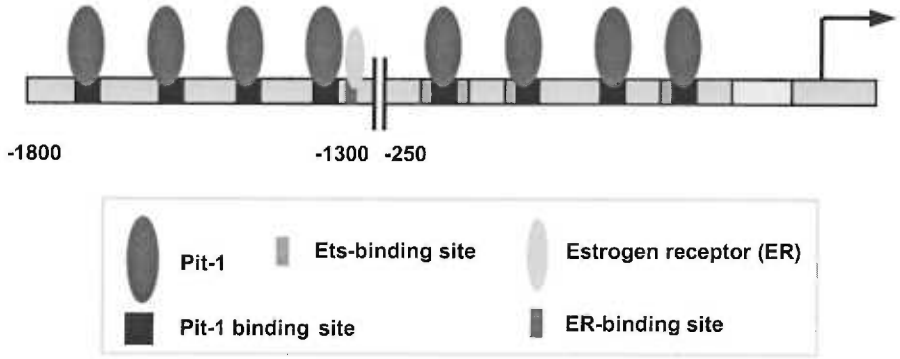


Figure 3. Structure of the prolactin promoter

In the 5'-flanking region of the rat prolactin gene, a sequence of about 2.5-kb contains two distinct regulatory regions: a proximal promoter located between -250 and +33 [16, 17] and a distal enhancer located between -1800 and -1300 [18]. Both regions can confer tissue-specific expression in pituitary cell lines [16-20]; synergistic interactions between the proximal promoter and the distal enhancer are necessary for the full physiological levels of expression as shown in transgenic mice experiments [21]. Identification and cloning of the pituitary-

specific transcriptional activator, Pit-1 [18, 22, 23], substantially expanded our knowledge of prolactin gene expression. There are four cis-elements in the proximal promoter (sites 1P to 4P) and four in the distal enhancer (sites 1D to 4D) that bind Pit-1. Pit-1 has an N-terminal transactivation domain and a POU homeodomain that binds to a consensus DNA sequence (AA/TA/TTATNCAT) [23]. Pit-1 expression is necessary for the development and proliferation of lactotroph, somatotroph and thyrotroph cells in the anterior pituitary gland [213], as immunohistological analysis reveals high expression of Pit-1 in these three cell types [213]. Furthermore, defects in the Pit-1 gene in the Snell and Jackson dwarf mice are characterized by decreased growth hormone, prolactin and TSHβ gene expression and a failure of lactotroph, somatotroph and thyrotroph proliferation [213]. Since Pit-1 expression is not restricted to the lactotroph cells, additional components may be required to confer tissue-specific expression of the prolactin gene in these cells.

Hormonal Regulation of Prolactin Gene Transcription

Prolactin gene transcription is stimulated by estrogen [24], cAMP [25], epidermal growth factor (EGF) [26], thyrotropin-releasing hormone [27] and several other peptide hormones and inhibited by dopamine [28, 25]. The positive transcriptional regulators of the prolactin gene act through different mechanisms. The steroid hormone estrogen induces prolactin gene transcription by binding to the estrogen receptor (ER), which subsequently binds to an imperfect palindromic estrogen response element (ERE) [24] located in the distal

enhancer, adjacent to the 1D Pit-1 site [29]. Mutagenesis studies demonstrate that multiple Pit-1 binding sites in both the distal and proximal regions of the prolactin gene are required for the estrogen response [31]. Nuclear ligation studies showed that binding of the estrogen receptor to the ERE site causes looping of the DNA, bringing the enhancer and promoter regions to juxtaposition via protein-protein interaction [30].

Pituitary adenylate cyclase-activating peptide (PACAP) [43, 44] and dopamine [28, 25] alter prolactin gene transcription through a cAMP-responsive pathway. Mutagenesis and linker-scanning studies provide evidence that Pit-1 binding sites are necessary for transcriptional responses to cAMP [47]. Presumably these responses to cAMP are mediated by the cAMP-dependent protein kinase (PKA), which has been shown to be sufficient to activate prolactin gene transcription by expression of the PKA catalytic domain [32]. The Rosenfeld group reported that Pit-1 is phosphorylated *in vivo* in response to cAMP treatment in pituitary cells and *in vitro* by PKA treatment [33]. Although Pit-1 phosphorylation enhances its ability to bind the prolactin promoter [34], heterologous expression of a Pit-1 mutant, in which the PKA phosphorylation sites are mutated, confers the similar response as the wild type Pit-1 [34, 24], suggesting that Pit-1 expression is insufficient to mediate the cAMP response. These findings also raise the possibility that Pit-1 may recruit another factor to Pit-1 binding sites in a PKA-dependent manner.

Growth factors such as insulin [35] and EGF [26] also stimulate prolactin gene transcription in GH_3 cells. These growth factors induce activation of the

Ras/Raf/MAPK pathway that in turn activates transcription factors, such as Elk-1 and c-Ets. It has been reported that transient expression of constitutively active Ras or Raf mutant is sufficient to induce prolactin transcription in GH_3 cells [36]. Furthermore, several studies suggest that there are several composite Ets/Pit-1 binding sites present in the proximal promoter of the prolactin gene; mutations of these Ets sites impair Ras-induced prolactin transcription [37, 38]. Moreover, coexpression of Ets-1 and Pit-1 synergistically enhances the prolactin promoter response to Ras activation [37]. These findings suggest that the pituitary-specific transcription factor Pit-1 can link the ubiquitous Ras/MAPK signaling pathway to pituitary-specific gene transcription by cooperating with a widely expressed transcription factor Ets-1, which is a substrate for MAPKs.

TRH Regulation of Prolactin Gene Transcription

TRH rapidly stimulates prolactin gene transcription in GH_3 cells [26, 39-41]. Measurable increases of the prolactin mRNA precursors occur within 1-2 min following addition of TRH and reach maximal stimulation at 20-40 min. After 1 hr of TRH exposure, prolactin gene transcription gradually attenuates, and by 36 hr only a 2-fold induction remains. The rapid onset of the transcriptional effects indicates that new protein synthesis is probably not required for mediating hormonal regulation of prolactin gene transcription, which is supported by cycloheximide studies. Therefore, TRH may activate prolactin transcription through second messenger pathways. In fact, the transcriptional effect of TRH on the prolactin gene can be reproduced by treatments combining calcium

ionophore and phorbol ester, indicating that calcium and PKC could be involved in TRH action, at least partially (see below) [41].

Binding of TRH to its receptor results in Ca^{2+} release from intracellular stores and Ca^{2+} influx from L-type calcium channels. Mobilization of calcium may be important for transcriptional regulation of the prolactin gene by TRH, since agents that disrupt cellular calcium metabolism inhibit TRH effects [41, 42]. Changes in calcium concentration might affect transcription through activating Ca^{2+} -dependent protein kinases. Previous studies showed that TRH can induce rapid phosphorylation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and can increase its activity in GH3 cells [12]. Expression of a constitutively active CaMKII mutant induces prolactin transcription [45]. Moreover, pretreatment of GH3 cells with a specific CaMKII inhibitor, KN62, partially inhibits TRH-induced prolactin gene transcription [46]. Together, these results provide evidence that CaMKII probably plays a role in mediating TRH responses. The response to the active CaMKII mutant is diminished when the distal enhancer of the prolactin gene is removed [45]. Further deletion studies showed that neither the proximal promoter nor the distal enhancer is sufficient to support a response to CaMKII; both regions are required to permit CaMKII-induced activation of prolactin transcription [46]. These findings suggest that transcriptional regulation of the prolactin gene by CaMKII is similar to that by estrogen, which involves communication between the distal enhancer and the proximal promoter and interaction of several transcription factors [30]. Mutagenesis studies also showed that mutations of the Pit-1 binding sites reduce responses to CaMKII, indicating

that Pit-1 may play a role in mediating the CaMKII responses [46]. It has been reported, however, that CaMKII does not phosphorylate Pit-1 [33]. Therefore, it is unclear which transcription factors are nuclear targets of CaMKII and how CaMKII mediates TRH-induced prolactin transcription.

TRH also increases intracellular levels of diacyglycerol leading to activation of PKC [7, 8]. The role for PKC in prolactin transcription was studied by exposing cells to the phorbol ester, PMA. Like TRH and EGF, PMA rapidly stimulates transcription within minutes of its addition to the GH cell cultures [41]. The PMA-responsive region of the prolactin promoter is restricted to the proximal promoter [20], which has been shown to be sufficient to mediate multihormonal responses [47]. Linker-scanning mutagenesis showed that mutations in five regions of the proximal promoter decrease both basal and TRH- or PMA-stimulated prolactin transcription; three of these regions are Pit-1 binding sites, suggesting that Pit-1 may be involved in TRH-induced prolactin transcription. It has been reported that TRH stimulates a transient phosphorylation of Pit-1, reaching a maximum in 5 min and returning to basal levels within 30 min [48]. These studies also showed that PKC mediates TRH-induced Pit-1 phosphorylation. However, chronic PMA treatment, which down-regulates PKC, neither blocks TRH-induced prolactin mRNA accumulation, as determined by nuclear run-on assay [49] nor inhibits activation of the prolactin promoter, as determined by transient transfection assay [48, 42]. Because PMA itself is a potent activator of prolactin transcription and the prolactin mRNA is relatively stable [50], these results are subject to alternative interpretations; indeed, chronic PMA treatment substantially increases

the basal level of prolactin transcription [48]. Therefore, whether PKC is involved in TRH-induced prolactin transcription still needs further investigation.

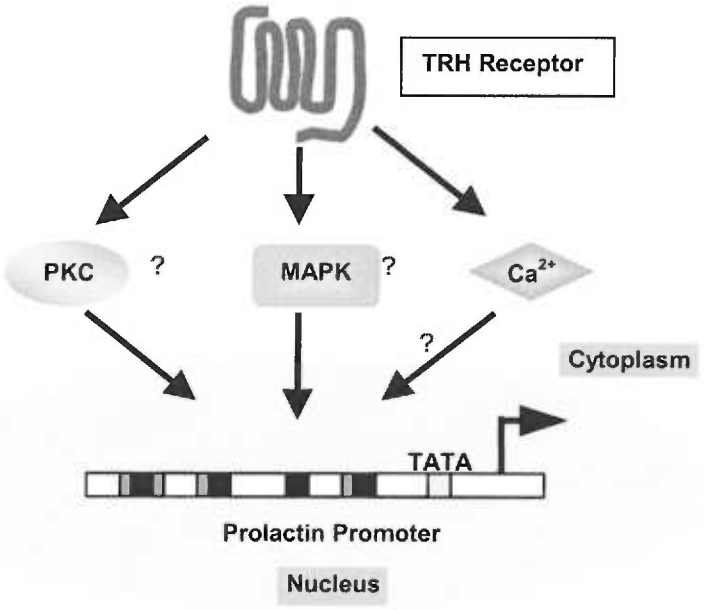


Figure 4. A model for possible pathways that could mediate TRH signal to the prolactin promoter

Recent studies have shown that TRH can induce MAPK activation in GH3 cells, and MAPK activation by TRH is, at least in part, PKC-dependent [13]. Activation of the MAPK pathway is sufficient to activate prolactin transcription [37, 38]. These studies suggest a possible role for MAPK to mediate TRH-induced prolactin gene transcription.

The MAPK Pathway

The MAPK pathway is a major signaling pathway that is initiated by many types of cell surface receptors. Activation of the MAPK pathway leads to activation of several transcription factors and other serine/threonine kinases that

contribute to gene expression, cellular proliferation, differentiation and cell cycle regulation [74]. The core components of the MAPK pathway are MAPK kinase kinase (Raf-1), MAPK kinase (MEK) and MAPK (or Erk).

Components of the MAPK Pathway

Raf-1 is a serine/threonine protein kinase, which contains a C-terminal catalytic domain and an N-terminal regulatory domain [51]. The N-terminal regulatory domain includes the following regions: a Ser/Thr-rich region, which contains sites for phosphorylation; a cysteine-rich Zn^{2+} finger-like region, which is necessary for activation, may be involved in phosphatidylserine binding [52]; and one or more regions that interact with the GTP-bound form of Ras [53, 54]. The interaction between Ras and Raf-1 is not sufficient for Raf-1 activation but is responsible for its recruitment to the plasma membrane [55, 56]. At the membrane, further events lead to full activation of Raf-1. Membrane-bound tyrosine kinases including Src phosphorylate Raf-1 on Tyr-340 and Tyr-341 [57, 52, 58]. Furthermore, treatment of Raf-1 with purified and membrane-associated tyrosine phosphatases inactivates Raf-1 [59], suggesting that tyrosine phosphorylation is important for Raf-1 activation. However, this activation mechanism may not apply to B-raf, a Raf-1 homolog that does not have tyrosine residues at similar position [60]. In addition to tyrosine phosphorylation, Raf-1 can be phosphorylated on multiple serine or threonine residues, which may be activating or inhibitory. For example, PKCα activates Raf-1 by phosphorylating Raf-1 on Ser-259 and Ser-499 *in vitro* [62]. Therefore, tyrosine and serine

phosphorylation of Raf-1 control its kinase activity once it reaches the plasma membrane. Raf-1 has several substrates. MEK1 and MEK2 are established substrates for Raf-1 [63, 64]. Another putative substrate for Raf-1 is IκB [65]; Raf-1 may transmit signals by releasing the cytoplasmic, active NFκB, which can then be translocated to the nucleus. Therefore, it's possible that Raf-1 participates in more than one signaling pathway.

MEK1 and MEK2 are highly similar in their primary amino acid sequences; both contain proline-rich sequences that are necessary for the interaction of MEK and Raf-1 [66]. Raf-1 phosphorylates MEK on both Ser-218 and Ser-222; phosphorylation of both serine residue is required for full MEK activity [67-71]. Activation of MEKs *in vivo* may also occur through inhibiting serine/threonine phosphatases, since in CV-1 cells, expression of SV40 small T antigen, an inhibitor of phosphatase PP2A, significantly stimulates MEK and MAPK activation [72]. MEK1 activity can also be negatively regulated by phosphorylation on Thr-286 and Thr-292 by Cdc2, a cyclin-dependent kinase [73]. The functional importance of this regulation is undefined.

MAPKs are the only substrates for MEKs that have been identified *In vitro* [74]. MEKs are dual-specific protein kinases that activate MAPKs by phosphorylating both tyrosine and threonine residues within a Thr-Glu-Tyr motif [75], which lies in the activation loop of the catalytic domain. Phosphorylation of both tyrosine and threonine residues of MAPKs is required for full activation [76]. Upon activation, MAPKs phosphorylate substrates on serine or threonine

residues within a proline-directed motif. Ser/Thr-Pro is the consensus sequence for substrate recognition by MAPKs [77-79].

Cellular Substrates of MAPKs

Targets of the MAPK signaling are located within many cellular compartments. Following activation, a fraction of MAPKs translocates into the nucleus [80-83] and phosphorylates several different transcription factors. Phosphorylation and activation of Ets proteins by MAPKs have been best illustrated. Several members of the Ets subfamily (Ets-1, Ets-2 and pointed P2) possess a highly conserved N-terminal regulatory domain and a C-terminal DNA-binding domain (Ets domain). MAPK phosphorylates a single threonine residue within the regulatory domain of Ets-1, Ets-2 and Pointed P2 [84]. In contrast, members of TCF subfamily (Elk-1and Net/Erp/Sap2) have an Ets domain at the N-terminus, followed by a protein-protein interaction motif and a transactivation domain at the C-terminus. The transactivation domain of the TCFs contains multiple serine and threonine residues that can be phosphorylated by MAPK [85-88]. Transcription mediated by these proteins is stimulated after they are phosphorylated by MAPKs. Ets proteins regulate specific gene transcription by interactions with other transcription factors at composite sites on DNA [88, 37, 38]. Other transcription factors that are phosphorylated by MAPK include c-Jun [89, 79], Fos [90], Myc [79] and STAT [91].

In addition to transcription factors, MAPKs are also able to phosphorylate downstream kinases such as S6 kinase $p90^{RSK}$ and MAPKAP kinase 2, which

phosphorylate ribosomal protein S6, the transcription factor c-Fos and a heat shock protein, respectively [74]. Several microtubule-associated proteins are also substrates for MAPKs, including MAP-1, MAP-2, MAP-4 and Tau. Phosphorylation of these proteins appears to regulate cytoskeletal rearrangements and cellular morphology [93]. Another set of MAPKs substrates are upstream proteins in the MAPK pathway, such as the EGF receptor [94, 95], the Ras exchange factor Sos [96], Raf1 [97] and MEK1 [98]. These modifications are likely involved in feedback regulation.

Activation of the MAPK Pathway by Receptor Tyrosine Kinases

Receptor tyrosine kinases, like the EGF, PDGF and FGF receptor, are single transmembrane proteins that possess intrinsic ligand-stimulated tyrosine kinase activity [99, 100]. Upon ligand binding, these receptor tyrosine kinases (RTKs) dimerize and transphosphorylate tyrosines within their cytoplasmic domains (Fig. 4). The resulting phosphotyrosine residues serve as docking sites for signaling molecules. For example, the adapter protein Shc binds to a specific phosphotyrosine on the EGF receptor via its phosphotyrosine-binding domain [101]. The association of Shc with the EGF receptor permits the tyrosine phosphorylation of Shc by the receptor itself or other tyrosine kinases such as Src [102]. This phosphorylation allows the binding of another adapter protein Grb2, which consists of one SH2 domain and two SH3 domains [103, 104]. The

SH2 domain mediates the association between Shc and Grb2. Grb2 can also bind directly to the EGF receptor through its SH2 domain [105]. The binding of

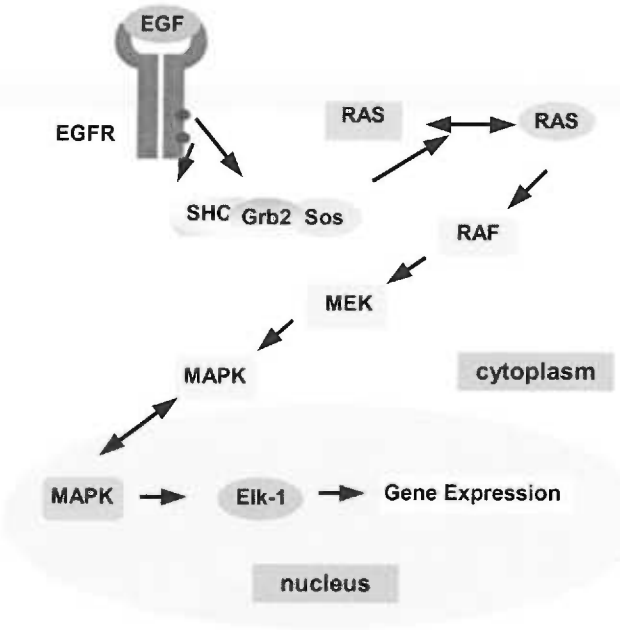


Figure 4. A diagram of receptor tyrosine kinase-induced signaling pathway

Grb2 with the EGF receptor also recruits Sos because the guanine nucleotide exchange factor Sos binds constitutively to the Grb2 SH3 domain [104]. The localization of Sos to the EGF receptor leads to exchange of Ras·GDP for GTP at the plasma membrane [106]. Ras·GTP interacts with Raf-1 and leads to activation of the MAPK pathway [53, 54, 107, 108]. Activation of the MAPK pathway by other receptor tyrosine kinases involves similar recruitment of adapter proteins leading to Ras activation.

Activation of the MAPK Pathway by G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) modulate the MAPK pathway at multiple levels, depending on the cell type and receptor involved. Thrombin and lysophosphatic acid (LPA) mediate activation of Ras [109], Raf-1 and MAPKs [110] in a pertussis toxin-dependent manner, indicating that $G_{i/o}$ family heterotrimeric G proteins are involved. In COS-7 cells expressing M_2 muscarinic receptor, which is coupled to G_i, carbachol induces Ras-dependent activation of MAPKs [111]. Ras and MAPK activation by the G_i -coupled receptors appear to be mediated by $\beta\gamma$ subunits, because transient expression of the $\beta\gamma$ subunits but not α_i subunit, results in the activation of MAPK in a Ras-dependent manner [112, 111]. In addition, expression of peptides or proteins that sequester the $\beta\gamma$ subunits is able to reduce the ability of G_i -coupled receptors to activate MAPK [113, 111]. Several studies also indicate that MAPK activation by the $\beta\gamma$ subunits may be mediated by a PKC-independent mechanism that involves a tyrosine kinase [114].

The signaling pathways coupling G_q-linked receptor to MAPK activation are more complicated. It has been reported that activation of MAPK is PKC-dependent but independent of $\beta\gamma$ and Ras and is insensitive to the tyrosine kinase inhibitor genistein [115, 116, 112]. These observations appear to be cell type-specific because, in cardiac myocytes, the G_q -coupled angiotensin II

receptor activates Ras through Shc/Grb2/Sos interaction in a PKC-independent manner [117]. In COS-7 cells expressing G_q-coupled M1 muscarinic receptors, expression of the dominant-negative Ras mutant or proteins that sequester $\beta\gamma$ subunits suppresses carbachol-induced MAPK activation [118, 111]. Furthermore, in Lyn- and Syk-deficient avian B cells (DT40), the M1 muscarinic receptor fails to activate both MAPK and MAPK kinase (MEK) [119]. The G_q agonist bradykinin activates PYK2, a brain-specific Ca^{2+}-sensitive tyrosine kinase, in PC12 cells [120]. These studies suggest that tyrosine kinases are essential for G_q-mediated activation of MAPK in some cells.

G_s-coupled receptors activate adenylyl cyclases via G_s, resulting in increased intracellular cAMP concentration, which in turn activates PKA [121] [122]. In some systems, PKA suppresses growth factor-induced MAPK activation [123-125]. This inhibition is associated with increased phosphorylation of Ser-43 in the Raf-1 regulatory domain, reducing Raf-1 affinity for Ras [124]. In rat pheochromocytoma PC12 cells, however, agonists of the cAMP signaling activate the MAPK pathway [126, 127]. This activation appears to result from high levels of B-raf expression in PC12 cells. cAMP activates the MAPK pathway though activating the guanine nucleotide exchange factor Rap1, which in turn activates B-raf in a Ras-independent manner [127].

Coupling GPCRs to the MAPK Pathway

The G protein-coupled endothelin [128], LPA [129], α_{2A}AR [130], angiotensin II [117], thrombin [131], and TRH [13] receptors mediate an agonist-dependent increase in tyrosine phosphorylation of Shc. LPA- and α_{2A}AR-mediated Shc tyrosine phosphorylation is sensitive to both pertussis toxin and a peptide that sequesters $\beta\gamma$ subunits [113], and can be mimicked by expression of the $\beta\gamma$ subunits [129]. Moreover, transient expression of dominant-negative Sos mutants inhibits Erk activation by the $\beta\gamma$ subunits and G_i-coupled α_{2A}AR. These

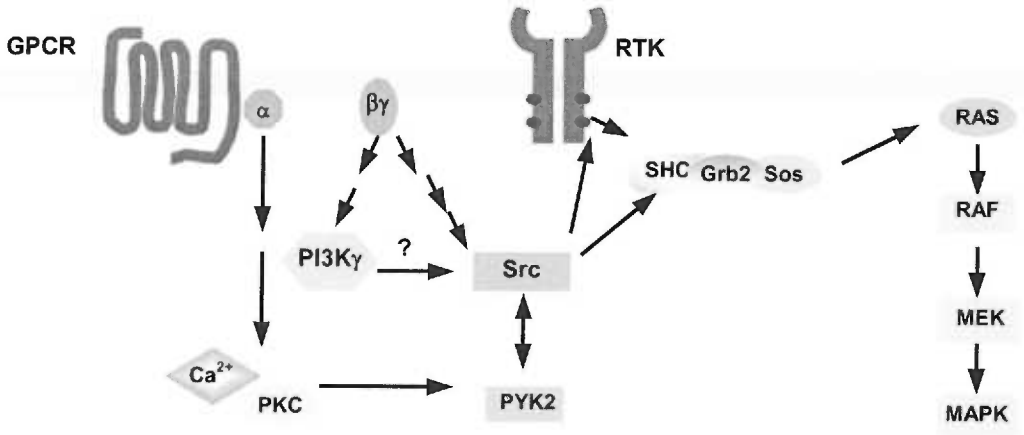


Figure 5. possible pathways that couple GPCRs to the MAPK pathway

findings suggest that G protein-coupled receptors utilize similar intermediates as receptor tyrosine kinases do to activate the MAPK pathway. The precise mechanisms are still unclear but appear to involve tyrosine kinases.

Coupling GPCRs to the MAPK Pathway by Non-Receptor Tyrosine Kinases

Tyrosine kinases can be activated by many GPCR agonists such as angiotensin II [117], bradykinin [120], TRH [132], endothelin [128], thrombin and LPA [109]. GPCR-induced MAPK activation can be attenuated by treatment with tyrosine kinase inhibitors, indicating that tyrosine kinases contribute to MAPK activation by GPCRs [109]. Recent studies have implicated several tyrosine kinases in linking GPCRs to MAPKs. These tyrosine kinases include Src family tyrosine kinases, such as Src [133], Fyn [117], Lyn and Syk [119], and a novel Ca^{2+}- and PKC- dependent kinase, PYK2 [120].

The molecular events whereby GPCRs stimulate Src family tyrosine kinases are still unclear. Several studies suggest that stimulation of phosphatidylinositol 3-kinase (PI3K) activity may play a role in G protein-mediated activation of MAPK and Src family tyrosine kinases in some cells. PI3K is known to associate with Src-family tyrosine kinase through their SH2 and SH3 domains [134, 135]. $\beta\gamma$-mediated PI3K activity has been described in neutrophils and platelets [136, 137]. Coexpression of CD-βARK, which sequesters free $\beta\gamma$ subunits [113], with PI3Kγ almost abolishes MAPK activation by PI3Kγ but not by a myristoylated form of PI3Kγ [138]. These findings indicate that one function of $\beta\gamma$ subunits is to localize PI3Kγ to the plasma membrane. In COS-7 cells, a specific PI3K inhibitor, wortmanin, inhibits M_2 muscarinic receptors and G$\beta\gamma$-

stimulated phosphorylation of Shc [130, 138]. However, wortmanin does not inhibit MAPK activation by transient expression of Sos and constitutively active mutants of Src, Ras and MEK [116], suggesting that the PI3K-dependent step may lie upstream of Src kinase activation. This view is consistent with recent findings that signaling from PI3Kγ to MAPK appears to require a tyrosine kinase, Shc, Grb2, Sos, Ras and Raf. Therefore, according to these studies, the following model is proposed [138]: activation of GPCRs set free the $\beta\gamma$ subunits, which recruit PI3Kγ to the plasma membrane. Interaction between the PI3K and a Src-like kinase enhances the tyrosine kinase activity, which in turn leads to interaction of the Shc/Grb2/Sos complex with the Ras pathway, resulting in increased MAPK activity.

In addition to PI3K, interactions between Src family tyrosine kinases and $\beta\gamma$-regulated tyrosine kinases might also contribute to regulation of activities of these tyrosine kinases. In PC12 cells, G_q -coupled receptors have been shown to activate the Ca^{2+} and PKC-dependent tyrosine kinase PYK2 [120]. PYK2 may activate the MAPK pathway through tyrosine phosphorylation of Shc and by recruitment of Grb2/Sos complex [120]. Further studies suggest that tyrosine phosphorylation of PYK2 leads to binding of the Src SH2 domain to Tyr-402 of PYK2 and activating Src [139]. Both PYK2 and Src are required for LPA- and bradykinin-induced MAPK activation. However, PKC activation and Ca^{2+} mobilization are unable to account for GPCR-mediated tyrosine phosphorylation in many non-neuronal cells [140, 141]. Bruton's tyrosine kinase (Btk), a member of a family of pleckstrin homology (PH) domain-containing tyrosine kinases, is

regulated by βγ subunits[142]. In hematopoietic cells, Btk interacts with the Src family kinases Fyn, Lyn and Hck [143]; Src-Btk interaction is associated with Btk activation [144]. However, this is unlikely to be a general mechanism by which G protein-coupled receptors regulate Src kinases, because this family of tyrosine kinases has limited tissue distribution and is not known to be involved in regulation of the MAPK pathway.

Coupling GPCRs to the MAPK Pathway by Receptor Tyrosine Kinase

The convergence of GPCR and RTK signaling pathways is supported by the following observations. In rat aortic smooth muscle cells, treatment with angiotensin II induces tyrosine phosphorylation of the PDGF receptor and formation of the Shc/Grb2 complex [145]. These effects are sensitive to inhibition by an antagonist of the angiotensin II type I receptor, indicating that angiotensin II mediates PDGF-independent activation of the PDGF receptor. Similar results were reported that endothelin-1, LPA and thrombin induce tyrosine phosphorylation of the EGF receptor and the EGF receptor-related RTK, HER2 [146]. The EGF receptor phosphorylation is accompanied with increased binding of both Shc and the EGF receptor to the GST-Grb2 fusion protein. AG1478, a specific kinase inhibitor of the EGF receptor, blocks GPCR-mediated phosphorylation of the EGF receptor and MAPK activation [146]. These studies suggest that the intrinsic tyrosine kinase activities of receptor tyrosine kinases may be modulated in a ligand-independent manner by GPCR-mediated signals

and that the RTKs themselves may function as scaffolds in GPCR signaling. Interestingly, the RTK that supports the GPCR signal can vary between cell types, possibly reflecting either relative abundance of RTKs or preferential coupling to particular RTKs. In L cells that lack the EGF receptor, transactivation of the PDGF receptor and MAPK activation by LPA are sensitive to the specific inhibitor of the PDGF receptor [147]; in contrast, in Rat-1 cells where the PDGF receptor is coexpressed endogenously with high levels of the EGF receptor, EGF receptor inhibition blocks LPA-stimulated MAPK activity.

The above findings suggest that cross-talk between RTKs and GPCRs is a general feature of GPCR signaling. However, little is known about the mechanisms by which GPCRs regulate RTK activities. Ca^{2+}, PKC and Src family tyrosine kinases have been proposed as early mediators of RTK transactivation. In PC12 cells, EGF receptor transactivation by either bradykinin or membrane depolarization is dependent on extracellular calcium [148, 149]. In rat vascular smooth muscle cells, however, angiotensin II-induced EGF receptor transactivation is only affected by intracellular Ca^{2+} mobilization [150]. In 293 cells stably transfected with the M_1 muscarinic receptor, carbachol-induced EGF receptor transactivation is solely PKC-dependent, and does not require a Ca^{2+} signal [151]. In contrast, PKC suppresses angiotensin II-induced tyrosine phosphorylation of the EGF receptor in GN4 rat liver epithelial cells [152]. These observations suggest that cell-specific mechanisms alter the role that PKC and Ca^{2+} play in regulating EGF receptor activation.

In addition to Ca^{2+} and PKC, some evidence suggests that RTK transactivation is mediated by $\beta\gamma$ subunits and Src family tyrosine kinases in some cells [150, 153]. Angiotensin II induces association of the catalytically active Src with the EGF receptor. This association is unaffected by the EGF receptor kinase inhibitor, indicating that Src activation precedes EGF receptor transactivation [150]. In COS-7 cells, overexpression of $\beta1\gamma2$ subunits leads to tyrosine phosphorylation of Shc, which associates with the tyrosine phosphorylated EGF receptor and HER2. Expression of a kinase-inactive Src mutant or the Src inhibitor kinase Csk attenuates $\beta\gamma$- and G_iCPR-mediated EGF receptor phosphorylation. However, G_iCPR- and Src-mediated increase in EGF receptor phosphorylation does not reflect increased EGF receptor autophosphorylation, which can be distinguished by an autophosphorylation-specific EGF receptor monoclonal antibody. Interestingly, treatment with a broad tyrosine phosphatase inhibitor, vanadate, increases the autophosphorylation of the EGF receptor [153], indicating that inactivating tyrosine phosphatases might be one of the mechanisms by which GPCRs activate RTKs.

This thesis seeks to understand the different signaling pathways which mediate responses to TRH. The studies will investigate the role of MAPK in TRH-induced transcription. Several approaches will be used to determine if MAPK is required for TRH effects on the prolactin promoter. In addition, the thesis also includes studies which examine the role of receptor tyrosine kinases, especially the EGF receptor, in mediating TRH-induced MAPK activation.

CHAPTER II

A ROLE FOR THE MITOGEN-ACTIVATED PROTEIN KINASES IN MEDIATING THE ABILITY OF TYROTROPIN-RELEASING HORMONE TO STIMULATE THE PROLACTIN PROMOTER

Introduction

The ability of TRH to stimulate prolactin synthesis and secretion involves the interaction of the hormone with a G protein-coupled receptor at the plasma membrane [4, 14]. The TRH receptor couples to G_q to stimulate the activity of phospholipase Cβ [5] leading to increased intracellular levels of diacylglycerol and inositol 1, 4, 5-trisphosphate [154]. Diacylglycerol activates protein kinase C (PKC), which is accompanied by redistribution of PKC from a soluble to a particulate subcellular fraction [8]. Inositol 1, 4, 5-trisphospate stimulates the release of Ca^{2+} from intracellular stores, resulting in a first phase of Ca^{2+} elevation [9]. A second phase of Ca^{2+} elevation is due to membrane depolarization, which leads to Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels [10]. Increases in intracellular Ca^{2+} lead to activation of a Ca^{2+}/calmodulin-dependent protein kinase [12]. It has also been shown that TRH can activate the mitogen activated protein kinase (MAPK) signaling pathway also known as the extracellular signal-regulated kinase (ERK) in GH_3 pituitary tumor

cells [13]. Thus multiple kinase pathways including PKC, Ca^{2+}/calmodulin-dependent kinase and MAPK are activated by TRH.

The specific signaling pathways that permit TRH to rapidly stimulate prolactin gene transcription [39, 41] have not been clearly defined. There is evidence that activation of PKC [155], Ca^{2+}/calmodulin-dependent protein kinases [45, 46] or MAPK [36] can stimulate the prolactin promoter. However, the roles that these pathways play in mediating TRH effects has not been resolved. There is evidence that PKC may not be required for TRH-induced activation of the PRL promoter [48, 49]. Although inhibition of Ca^{2+}/calmodulin-dependent protein kinases was found to reduce the ability of TRH to activate the PRL promoter, the DNA elements that are required for responsiveness to specific Ca^{2+}/calmodulin-dependent protein kinases do not co-localize with TRH-responsive DNA elements [46]. Thus, there are questions concerning the roles that PKC and Ca^{2+}/calmodulin-dependent protein kinases play in mediating TRH effects on the PRL promoter.

In the present study we have examined a possible role for the MAPK pathway in mediating TRH effects on the PRL promoter. We have used several approaches to determine if MAPK activity is required for TRH effects on the prolactin promoter. We have also assessed the role that PKC activation and Ca^{2+} signaling play in activating the MAPK pathway in GH_3 cells.

Materials and Methods

Materials

Cell culture media and supplies were purchased from GIBCO BRL. Radioisotopes and reagents for enhanced chemiluminescent detection of immunocomplexes were purchased from DuPont New England Nuclear. Anti-ERK2 antibody, anti-phospho-ERK antibody, agarose beads-conjugated anti-ERK2 antibody and horseradish peroxidase (HRP)-coupled anti-mouse IgG antibody were obtained from Santa Cruz Biotechnology. The MEK inhibitor, PD98059, was purchased from Alexis Corporation; PKC inhibitor, GF109203X, from Calbiochem; nimodipine from Research Biochemicals International; EGF from Boehringer Mannheim; TRH from Peninsula Laboratories.

Reporter genes and expression vectors

A reporter gene containing the proximal 255 base pairs of the 5'-flanking region of the rat prolactin gene was prepared by polymerase chain amplification of the appropriate region from the prolactin gene [156] which was inserted upstream of the firefly luciferase coding sequence in the promoter-less construct, pLuc-Link [157]. PRL reporter genes containing mutations in specific Ets factor binding sites were prepared by oligonucleotide-directed mutagenesis. The specific mutations involved mutation of the Ets site at -211 to -208 with the sequence TTCC to the sequence TGAA, the Ets site at -162 to -159 from TTCC to AGGC and the dual Ets site at -75 to -66 from GGAAgaGGAT to GGCCgaTTAT. A luciferase reporter gene containing 5 GAL4 binding sites

upstream of a minimal promoter as well as an expression vector for a Gal4-Elk1 fusion protein have been described previously [158, 159]. A reporter gene, pRL, expressing *Renilla* luciferase was purchased from Promega.

A mammalian expression vector for kinase-defective MEK1 mutant [71] was generously provided by Dr. Edwin G. Krebs. An expression vector encoding a constitutively active form of MEK1 was generated by replacing Ser 218 and Ser 222 by glutamic acid and cloned into pCDNA3 vector [69].

Cell Cultures and Transfections

GH_3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% horse serum, 2.5% fetal bovine serum, 100U/ml penicillin, and 100 μg/ml streptomycin. For transient transfection assays, 2.5×10^5 cells per well were planted in 6-well plates 1 day before the transfection. DNA was introduced into these cells using lipofectamine (GIBCO BRL) in serum-free medium according to a protocol provided by the manufacturer. In each experiment the total amount of transfected DNA per culture dish was constant (usually at 2 μg). After 5 h of treatment with the lipofectamine/DNA mixture, an equal volume of serum-containing culture medium was added. After an additional 18 h, the cells were transferred to serum-free medium. TRH treatments were applied 24 h after transfer to serum-free medium and the cells were then lysed 5 to 6 h later for reporter gene analysis. In most experiments cells were transfected with a firefly luciferase reporter gene and as an internal standard for transfection efficiency a control plasmid which expresses *Renilla* luciferase.

Renilla luciferase requires a different substrate than firefly luciferase and can be assayed independently. Cells were lysed and the activities of firefly and *Renilla* luciferase determined using a protocol and reagents from Promega. Total firefly luciferase light units were normalized to total *Renilla* luciferase activity. The results of transfection studies are reported as means ± standard error of the mean for several separate transfections (individual culture dishes) which were performed as part of the same experiment.

Immunoblotting

For analysis of protein expression, cells were grown to approximately 80% confluency, transferred to serum-free medium and then treated with inhibitors and agonists as indicated. After treatments, the cells were washed twice with ice-cold 0.15 M NaCl, 0.01 M $NaPO_4$ (pH 7.4). The cells were lysed for 10 min on ice in 20 mM Tris, pH 7.5, 1% Triton X-100, 10% glycerol, 50 mM β-glycerolphosphate, 2 mM EGTA and 1 mM dithiothreitol. The lysates were centrifuged for 20 min at 12,000 x g. The supernatants (100 μg protein) were adjusted to contain 1% sodium dodecyl sulfate and 5% 2-mercaptoethanol and heated for 3 min in a boiling water bath before electrophoresis on a sodium dodecyl sulfate-containing polyacrylamide gel [160]. Proteins were then transferred by electroblotting to a polyvinylidene difluoride membrane. The membranes were incubated in 0.15 M NaCl, 0.01 M $NaPO_4$ (pH 7.4) containing 3% bovine serum albumin and 0.01% Tween 20 for 1 h and then incubated with a primary antibody for 1 h. After six washes with 0.15 M NaCl, 0.01 M NaPO4

(pH 7.4), 0.01% Tween 20, horseradish peroxidase-conjugated secondary antibody was incubated with the blot for 30 min. After extensive washing, the proteins were detected by using the enhanced chemiluminescence system (DuPont).

ERK immunocomplex kinase assay

Cells were treated and lysed as described for immunoblotting. Cell extracts were incubated with agarose-conjugated anti-ERK2 antibody for 1-2 h. Immunoprecipitates were washed once in lysis buffer, twice in 500 mM NaCl, 100 mM Tris-HCl pH 7.5, 0.1% Triton X-100 and 2.5% sucrose and once in kinase assay buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1% Triton X-100 and 2 mM EGTA). In vitro kinase assays were carried out for 25 min at 30° C in 20 μl of kinase assay buffer supplemented with 1 μCi of [γ-^{32}P] ATP and 5 μg glutathione S-transferase fusion protein which includes the carboxy-terminal transcriptional activation of Elk1 (residues 307-428) including several MAPK phosphorylation sites [161]. The GST-Elk1 fusion protein was expressed in E. coli and purified using glutathione-sepharose beads [162]. The kinase reactions were stopped by adding sodium dodecyl sulfate to 1% (w/v) and the reactions were resolved on a denaturing, 10% polyacrylamide gel and the phosphorylated proteins were detected by autoradiography.

Results

Activation of MAPK Can Stimulate Prolactin Promoter Activity

Previous studies have shown that constitutively active forms of Ras or Raf can activate the prolactin promoter suggesting that activation of the MAPK signaling pathway is sufficient to stimulate prolactin transcription [36, 38]. While perhaps unlikely, it is possible that Ras or Raf may lead to signaling events other than activation of MAPK [163, 164]. To further assess the ability of the MAPK pathway to alter prolactin promoter activity, we tested the effects of a constitutively active form of MAPK kinase (MAPKK), also known as MAPK/ERK kinase (MEK). As the MAP kinases, ERK1 and ERK2, are the only known substrates of MEK [74], this approach allows a further test of the ability of MAPK to regulate the prolactin promoter. The expression vector for constitutively active MEK produced substantial activation of the prolactin reporter gene (Fig. 1A) and had little or no effect on the thymidine kinase minimal promoter (Fig. 1B). Thus, MAPK activation selectively stimulates prolactin promoter activity.

TRH Induces Prolonged MAPK Activation

In some systems, sustained rather than transient activation of a signaling pathway is required to induce long-term responses. For instance, in PC12 cells, sustained MAPK activation is associated with differentiation [80]. Although previous studies have shown that TRH can rapidly activate MAPK [13], these studies examined very early time points and did not determine whether TRH has more prolonged effects on the kinase activities. To determine whether TRH-induced MAPK activation is a reasonable candidate for mediating long-term

transcriptional effects of TRH, the time course of MAPK activation by TRH in GH_3 cells was determined (Fig. 2). To assess MAPK activation, an immuno-complex assay was used. For this assay, cell lysates were immunoprecipitated with an antibody to ERK2 and then the immunoprecipitated proteins were incubated with [^{32}P] ATP and GST-Elk1 as the kinase substrate. The immuno-complex assay demonstrated that TRH-induced ERK2 activation was maximal at the earliest time point examined, 2.5 min and declined at later time points (Fig 2A). However, it is important to note that at all TRH time points, including the 75 minute treatment, ERK2 activity of TRH-treated samples was greater than that of the untreated control. This activation at later time points has been observed in several different experiments (data not shown). The use of anti-phospho-ERK antibodies to detect the activated form of ERKs also yielded results consistent with sustained activation of MAPK. MAPK is activated by phosphorylation of both threonine and tyrosine residues [165]. Antibodies directed specifically against the phosphorylated forms of MAPK detect both ERK1 and ERK2. Increased phosphorylation of ERK1 and ERK2 was detectable for at least an hour (Fig. 2B). Analysis of total, immunoreactive ERK1 and ERK2 indicated that TRH-induced increases in ERK phosphorylation was not due to increases in the amount of these kinases (Fig. 2C). These results provide evidence that TRH induces an initial burst of MAPK activation followed by a lower, but clearly detectable prolonged phase of activation.

Activation of MAPK has been shown to lead to phosphorylation and activation of Elk1, a member of the Ets family of transcription factors, and a

component of the serum response factors [161]. To determine whether TRH-induced MAPK activation is sufficient in magnitude and duration to alter a transcriptional event, we examined the activation of a GAL4-Elk1 fusion (Fig. 3). TRH stimulated expression of the GAL4-luciferase reporter gene more than 20-fold. Substitution of serine 383 of Elk1 with alanine, which disrupts a critical MAPK phosphorylation site [161, 166], strongly diminished TRH-induced activation of Elk1 suggesting that the transcriptional response likely involves direct phosphorylation of Elk1 by MAPK. These results suggest that TRH treatment of GH_3 cells activate MAPK in a manner, which is sufficient to modulate a transcriptional response.

MAPK Activation is Necessary for TRH-induced Increases in Prolactin Promoter Activity

A kinase-defective, interfering mutant form of MEK was used to investigate the functional role of MAPK. MEK activates MAPK by phosphorylating MAPK at both threonine and tyrosine residues [165]. The mutant MEK was made by substituting an alanine for a lysine residue within the ATP binding site of the enzyme. Although the mutant MEK can be phosphorylated by Raf, it cannot phosphorylate and activate MAPK [67,167], and therefore interferes with signal transduction. To determine an effective concentration of mutant MEK expression vector, increasing concentrations of the expression vector were tested for their ability to reduce TRH-induced GAL4-Elk activation (Fig. 4A). The MEK mutant reduced both basal and TRH-induced activation of Gal4-Elk1 in a dose-

dependent manner (Fig. 4A) suggesting that the MEK mutant can interfere with endogenous MEK signaling. Based on the titration study, the ability of 2 μg of the mutant MEK expression vector to inhibit TRH effects on prolactin-luciferase (Fig. 4B), GAL4-Elk (Fig. 4C) or the thymidine kinase-luciferase reporter gene (Fig.4 D) was compared. This concentration of the mutant MEK vector had similar effects to reduce TRH-induced activation of a prolactin-luciferase reporter gene and GAL4-Elk1. In contrast, the mutant MEK had little or no effect on the thymidine kinase promoter (Fig. 4D), suggesting that the inhibitory effects on the prolactin promoter are not due to non-specific inhibition of transcription.

PD98059 is a selective inhibitor of MEK1 and MEK2 [168, 169]. This inhibitor blocks MAPK activation in several cell types and blocks processes such as neurite outgrowth in nerve growth factor-treated PC12 cells [170]. We first tested whether this inhibitor was able to block TRH-induced MAPK activation in GH_3 cells (Fig. 5). Treatment of GH_3 cells with 100 μM PD98059, almost completely blocked TRH-induced ERK2 activation. The effects of PD98059 on TRH-induced activation of the prolactin promoter and GAL4-Elk were then examined (Fig. 6). PD98059 substantially reduced both prolactin promoter and Gal4 promoter activation by TRH. The results of studies using an expression vector for an interfering, kinase-defective MEK as well the PD98059 studies suggest that MAPKs are required for TRH-induced activation of the prolactin promoter.

DNA Elements that Contribute to TRH-Responsiveness of the Prolactin Promoter Co-localize with MAPK-Responsive Elements

Several binding sites for members of the Ets family of transcription factors have previously been identified as important for mediating responses to activation of the MAPK pathway [36,38]. To determine if Ets binding sites are also important for TRH-responsiveness, we prepared reporter genes containing block mutations which disrupt specific Ets binding sites within the prolactin promoter (Fig. 7A). The wild type and mutant prolactin reporter constructs were transfected into GH_3 cells and compared for responses to TRH (Fig. 7B) or an expression vector for activated MEK (Fig. 7C). In general, the specific Ets mutations had a similar pattern of effects on responsiveness to TRH and activated MEK. Consistent with previous reports [37], disruption of an Ets site located at -211 to -208 had the greatest effect to reduce responsiveness to MEK and also had the greatest effect to reduce TRH-induced reporter gene activity. The Ets sites which were tested are those which have been reported in previous work to have a possible involvement in regulating the PRL gene. There are other possible Ets binding sites within the proximal region of the PRL gene. At the present it would be premature to make conclusions about the role of non-Ets factors in mediating responses to TRH and MAPK. None the less, the similar pattern of effects of Ets mutations provides evidence that DNA elements necessary for TRH responsiveness co-localize with DNA elements required for MAPK-responsiveness.

TRH-Induced Activation of MAPK and the Prolactin Promoter is Mediated by PKC and Ca^{2+} Influx

Previous studies have used chronic treatment with phorbol esters to provide evidence that PKC plays a role in TRH-induced MAPK induction [13]. To further test the role of PKC in mediating TRH effects on MAPK activity, we selected the PKC inhibitor, GF109203X [171] and determined ERK2 activity using an immunocomplex assay (Fig. 8). As expected, GF109203X substantially blocked PMA-induced ERK2 activation, indicating that the inhibitor successfully blocked PKC-dependent MAPK activation. The inhibitor partially reduced TRH-stimulated ERK2 activation and had little or no effect on the ability of EGF to activate ERK2. While the ability of GF109230X to reduce TRH-induced MAPK activation is somewhat modest, it has been consistently observed in several experiments (for instance see also Fig. 9). These results suggest that TRH appears to induce MAPK activation through both PKC-dependent and PKC-independent pathways.

TRH also leads to the entry of extracellular Ca^{2+} through L-type voltage-sensitive Ca^{2+} channels resulting in a sustained, plateau-like elevation of intracellular Ca^{2+} [172]. A role for Ca^{2+} influx in TRH-mediated activation of the prolactin promoter has previously been described [42, 173, 174]. As Ca^{2+} influx has been shown to lead to MAPK activation in some cells [120, 149,175-177], it seemed possible that Ca^{2+} influx might contribute to TRH-induced MAPK induction. To explore this possibility, GH_3 cells were treated with nimodipine, a blocker of L-type Ca^{2+} channels [10]. Nimodipine slightly reduced ERK2

activation at all time points examined (Fig. 9). At the later time points, from 10 min to 60 min, ERK2 activation was approximately half that of control cells. Similar results have been observed in several different experiments (data not shown). The PKC inhibitor, GF109203X, had a different time course of effects on ERK2 phosphorylation. GF109203X substantially reduced TRH-induced ERK2 activation at the earliest time point, 2.5 min, and had relatively little effect at the later time points. The combination of nimodipine plus GF109203X appeared to reduce ERK2 activation more than either treatment alone. Similar results were obtained when ERK1 and ERK2 activation was assessed by immunoblotting with a phospho-ERK antibody (data not shown). These findings suggest that both PKC activation and Ca^{2+} influx may play a role in TRH-induced MAPK activation. PKC appears to play a major role at early times after TRH stimulation while Ca^{2+} influx plays a more important role at later time points.

Inhibitor studies were then used to test the role of Ca^{2+} influx and PKC activation in mediating TRH-induced prolactin promoter activation. GH_3 cells were pretreated with nimodipine, GF109203X or a combination of the inhibitors and the ability of TRH to stimulate the expression of a prolactin-luciferase reporter gene was examined (Fig. 10). Both GF109203X and nimodipine treatment rather strongly blunted the ability of TRH to stimulate prolactin promoter activity and the combination of the two inhibitors almost completely blocked TRH effects. These experiments provid evidence that TRH-induced PKC activation and Ca^{2+} influx contribute to MAPK induction and activation of the prolactin promoter. To further test the role of Ca^{2+} influx-induced MAPK

activation in modulating prolactin gene expression, the effects of the MEK inhibitor, PD98059 on Ca^{2+} influx-stimulated prolactin promoter activity was examined (Fig. 11). Control or PD98059-treated GH_3 cells were stimulated with the Ca^{2+} channel agonist, Bay K8644, which has been demonstrated to stimulate prolactin promoter activity [42,178]. The ability of PD98059 to substantially reduce Ca^{2+} influx-stimulated prolactin promoter activity provides evidence that MAPK plays a role in mediating Ca^{2+} effects on transcription in this system.

Discussion

These studies provide evidence that TRH induced MAPK activation likely plays a role in regulating transcription of the prolactin gene. Previous studies have shown that activated Ras can stimulate prolactin promoter activity [36,38], implying that activation of the MAPK pathway is sufficient to stimulate prolactin gene expression. Our studies demonstrate that an expression vector for constitutively active MEK also increases prolactin promoter activity. This result offers additional evidence that the MAPK cascade is indeed sufficient to activate the prolactin promoter. Our work also extends the view of previous studies, which demonstrated that TRH can activate MAPK. Our findings demonstrate that TRH can induce prolonged activation of MAPK. TRH-induced MAPK activation was observed for at least an hour, consistent with the ability of TRH to stimulate prolonged activation of prolactin gene transcription, which persists for hours [39]. The finding that TRH can lead to activation of the GAL4-Elk1 transcription factor offers additional evidence that TRH can alter a transcriptional

event and also implies that TRH probably induces nuclear localization of MAPK. Importantly, transfection of a kinase-defective, interfering MEK mutant or addition of the MEK inhibitor, PD98059, was found to suppress TRH-induced prolactin promoter activity. Thus our studies provide evidence that TRH activates the MAPK pathway and that MAPK activation is sufficient and necessary for activation of the prolactin promoter.

The finding that DNA sequences of the prolactin promoter which are required for TRH responsiveness, co-localize with DNA sequences required for MAPK-responsiveness reinforces the view that MAPK plays a role in mediating transcriptional responses to TRH. Previous studies have provided evidence that binding sites for members of the Ets family of transcription factors are important for MAPK- and multihormonal-responsiveness of the prolactin promoter [37,38,179,180]. Therefore, we tested TRH- and MAPK-responsiveness of reporter genes with clustered point mutations that disrupted several consensus Ets bindings sites. Disruption of an Ets site at position -211 to -208 of the prolactin promoter had a substantial effect to reduce both TRH- and MAPK-responsiveness. This Ets site was previously found to be important for ras-responsiveness [179]. Interestingly, although we previously used multimers of synthetic binding sites to demonstrate that an Ets site at position -162 to -159 is capable of responding to MAPK activation, this element was not required for TRH- or MAPK-responsiveness within the context of the normal prolactin promoter. It is not clear why the upstream Ets binding site at -211 to -208 plays the predominant role in mediating MAPK responsiveness.

It is probably important that TRH effects on MAPK persist for at least an hour. The kinetics of MAPK activation can influence nuclear translocation of MAPK and therefore alter access to nuclear substrates [80]. Therefore the kinetics of MAPK activation can have important effects on physiological responses. For instance in PC12 cells, NGF causes sustained MAPK activation, nuclear translocation of MAPK and induces differentiation [69]. In contrast, EGF results in a transient cytoplasmic activation of MAPK which fails to induce differentiation of PC12 cells [80].

Our studies provide evidence that a PKC-dependent pathway contributes to TRH effects on both MAPK activation and stimulation of the prolactin promoter. The PKC inhibitor, GF109203X partially inhibited TRH induced activation of the MAPK and prolactin promoter activity providing evidence that both PKC-dependent and PKC-independent pathways mediate TRH effects. Our finding that PKC appears to play a role in mediating TRH effects on prolactin gene expression differs from earlier studies which concluded that PKC was not involved [42]. The previous studies used chronic phorbol ester treatment to deplete cells of PKC activity while we used GF109203X. Phorbol ester treatment is a very potent activator of the prolactin promoter, which stimulates transcription for more than 24 hours making it difficult to interpret the effects of PKC depletion [48]. The use of GF109203X avoids the complication of chronic phorbol ester treatment, which both activates and depletes PKC activity. GF109203X inhibits the activity of most PKC isoforms [171,181]. It is possible that differences in the

effects of GF109203X as compared with that of chronic PMA treatment reflect differential inhibition of specific subsets of PKC isozymes.

The present studies also provide evidence that Ca^{2+} influx plays a role in mediating TRH effects on both MAPK activation and prolactin gene expression. TRH treatment stimulates a rapid transient increase in cytosolic Ca^{2+} as well as sustained, plateau-like elevation in Ca^{2+} levels, which is dependent on influx through Ca^{2+} channels [154]. Although previous studies have shown that the L-type Ca^{2+} channel blockers can inhibit the ability of TRH to activate the prolactin promoter [42, 174], the signaling mechanisms that permit Ca^{2+} influx to regulate transcription have not been identified. Our finding that nimodipine reduces TRH-induced MAPK activation suggests that MAPK is likely at least part of the mechanism mediating Ca^{2+} responsiveness. Nimodipine effects on TRH-induced MAPK activation were most prominent at later time points consistent with the known contribution of Ca^{2+} channels to the later, plateau phase of Ca^{2+} elevation [154]. Furthermore, the MEK inhibitor PD98059, blunted the ability of Ca^{2+} influx to increase prolactin promoter activity, providing evidence that MAPK plays a role in mediating Ca^{2+} effects on prolactin transcription.

There are many possible mechanisms that may permit Ca^{2+} influx to activate the MAPK pathway in GH_3 cells. In some cells, Ca^{2+} influx leads to tyrosine phosphorylation of the EGF receptor in a ligand independent manner and subsequent activation of the MAPK by Shc, Grb2, the guanine nucleotide exchange factor Sos1 and Ras [149]. The mechanism responsible for ligand-independent tyrosine phosphorylation of the EGF receptor is not well

established. A Ca^{2+}-responsive tyrosine kinase such as PYK2 [120] would be a candidate for mediating this response. However, we have been unable to demonstrate the presence of PYK2 in GH_3 cells (Y. -H. Wang, unpublished studies). Src transformation of fibroblasts does lead to tyrosine phosphorylation of the EGFR [176]. In addition, targeted gene disruption of the Src family member Fyn suggests that the Fyn protein may play a role in calcium-dependent responses in the nervous system, such as synaptic potentiation and memory formation [182]. Thus, it remains quite possible that some member of the Src family of tyrosine kinases may contribute to Ca^{2+} effects on MAPK activation. Another mechanism that might mediate Ca^{2+} effects on MAPK activation would involve a Ca^{2+}-sensitive guanine nucleotide exchange factor, such as Ras-GRF [175]. Finally, Ca^{2+} influx may activate MAPK through Ca^{2+}/calmodulin-dependent kinases [177].

Our studies suggest a model of prolactin gene expression in which TRH generates two different signals, PKC activation and increased intracellular Ca^{2+} levels that converge to activate MAPK. MAPK likely influences transcription of the prolactin gene through phosphorylation of an Ets transcription factor, probably Ets-1 [37, 183]. While this broadly outlines a signaling pathway there are many aspects of this pathway which have not been identified. In addition, it remains possible that pathways other than MAPK activation also contribute to TRH effects on the prolactin promoter. For instance, TRH activates Ca^{2+}/calmodulin-dependent kinase type II in GH_3 cells [12] and inhibitor studies using KN-62 suggest that a Ca^{2+}/calmodulin-dependent protein kinase

participates in TRH-induced prolactin gene expression [46]. However, KN-62 has little or no effect on TRH-induced MAPK activation (Y.-H. Wang, unpublished result). Thus a Ca^{2+}/calmodulin dependent protein kinase pathway which is independent of the MAPK may also contribute to TRH-induced prolactin gene expression. Although an expression vector for a constitutively active form of Ca^{2+}/calmodulin dependent protein kinase type II can activate prolactin reporter genes which contain the distal enhancer region, removal of this region almost completely eliminates responsiveness [46]. In contrast, the distal enhancer is not required for TRH responsiveness [42, 178]. Although Ca^{2+}/calmodulin dependent protein kinase type II may not be sufficient to activate the proximal promoter, it remains possible that it acts in concert with the MAPK pathway to modulate the prolactin promoter. Thus the ability of TRH to modulate transcription of the prolactin gene may depend on a complex interaction between PKC, MAPK and perhaps of Ca^{2+}/calmodulin dependent protein kinase signaling pathways.

Figure 1. Activation of the MAPK pathway can stimulate prolactin promoter activity.

Reporter genes containing 255 base pairs of the proximal region and promoter of the rat prolactin gene linked to firefly luciferase (A) or the herpes simplex virus thymidine kinase promoter linked to luciferase (B) were transfected with a control empty vector (control) or an expression vector for constitutively active MEK (Activated MEK). The cells also received a control reporter gene that expresses *Renilla* luciferase. The cells were collected 48 h after transfection and firefly and *Renilla* luciferase activity was determined. The values obtained for *Renilla* luciferase activity were used to correct for transfection efficiency. Firefly luciferase values are the average ± standard error of three separate transfections.

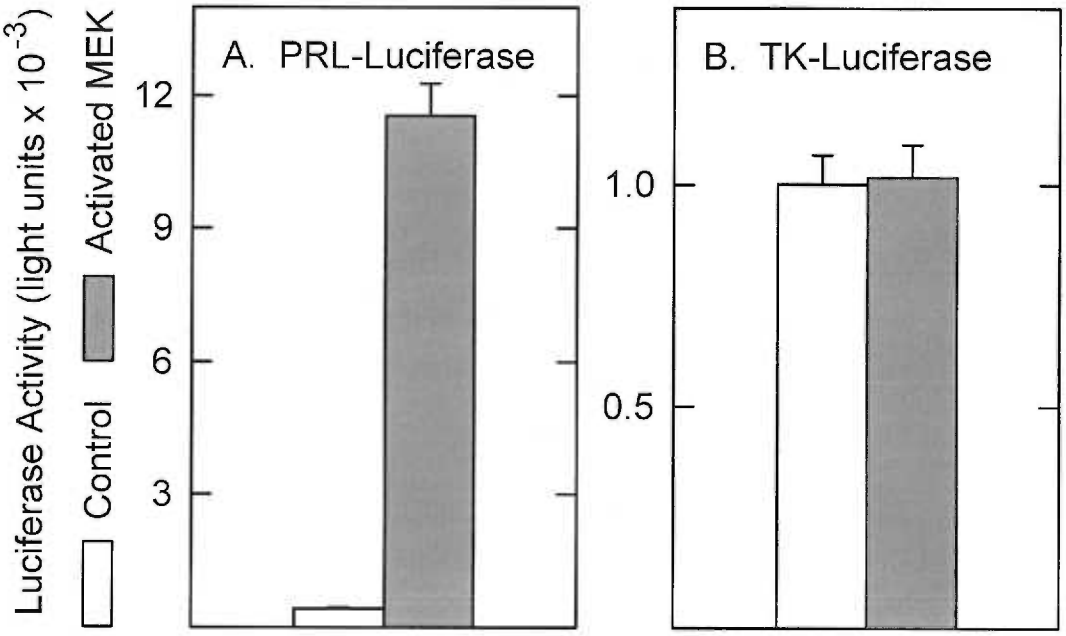

Luciferase Activity (light units x 10^{-3})
Control
Activated MEK
A. PRL-Luciferase
12
9
6
3
B. TK-Luciferase
1.0
0.5

Figure 2. Time course of TRH-stimulated MAPK activation.

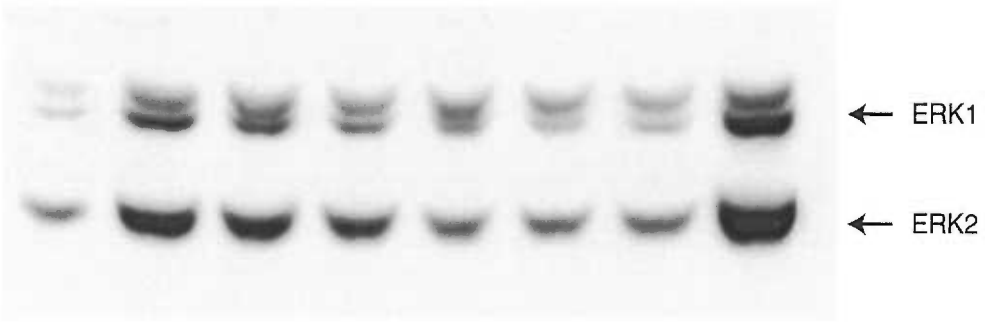
MAPK activation was determined by an immunocomplex assay (A) or by immunoblotting with an antibody to phospho-ERK (B). GH_3 cells were treated with 100 nM TRH for the indicated times and cell lysates were prepared. For the immunocomplex kinase assay, the cell lysates were immunoprecipitated with agarose bead-conjugated anti-ERK2 antibody. The ERK2 immunoprecipitate was then incubated with [^{32}P] ATP and a GST-Elk1 fusion protein was used as a substrate. The phosphorylated proteins were then resolved by denaturing gel electrophoresis. For the immunoblot analysis of MAPK activation, cell lysates were resolved by denaturing gel electrophoresis, transferred to a membrane and phospho-ERK visualized by immunostaining. To assess total levels of MAPK, the blot was reprobed with an antibody that detects both the activated and non-activated forms of ERK2 and more weakly detects both forms of ERK1.

A. Erk2 Immunocomplex Assay

TRH Treatment (min)									EGF 5 min
0	2.5	5	10	15	30	45	60	75	

TRH Treatment (min)							EGF 5 min
0	2.5	10	30	45	60	75	

B. Phospho-ERK Immunoblot



C. ERK Immunoblot

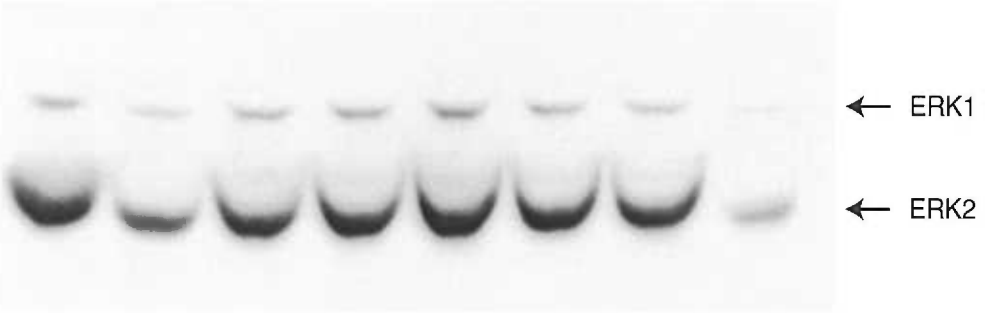


Figure 3. TRH treatment of GH_3 cells can increase the activity of a MAPK-responsive transcription factor.

GH_3 cells were transfected with plasmids encoding either GAL4-Elk1 or mutant GAL4-Elk1, in which serine-383, which is part of an important MAPK phosphorylation site, was mutated to alanine (GAL4-ElkS383A). The cells also received a firefly luciferase reporter gene containing 5 GAL4-binding sites upstream of a minimal promoter as well as a control reporter gene that expresses *Renilla* luciferase. At 48 h after transfection the cells were treated with 100 nM TRH or 10 nM EGF and then collected 6 h later for analysis of luciferase activity. Firefly luciferase values are the average ± standard error of three separate transfections that have been corrected for transfection efficiency.

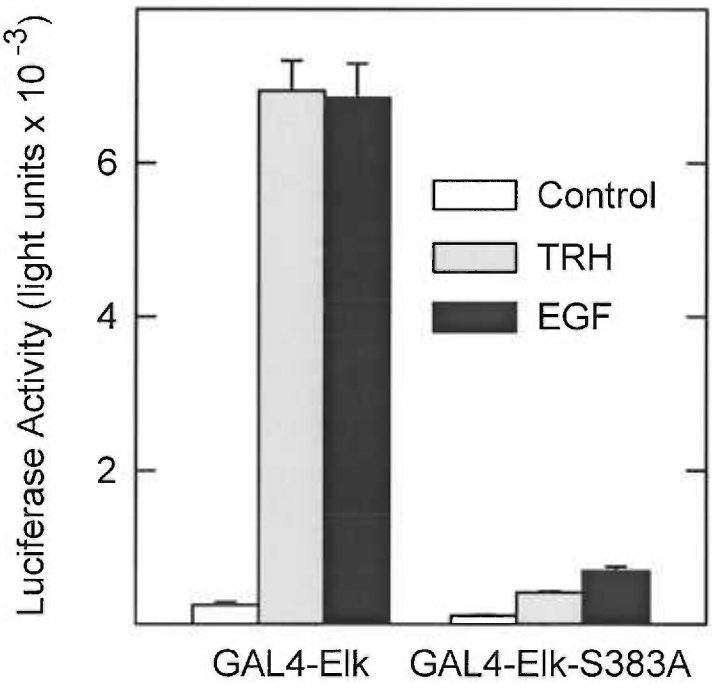

Luciferase Activity (light units x 10 -3)
6
4
2
Control
TRH
EGF
GAL4-Elk
GAL4-Elk-S383A

Figure 4. Inhibition of MAPK reduces TRH-induced prolactin promoter activity.

GH_3 cells were transfected with an expression vector for an interfering, kinase-deficient mutant MEK (MEK mutant) to inhibit activation of MAPK. To determine an effective concentration of mutant MEK expression vector, increasing concentrations of the expression vector were tested for their ability to reduce TRH-induced GAL4-Elk activation (A). Based on the titration study, the ability of 2 μg of the mutant MEK expression vector to inhibit TRH effects on prolactin-luciferase (B), GAL4-Elk (C) or thymidine kinase-luciferase reporter gene (D) was examined. Cells were treated with TRH at 48 h after transfection and then collected for analysis of luciferase activity 6 h later. All values are means ± standard error for three separate transfections.

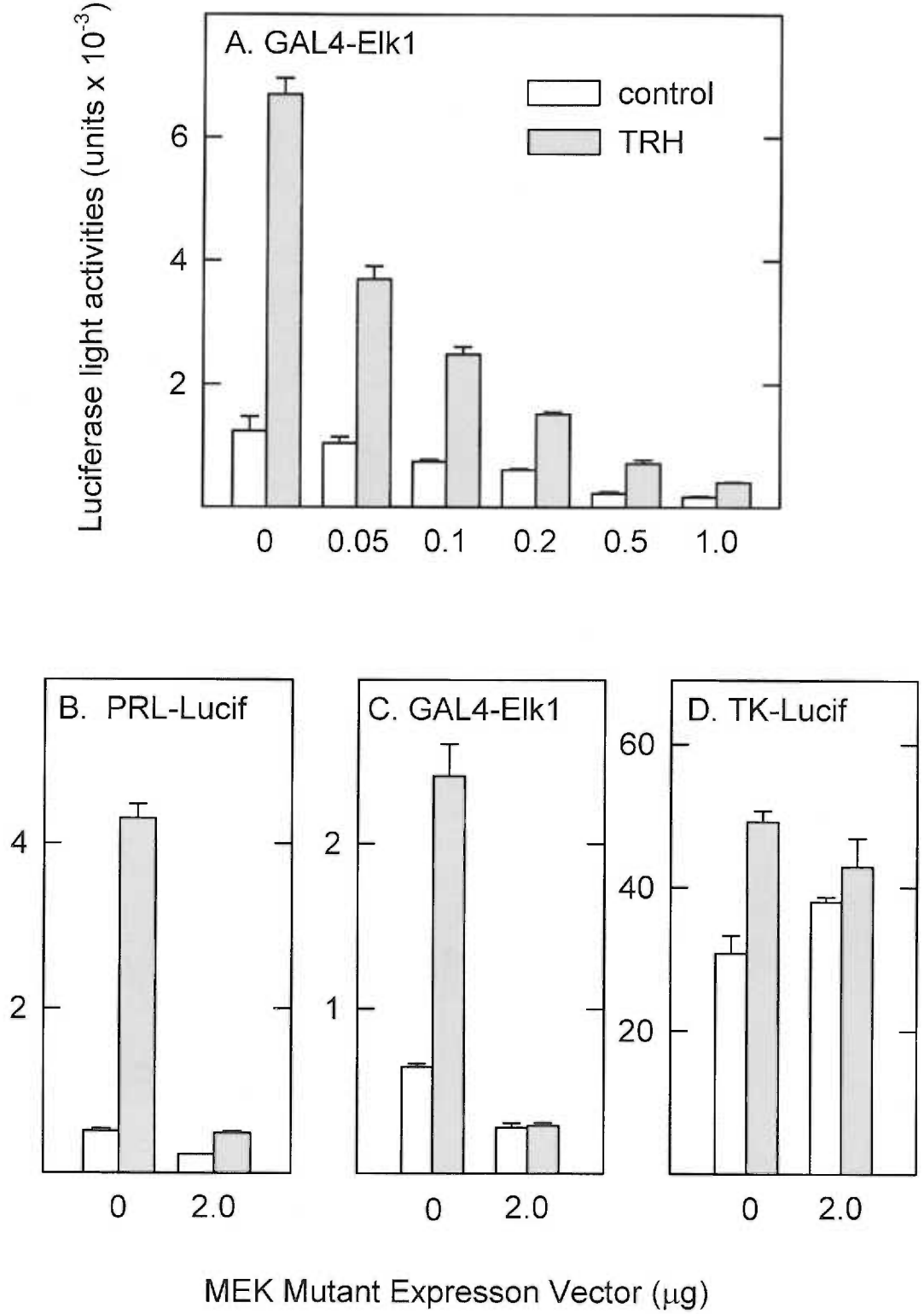

A. GAL4-Elk1
control
TRH
Luciferase light activities (units x 10^{-3})
6
4
2
0
0.05
0.1
0.2
0.5
1.0
B. PRL-Lucif
4
2
0
2.0
C. GAL4-Elk1
2
1
0
2.0
D. TK-Lucif
60
40
20
0
2.0
MEK Mutant Expresson Vector (μg)

Figure 5. The MEK inhibitor PD98059 blocks TRH-induced activation of MAPK.

GH_3 cells were untreated or pretreated with 100 μM PD98059 for 1 h and then treated with 100 nM TRH or no additional treatment for 6 h. The cells were lysed and ERK2 activity was assessed by immunocomplex kinase assay using GST-Elk1 as a substrate.

Pretreat	None		PD98059	
TRH	-	+	-	+

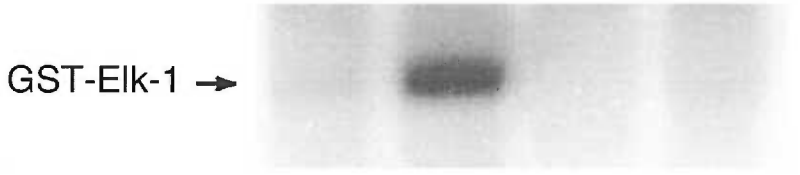


Figure 6. The MEK inhibitor PD98059 reduces TRH-induced activation of the prolactin promoter.

GH_3 cells were transfected with either the 255PRL-luciferase construct (A) or an expression vector for GAL4-Elk1 plus a reporter gene containing 5 GAL4 binding sites upstream of a minimal promoter linked to luciferase (B). At 48 h after transfection the cells were untreated (control) or treated with 100 μM PD98059 as indicated and after incubation for an additional h, half of the cultures were treated with 100 nM TRH. Cells were harvested 6 h after TRH treatment and luciferase activity determined. All values are means ± standard error for three separate transfections that have been corrected for transfection efficiency.

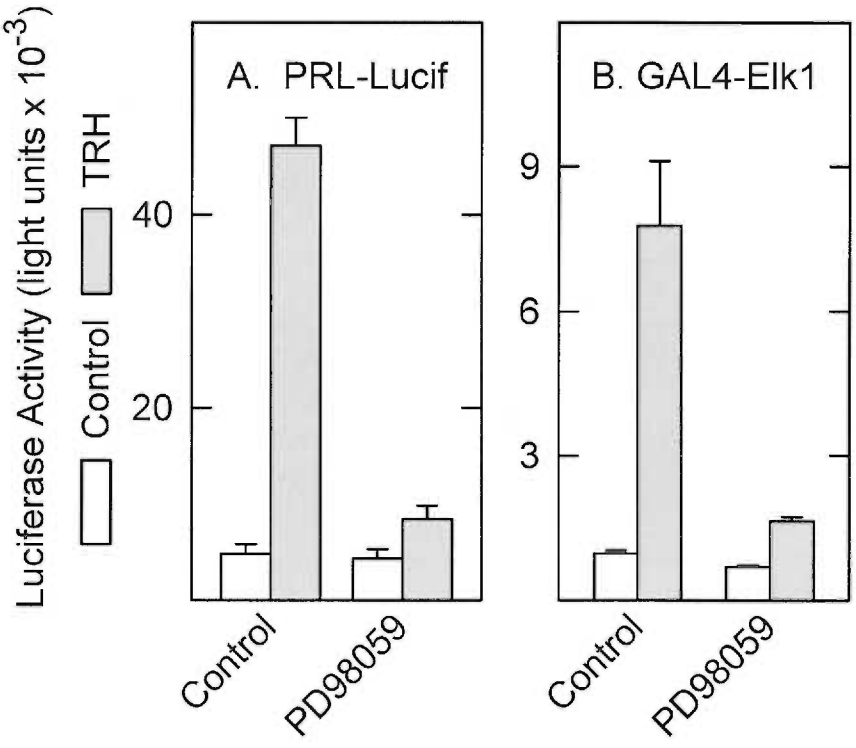

Luciferase Activity (light units x 10^{-3})
Control
TRH
A. PRL-Lucif
B. GAL4-Elk1
40
20
9
6
3
Control
PD98059
Control
PD98059

Figure 7. Co-localization of TRH- and MAPK-responsive DNA elements in the prolactin promoter.

Specific Ets sites (solid boxes) which are adjacent to binding sites for Pit-1 (gray boxes) in prolactin promoter were mutated as indicated (A). Reporter genes containing Ets sites mutations in the context of the 255PRL-luciferase reporter gene (100 ng) were transfected into GH_3 cells and tested for responsiveness to 100 nM TRH treatment for 6 h (B) or transfection of 150 ng of an expression vector encoding a constitutively active form of MEK (C). All values are means ± standard error for three separate transfections which have been corrected for variations in transfection efficiency.

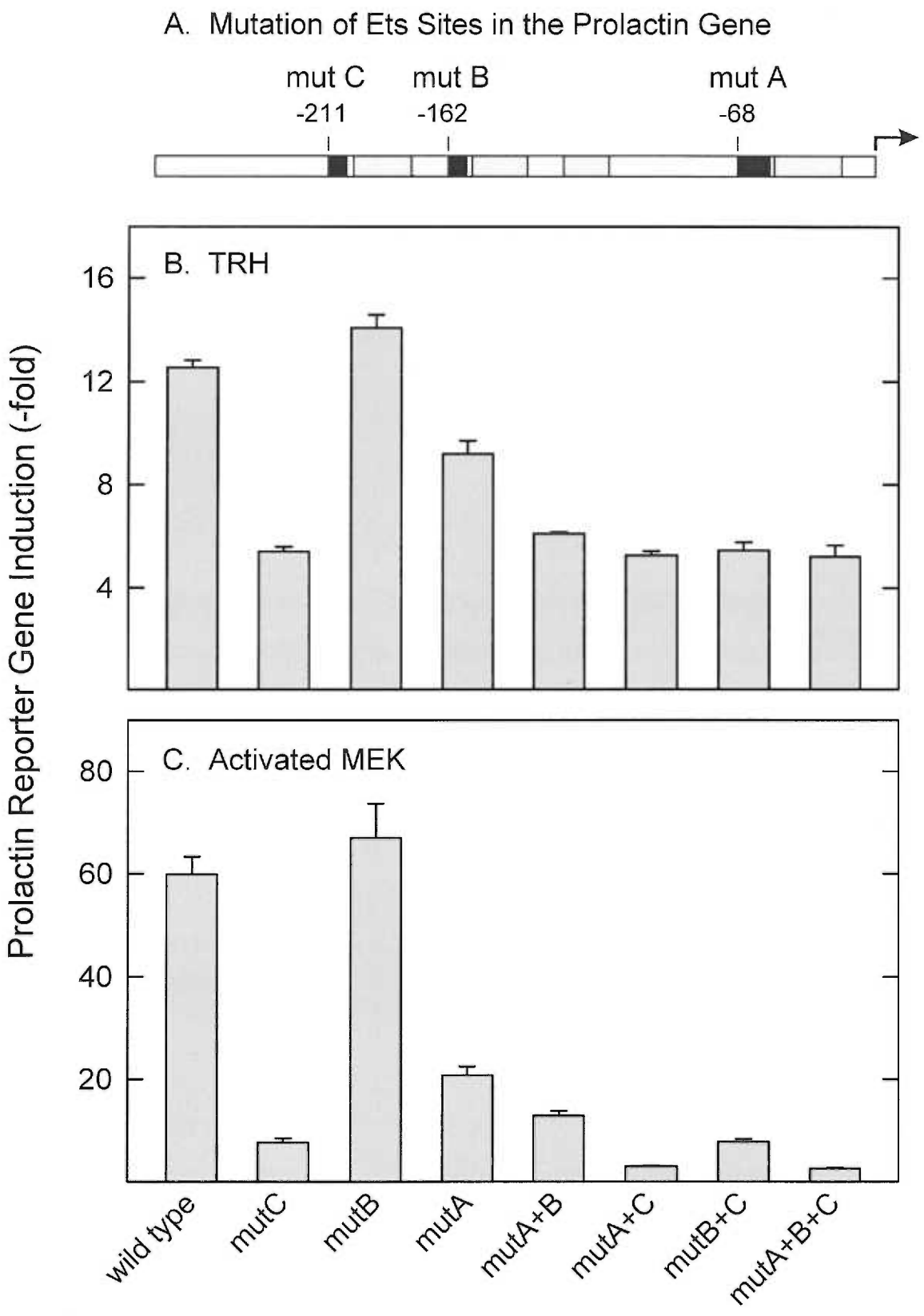

A. Mutation of Ets Sites in the Prolactin Gene
mut C
-211
mut B
-162
mut A
-68
B. TRH
C. Activated MEK
Prolactin Reporter Gene Induction (-fold)
16
12
8
4
80
60
40
20
wild type
mutC
mutB
mutA
mutA+B
mutA+C
mutB+C
mutA+B+C

Figure 8. The PKC inhibitor, GF109203X, reduces TRH-induced activation of MAPK.

GH_3 cells were untreated or treated with 5 μM GF109203X for 20 min, and subsequently treated with 100 nM TRH for 2.5 min, 10 nM EGF for 5 min or 100 nM PMA for 10 min. ERK2 activity was assessed by immunocomplex kinase assay.

Pretreat	None				GF109203X			
Treatment	-	PMA	TRH	EGF	-	PMA	TRH	EGF

Figure 9. Analysis of the time course of TRH-induced MAPK activation after treatment with the Ca^{2+} channel blocker, nimodipine, or the PKC inhibitor, GF109203X.

GH_3 cells were pretreated with 500 nM nimodipine or 1 μM GF109203X for 20 min and then treated with 100 nM TRH for the indicated time and cell lysates were prepared. ERK2 activity was determined by an immunocomplex kinase assay.

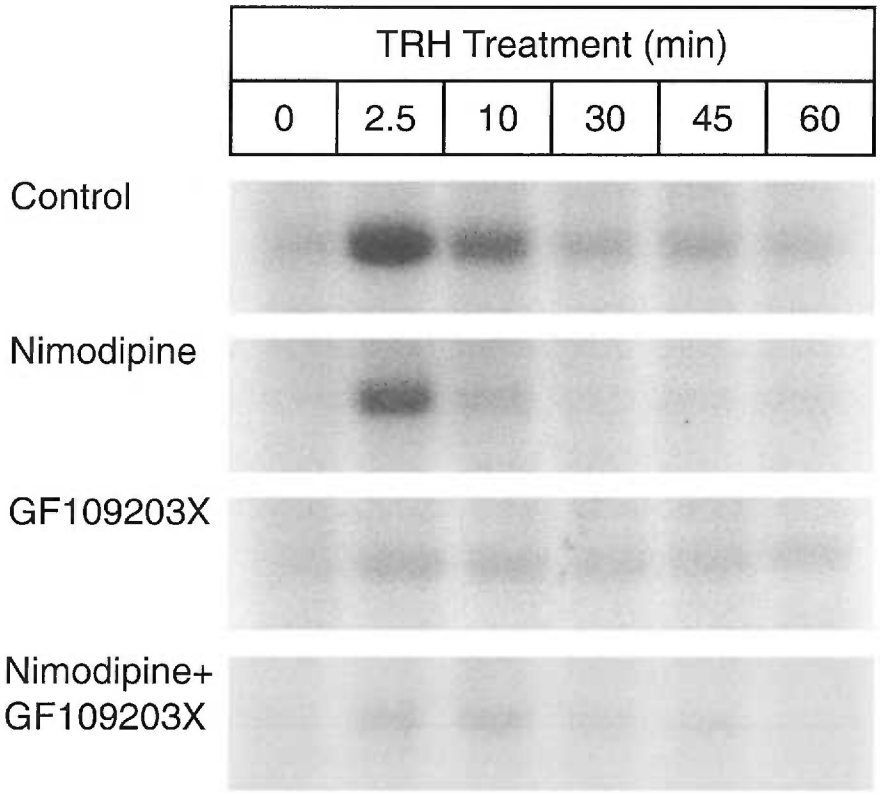

TRH Treatment (min)
0
2.5
10
30
45
60
Control
Nimodipine
GF109203X
Nimodipine+
GF109203X

Figure 10. TRH-induced activation of the prolactin promoter is inhibited by treatment with GF109203X and nimodipine.

GH_3 cells were transfected with the 255PRL-luciferase reporter gene. At 48 h after transfection, the cells were treated with 500 nM nimodipine or 1μM GF109203X for 20 min and then half of the cultures were treated with 100 nM TRH for 6 h. All values are means ± standard error for three separate transfections that have been corrected for transfection efficiency.

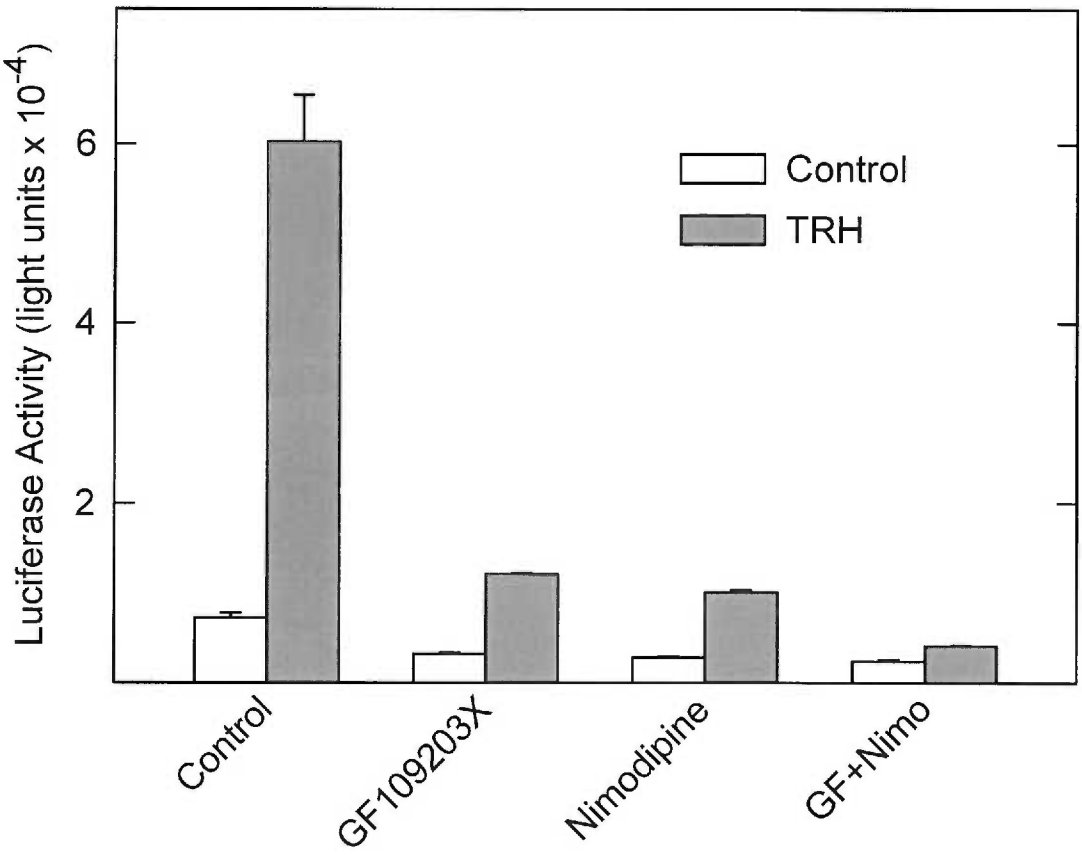
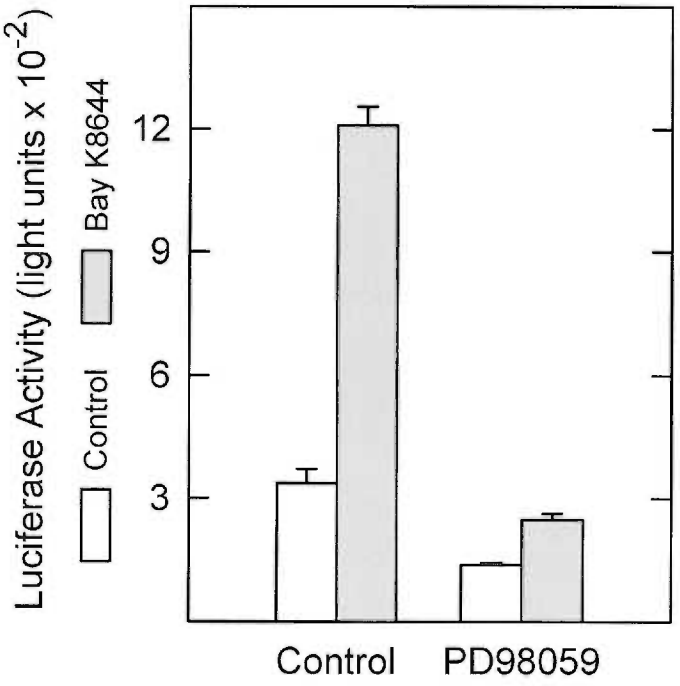

Luciferase Activity (light units x 10^{-4})
6
4
2
Control
TRH
Control
GF109203X
Nimodipine
GF+Nimo

Figure 11. Ca^{2+} influx-induced activation of the prolactin promoter is inhibited by treatment with the MEK inhibitor, PD98059.

GH_3 cells were transfected with the 255PRL-luciferase reporter gene. At 48 h after transfection, the cells were treated with 100 μM PD98059 for 1 h and then half of the cultures were treated with 1 μM Bay K8644 for 6 h. All values are means ± standard error for three separate transfections that have been corrected for transfection efficiency.


Luciferase Activity (light units x 10^{-2})
Control
Bay K8644
12
9
6
3
Control
PD98059

CHAPTER III

A ROLE FOR THE EPIDERMAL GROWTH FACTOR RECEPTOR AND HER2 IN MEDIATING THE EFFECTS OF THYROTROPIN-RELEASING HORMONE ON THE MITOGEN-ACTIVATED PROTEIN KINASE AND GENE TRANSCRIPTION

Introduction

The thyrotropin-releasing hormone (TRH) receptor is a seven-transmembrane, G_q-coupled receptor [14]. Activation of the TRH receptor stimulates the activity of phospholipase Cβ [5], leading to the production of diacylglycerol and inositol-1, 4, 5-trisphosphate [154]. Increased intracellular levels of diacylglycerol and inositol-1, 4, 5-trisphosphate result in activation of protein kinase C (PKC) and mobilization of intracellular calcium [9]. TRH-induced signaling events include activation of the mitogen-activated protein kinase (MAPK) in both PKC-dependent and PKC-independent manner [13, 184]. While the mechanisms which mediate TRH effects on MAPK activation have not been completely determined, it has been observed that TRH induces rapid tyrosine phosphorylation of Shc leading to activation of Ras [13]. This finding suggests that TRH induced activation of MAPK may involve activation of a tyrosine kinase which in turn leads to activation of Ras and the MAPK cascade. Thus the

signaling pathways which allow the G_q-coupled TRH receptor to activate MAPK may overlap with the well-studied pathways through which receptor tyrosine kinases activate Ras and MAPK cascade [100].

Interestingly, there is evidence that TRH and EGF have overlapping activities in GH_3 cells [185, 186]. Both EGF and TRH can stimulate prolactin synthesis and inhibit growth hormone synthesis in GH3 cells [186]. Long-term incubation with either TRH or EGF can induce similar morphological changes in GH3 cells [185, 186]. The similar effect of TRH and EGF in GH3 cells supports the possibility that the two signaling pathways may converge at some point.

In the present study we have further examined the possibility that TRH may activate a tyrosine kinase in GH_3 cells. We report that TRH induces tyrosine phosphorylation of the EGF receptor. Activation of the EGF receptor by TRH is accompanied by tyrosine phosphorylation of the related receptor tyrosine kinase, HER2 as well as the adapter proteins Grb2 and Shc. Blocking TRH-induced EGF receptor activation was found to reduce activation of the MAPK cascade and specific transcripitonal events demonstrating the necessary role for the EGF receptor in mediating downstream effects of TRH.

Materials and Methods

Materials

Cell culture media and supplies were purchased from GIBCO BRL. Radioisotopes and enhanced chemiluminescent reagents (ECL) were purchased from DuPont New England Nuclear. Anti-Erk polyclonal antibodies, anti-

phospho-Erk monoclonal antibody, anti-phosphotyrosine monoclonal antibody (PY99), anti-human EGF receptor polyclonal antibody, anti-HER2 polyclonal antibody, agarose-conjugated anti-Myc antibody, agarose-conjugated GST-Grb2 and horseradish peroxidase (HRP)-coupled secondary antibodies were obtained from Santa Cruz Biotechnology. Anti-Shc polyclonal antibody was purchased from Transduction Laboratory. Anti-rat EGF receptor antibodies were kindly provided by Dr Shelton Earp (University of North Carolina at Chapel Hill) or purchased from Calbiochem Inc.. The PKC inhibitor, GF109203X and Src-like tyrosine kinase inhibitor, PP1, were purchased form Calbiochem. Nimodipine and AG1478 were from Research Biochemicals International; EGF was obtained from Boehringer Mannheim. TRH was purchased from Peninsula Laboratories.

Cell Culture and Transfections

GH_3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% horse serum and 2.5% fetal bovine serum, 100U/ml penicillin, and 100 μg/ml streptomycin. For transient transfection assays, 2.5 or 5 $\times 10^5$ cells per well were planted in 6-well plates one day before the transfection. DNA was introduced into these cells using the lipofectamine (GIBCO BRL) according to a protocol provided by the manufacturer. In each experiment, the total amount of transfected DNA per well was maintained as a constant (usually 2 μg) by addition of empty expression vector (either pCDNA3a or pRK5). After 5 hr of treatment with the lipofectamine/DNA mixture, an equal volume of serum-

containing culture medium was added. After an additional 18 hr, the cells were cultured in serum-free medium for a further 24 hr until lysis.

For reporter gene assays, two different luciferase reporters that can be assayed independently were used in each experiment. To assess transcriptional activation, firefly luciferase constructs [187] were used which contain five Gal4 binding sites upstream of a minimal promoter [158, 159], the proximal 255 bp of the 5' flanking region of the prolactin gene [184] or a minimal herpes simplex virus thymidine kinase promoter [20]. To correct for transfection efficiency, the cells were also transfected with pRL, which expresses *Renilla* luciferase. *Renilla* luciferase requires a different substrate than the firefly luciferase and can be assayed independently. The activities of firefly and *Renilla* luciferase were determined using a protocol and reagents from Promega. Total firefly luciferase light units were normalized to total Renilla luciferase activity.

Immunoprecipitation and immunoblotting

Cells were lysed for 10 min on ice in lysis buffer (150 mM NaCl, 20 mM Tris pH7.5, 1% Triton X-100, 1 mM EDTA, 50 mM β-glycerolphosphate, 10 mM NaF and 10%(v/v) glycerol) with freshly added 1mM Na_3VO_4 and 1x Complete™ proteinase inhibitor (Boehringer Mannheim). The cell lysates were centrifuged for 10 min at 12,000 x g. For immunoprecipitation experiments, 1 mg of cell lysate was immunoprecipitated by incubation with the appropriate antibody overnight at 4°C. Twenty μl of protein A/G agarose beads were then added for

an additional 45 mins. Immune complexes were washed three times with the lysis buffer and resuspended in SDS sample buffer (62.5mM Tris-HCl, pH6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.05% (w/v) bromophenol blue). For immunoblotting, samples were subjected to denaturing-polyacrylamide gel, transferred to PVDF membrane (Millipore) and incubated with the selected antibody. Immunoblots were developed with the ECL system. For reprobing of immunoblots, the membrane was stripped in 62.5 mM Tris pH 6.8, 2% SDS, 100 mM β-mercaptoethanol at 50°C for 30 min.

Immunocomplex kinase assay

Immunoprecipitates were prepared as described above. For the Erk kinase assay, immunoprecipitates were washed once in the Erk kinase assay buffer (20 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.1 % Triton X-100 and 2 mM EGTA). The kinase assays were carried out for 25 min at 30°C in 20 μl of Erk kinase assay buffer supplemented with 1 μCi of [γ-^{32}P] ATP and 5 μg GST fusion carboxy-terminal Elk1 [184]. For the EGF receptor kinase assay, the immunoprecipitates were washed once with EGFR kinase buffer (20 mM Hepes pH 7.3, 10 mM $MnCl_2$, 1 mM DTT, 0.2 mM Na_3VO_4). The kinase assays were carried out for 10 min at 30°C in 20 μl of the EGFR kinase assay buffer in the presence of 5 μCi of [γ-^{32}P] ATP. For measuring the EGF receptor's kinase activity by using an exogenous substrate, 5 μg of myelin basic protein (MBP) for each sample was added to the reaction mixture. The kinase reactions were stopped by adding SDS sample

buffer and the reactions were resolved on an SDS polyacrylamide gel and the phosphorylated proteins were detected by autoradiography and quantitated by Phosphoimager.

Results

TRH-induced MAPK activation requires Ras

As a starting point for further analysis of the signaling pathways mediating TRH effects on MAPK activation, we chose to examine the role for Ras in mediating this response. Previous studies have shown that both TRH and EGF can lead to a two-fold increase in the percentage of Ras which is bound with GTP [13]. While this data strongly supports a possible role for Ras in mediating TRH-induced activation of MAPK, it does not determine if Ras is required for this response. To address this question, GH_3 cells were transiently transfected with an expression vector for a dominant-negative mutant form of Ras (N17Ras) and epitope-tagged Erk2. The cells were treated with TRH or EGF and MAPK activity was determined by an immunocomplex kinase assay. Transfection of the N17Ras mutant substantially reduced both EGF- and TRH-induced MAPK activation (Fig. 1). These findings offer evidence that Ras is required for full TRH-induced MAPK activation.

TRH stimulates tyrosine phosphorylation of the EGF receptor and recruitment of adapter proteins

A number of studies have shown that G protein-coupled receptors can activate Ras and the MAPK cascade through activation of tyrosine kinases [129,133,153,188]. As an initial step to explore possible regulation of tyrosine kinase activity by TRH, we compared the effects of TRH and EGF on phosphotyrosine containing proteins in GH_3 cell lysates. As expected, EGF treatment strongly increased the phosphotyrosine content of several proteins (Fig 2A). The effects of TRH treatment were subtle, but TRH appeared to also stimulate an increase in the phosphotyrosine content of several proteins. In particular, increased phosphotyrosine content was detected for proteins of approximately 185, 170 and 66 Kda. These findings are consistent with an effect of TRH to either activate a tyrosine kinase or inhibit a phosphotyrosine phosphatase.

The TRH-inducible 170-KD band comigrated with an EGF-inducible band. As this is the appropriate size for the EGF receptor, this finding suggests that TRH may stimulate phosphorylation of the EGF receptor. To directly test this possibility, GH3 cells were treated with TRH for varying times and then EGF receptor phosphorylation was evaluated by immunoprecipitation of the receptor followed by immunobotting with an anti-phosphotyrosine antibody (Fig. 2B). TRH treatment was found to increase the phosphotyrosine content of the EGF receptor at the earliest time point examined (2.5 min) and the phosphotyrosine content was elevated for at least an hour. Atlhough EGF was much more active

than TRH in stimulating EGF receptor phosphorylation, TRH stimulated an easily detectable increase in EGF receptor phosphotyrosine content. To determine if the effect of TRH on the EGF receptor were mediated by the TRH receptor, we treated Rat-1 fibroblast cells, which have not been reported to express TRH receptors, with TRH and EGF. As expected, EGF but not TRH was able to stimulate EGF receptor phosphorylation in this cell line (Fig. 2C).

To investigate the nature of the TRH-induced tyrosine phosphorylation of the EGF receptor, we tested whether a potent and specific inhibitor of the EGF receptor, tyrophostin AG1478 [189,190], had an effect on TRH-induced EGF receptor tyrosine phosphorylation. AG1478 treatment substantially reduced TRH-induced tyrosine phosphorylation of the EGF receptor (Fig. 3A). As AG1478 interacts with the ATP-binding site on the EGF receptor and blocks the binding of ATP [189,190], this finding suggests that TRH stimulates EGF receptor autophosphorylation. To further investigate whether TRH-induced tyrosine phosphorylation of the EGF receptor reflects activation of the receptor's intrinsic kinase activity, we performed kinase assays on EGF receptor immunoprecipitates using myelin basic protein as a substrate [191,192]. TRH stimulated EGF receptor kinase activity, and the TRH-induced increase in kinase activity was blocked by AG1478 treatment (Fig. 3B). Similar results were obtained when EGF receptor autophosphorylation was assessed (data not shown).

Recruitment and phosphorylation of adapter proteins are key events for mediating the ability of the EGF receptor to activate the Ras/MAPK pathway

[102, 103, 193]. Treatment of GH3 cells with TRH or EGF resulted in the apparent interaction of the tyrosine-phosphorylated EGF receptor with a glutathione-S-transfeRase (GST) Grb2 fusion protein (Fig. 4A). As observed for TRH-induced phosphorylation of the EGF receptor, TRH was much less effective than EGF treatment in stimulating the interaction of Grb2 and the EGF receptor. We also examined phosphorylation of Shc (Fig. 4B). Following TRH treatment, tyrosine-phosphorylated proteins of 185K, 66K and 52K were immunoprecipitated with antisera to Shc (Fig. 5C). The 52 and 66 KDa protein represent isoforms of Shc, consistent with previous studies demonstrating TRH-induced tyrosine phosphorylation of Shc [13]. The 185 KDa phospho-protein, which was co-immunoprecipitated with Shc (Fig. 4A) and comigrated with HER2 immunoprecipitates (data not shown), likely represents the HER2 receptor tyrosine kinase (see below).

TRH Treatment Leads to Tyrosine Phosphorylation of HER2

In general, EGF receptor signaling is rapidly down-regulated due to receptor-mediated endocytosis [194]. In contrast, TRH-induced activation of the EGF receptor persists for approximately an hour, implying that some event modulates the time course of activation. HER2, also known as ErbB2 or neu, is a member of the ErbB family of receptor tyrosine kinases and is most closely related to the EGF receptor (ErbB1) [195]. HER2 can form heterodimers with the EGF receptor and enhance EGF-induced tyrosine phosphorylation of the EGF

receptor and potentiate and prolong EGF-induced signal transduction [196-200]. To explore the possibility that HER2 may play a role in TRH-induced EGF receptor activation, we examined TRH effects on HER2 phosphorylation. Treatment with TRH resulted in an increase in the phosphotyrosine content of HER2, which persisted for at least an hour (Fig. 5A). To determine whether TRH-induced tyrosine phosphorylation of HER2 was mediated by the EGF receptor, we treated GH3 cells with the specific EGF receptor inhibitor AG1478. At a concentration of 250 nM, AG1478 strongly inhibited TRH-induced tyrosine phosphorylation of HER2 (Fig. 5B). As AG1478 inhibits HER2 only at much higher concentrations (more than 100μM) [190], these data suggest that TRH-induced HER2 activation depends on activation of the EGF receptor. These findings provide evidence that TRH induces HER2 phosphorylation in GH_3 cells and the activation of HER2 may contribute to prolonged phosphorylation of the EGF receptor.

Tyrosine phosphorylation of the EGF receptor by TRH is not mediated by PP1-sensitive tyrosine kinases

Previous studies have shown that activation of Src family nonreceptor tyrosine kinases by G_i-coupled receptors can account for tyrosine phosphorylation of both EGF receptor and Shc [153]. To study the mechanism of TRH-induced tyrosine phosphorylation of the EGF receptor, we pretreated cells with PP1, a specific inhibitor for the Src family tyrosine kinases [201]. Although

PP1 reduced the phosphotyrosine content of several proteins (data not shown), it had little effect on tyrosine phosphorylation of the EGF receptor (Fig. 6). These results suggest that tyrosine phosphorylation of the EGF receptor by TRH is not mediated by PP1-sensitive tyrosine kinases.

Tyrosine phosphorylation of the EGF receptor by TRH is a PKC and Ca^{2+}-dependent response

Activation of phospholipase Cβ by the TRH receptor leads to increased intracellular concentration of diacylglycerol and subsequent activation of PKC. To determine if PKC lies in the pathway between the TRH receptor and the EGF receptor, we pretreated cells with GF109203X, a specific PKC inhibitor [171]. Treatment of GH_3 cells with the PKC inhibitor reduced the subsequent TRH-induced tyrosine phosphorylation of the EGF receptor (Fig. 7A and 7B). Thus, PKC activation appears to be necessary for TRH-induced EGF receptor transactivation.

TRH treatment results in Ca^{2+} release from intracellular stores and Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels [10]. To determine if changes in Ca^{2+} concentration have an effect on TRH action, cells were treated with the Ca^{2+} chelator EGTA, prior to addition of TRH. EGTA treatment reduced tyrosine phosphorylation of the EGF receptor by TRH (Fig. 7A and 7B). Surprisingly, treatment with nimodipine, an L-type Ca^{2+} channel blocker [10], had little effect on TRH-induced activation of the EGF receptor (Fig. 7C) although our

previous studies have shown that nimodipine can reduce TRH-induced MAPK activation [184]. Furthermore, treatment of GH3 cells with TMB-8 [10], an intracellular Ca^{2+} chelator, also had little effect on TRH-induced tyrosine phosphorylation of the EGF receptor (Fig. 7C). These findings suggest that increases in intracellular Ca^{2+} concentrations from either Ca^{2+} influx or Ca^{2+} release from intracellular stores are not required for TRH effects on EGF receptor activation. Nonetheless, TRH-induced EGF receptor transactivation is sensitive to the changes in extracellular Ca^{2+} concentration.

EGF Receptor Activity is Required for TRH-induced MAPK Activation and Specific Gene Transcription

To test the role that EGF receptor phosphorylation plays in mediating TRH effects on MAPK activation, we examined the effects of AG1478 on MAPK activation. As determined by immunoblotting with an anti-phospho-Erk antibody, TRH-induced MAPK activation was reduced by pretreatment with AG1478 (Fig. 8A). To further examine the role that the EGF receptor plays, we used an expression vector for a kinase-defective mutant of the EGF receptor (HERK721A) [202]. The HERK721A expression vector was transfected into GH_3 cells with an expression vector for Myc-tagged Erk2 and Erk2 activity was then determined by an immunocomplex assay (Fig. 8B). TRH-induced Erk2 activation was substantially reduced by the HERK721A expression vector. But Erk2 activation by EGF remained unaltered. This may be caused by a relatively high

concentration of the ligand (10 nM), that overcomes the inhibitory effect by shifting the ligand-receptor and receptor-receptor equilibrium [202, 99]. The HERK721A mutant inhibited EGF-induced Myc-tagged Erk2 activation when we used a lower concentration of the ligand (100 pg/ml) (Fig. 8C). These results provide evidence that EGF receptor activity is required for full TRH effects on the MAPK pathway.

To determine the functional importance of TRH-induced tyrosine phosphorylation of the EGF receptor, we examined the effects of AG1478 on specific transcriptional responses to TRH. Previous studies have shown that activation of the MAPK pathway is essential for TRH effects on the transcriptional activity of a GAL-Elk1 fusion protein as well as full induction of the prolactin promoter [184]. Transfection of an expression vector for the kinase-defective EGF receptor, substantially reduced TRH-induced activation of Gal4-Elk1 activity (Fig. 9A) and more modestly reduced induction of the prolactin promoter (Fig. 9B). As expected, neither TRH nor the expression vector for the mutant EGF receptor affected the activity of thymidine kinase reporter gene (Fig. 9C). These data indicate that the EGF receptor's kinase activity is required for the full induction of transcriptional responses by TRH. The partial inhibition on the prolactin promoter also suggests that the EGF receptor pathway is not entirely responsible for TRH-induced activation of this transcriptional response.

Discussion

These studies provide evidence that TRH-induced phosphorylation and activation of the EGF receptor plays a role in activation of the MAPK cascade leading to specific transcriptional events. An increasing body of work has shown that tyrosine kinases may play a role as downstream components of some G protein-coupled receptor pathways [119, 120, 139, 146]. In the present study, we demonstrate that TRH can stimulate tyrosine phosphorylation of the EGF receptor in a time-dependent manner. The use of specific inhibitors has provided evidence that TRH-induced activation of the EGF receptor is required for several downstream events including phosphorylation of adapter proteins, activation of the MAPK pathway and stimulation of Elk1 transcriptional activation and full activation of prolactin gene transcription.

The ability of TRH to activate the EGF receptor appears to involve autophosphorylation of the receptor. TRH-induced tyrosine phosphorylation of the EGF receptor and downstream activation of MAPKs are inhibited by tyrphostin AG1478, which specifically inhibits the EGF receptor's kinase activity by competing for ATP binding. Similar results were obtained when a kinase-inactive mutant of the EGF receptor was used to block the receptor signaling. AG1478 was also found to reduce TRH-induced tyrosine phosphorylation of HER2 and Shc. These findings provide evidence that TRH induced activation of the EGF receptor and downstream signaling events shares many similarities with EGF-induced activation of its receptor [102, 103, 193]. Consistent with the view that the TRH signaling pathway involves the EGF receptor positioned upstream

of the Ras-dependent MAPK pathway, expression of a dominant-negative Ras mutant reduced TRH-induced MAPK activation.

TRH-induced phosphorylation of the EGF receptor is accompanied by phosphorylation of the related receptor tyrosine kinase, HER2. HER2 is a preferred partner of all members of the ErbB family [203] and HER2 can enhance signaling by the EGF receptor in several ways. HER2 can increase the duration of EGF-induced tyrosine phosphorylation of the EGF receptor by slowing the relatively fast endocytosis rate of the EGF receptor [204]. HER2 is very efficient in coupling to the MAPK pathway [204] and HER2 can potentiate EGF-induced MAPK activation by recruiting different SH2-containing substrates to the heterodimer complexes [199, 205]. Because TRH-induced HER2 tyrosine phosphorylation depends on EGF receptor activity, it is possible that the EGF receptor and HER2 form heterodimers in GH3 cells. The heterodimerization between the EGFR and HER2 may explain why TRH can induce the prolonged activation of the EGF receptor and why the EGF receptor has such profound effect on TRH-induced MAP kinase activation, even though TRH only activates a small portion of EGF receptors, compared with EGF.

The molecular events that lead to TRH-induced phosphorylation of the EGF receptor are unclear, although they appear to involve both PKC and extracellular Ca^{2+} in some way. A requirement for PKC activity has been shown by the ability of the PKC inhibitor GF109203X to attenuate TRH-induced tyrosine phosphorylation of the EGF receptor. However, the manner in which PKC contributes to activation of the EGF receptor is unclear. It has been shown that

PKC can directly phosphorylate the EGF receptor on threonine 654 [206, 207]. However, this does not lead to EGF receptor activation but rather inhibits tyrosine kinase activity of the receptor and reduces high affinity EGF binding [207-209]. It may be that specific PKC isozymes have different effects on the EGF receptor accounting for this apparent discrepancy. It is also possible that PKC may activate the EGF receptor through inactivating a protein tyrosine phosphatase. It has been shown that radiation, oxidants and alkylating agents can alter the phosphorylation of receptor tyrosine kinases through effects on protein phosphatases [210]. Interestingly, TRH-induced EGF receptor phosphorylation requires the presence of extracellular Ca^{2+}. However, neither Ca^{2+} release from intracellular stores nor Ca^{2+} influx is required for the EGF receptor phosphorylation. Studies in several different cell lines have revealed very different effects of PKC and Ca^{2+} on tyrosine phosphorylation of the EGF receptor. Ca^{2+} influx through voltage-sensitive Ca^{2+} channels is sufficient to induce tyrosine phosphorylation of the EGF receptor in PC12 cells [149]. In rat vascular smooth muscle cells, angiotensin II-induced EGF receptor phosphorylation is only affected by intracellular Ca^{2+} mobilization [150]. In 293 cells expressing a transfected m1 acetylcholine receptor, carbachol-induced EGF receptor phosphorylation is solely PKC-dependent, and does not require a Ca^{2+} signal [151]. In contrast, PKC suppresses angiotensin II-induced tyrosine phosphorylation of the EGF receptor in GN4 rat liver epithelial cells [152]. These very different observations suggest that cell-specific mechanisms apparently alter the role that PKC and Ca^{2+} play in regulating EGF receptor activation.

It is striking that although TRH and EGF share many signaling events in common, there appears to be a substantial quantitative difference in the activation of specific signaling steps. For instance, EGF has a much greater effect than TRH on phosphorylation of the EGF receptor, HER2, Shc and Grb2. Nonetheless, TRH-effects on EGF receptor phosphorylation are clearly important as the EGF receptor tyrosine kinase inhibitor AG14178 substantially reduced TRH-induced MAPK activation as well as transcriptional responses. What accounts for this apparent discrepancy between weak signal activation and an important functional role? One aspect may be the prolonged time course of TRH effects on EGF receptor activation as opposed to the rapid down regulation of the EGF receptor, which occurs after EGF treatment. Another aspect may involve an additive effect of multiple TRH-induced signaling events. For instance, TRH treatment results in Ca^{2+} influx through voltage-gated Ca^{2+} channels [211]. Although the present findings suggest that Ca^{2+} influx is not required for EGF receptor phosphorylation, previous studies have provided evidence that Ca^{2+} influx does contribute to MAPK activation in GH_3 cells [184]. Thus, TRH appears to initiate separate signaling pathways involving EGF receptor phosphorylation and Ca^{2+} influx that converge on MAPK activation. It seems likely that additive and possibly synergistic interaction of the EGF receptor/Ras pathway and the Ca^{2+} influx pathway contributes to the full MAPK response.

The present findings provide new insights into the overlapping biological activities of EGF and TRH in GH3 cells. Both EGF and TRH stimulate prolactin

synthesis [185, 186] and prolactin gene transcription [26, 39] in GH_3 cells. TRH and EGF effects on expression of the prolactin gene are mediated via multiple, common DNA elements [212]. Previous studies have shown that both EGF and TRH can induce tyrosine phosphorylation of Shc and activate the MAPK pathway [13]. The present study reveals a new level of signal convergence that occurs at the level of the plasma membrane. As both TRH and EGF activate the EGF receptor and several common downstream signaling steps, it is not surprising to find that TRH and EGF share some biological actions.

Figure 1. TRH-induced ERK activation is Ras-dependent.

GH_3 cells were transiently transfected with an expression vector for Myc-Erk2 (0.5 μg), with or without the RasN17 expression vector (1.5 μg). Cells were left untreated or treated with 100 nM TRH for 2.5 min, 10 nM EGF for 5 min or 100 nM PMA for 10 min. Myc-Erk2 was immunoprecipitated with the agarose-conjugated anti-Myc antibody (9E10) and assayed for Erk2 activity using GST-Elk as a substrate. The expression level of Myc-Erk2 was analyzed by immunoblotting Myc immunoprecipitates with the anti-Erk2 antibody.

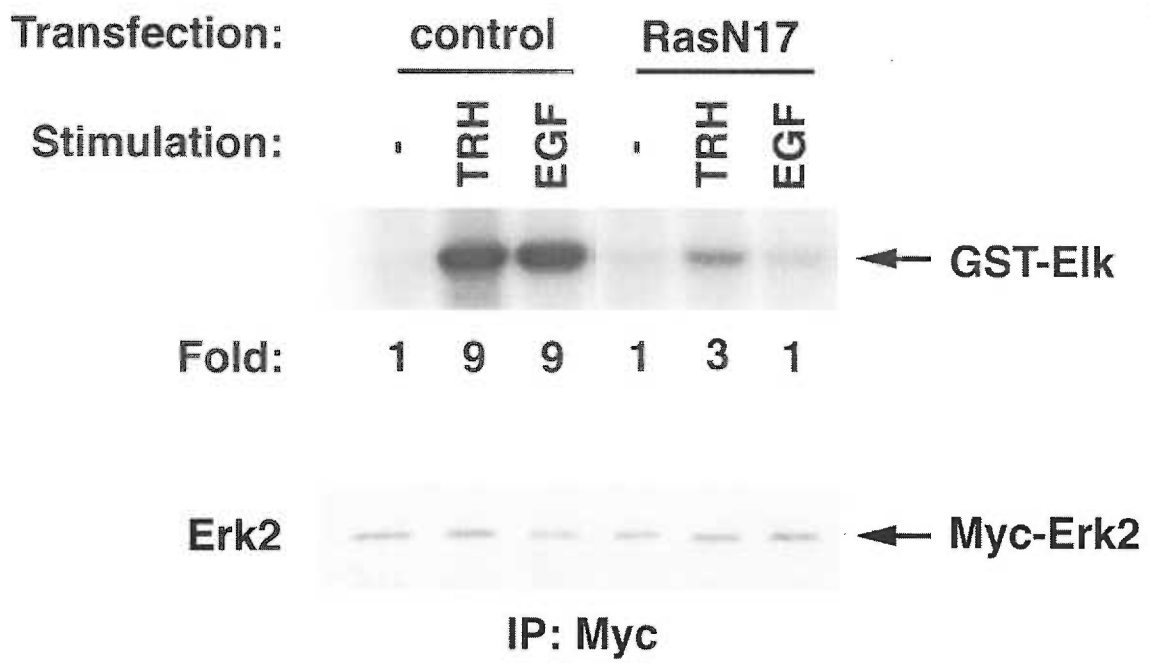

Transfection:
control
RasN17
Stimulation:
-
TRH
EGF
-
TRH
EGF
GST-Elk
Fold:
1
9
9
1
3
1
Erk2
Myc-Erk2
IP: Myc

Figure 2. TRH induces activation of tyrosine kinases in GH3 cells.

A. GH3 cells were serum-starved for 48 hr. Cells were treated with or without 100 nM TRH for 2.5 min and lysed. One hundred μg of protein from each sample were resolved by 8% SDS -PAGE. The membrane was immunoblotted with the anti-P-tyrosine antibody (PY99). B. GH3 cells were treated with TRH at different time points. Cell lysates were immunoprecipitated (IP) with the anti-rat EGF receptor antibody (provided by Dr. Earp), followed by immunoblotting with the anti-P-tyrosine antibody and reprobing with the anti-EGF receptor antibody. C. Rat-1 cells were serum-starved for 24 hr. Cells were treated as above. The EGF receptor immunoprecipitates were blotted with PY99 and then reprobed with the anti-EGF receptor antibody (Calbiochem).

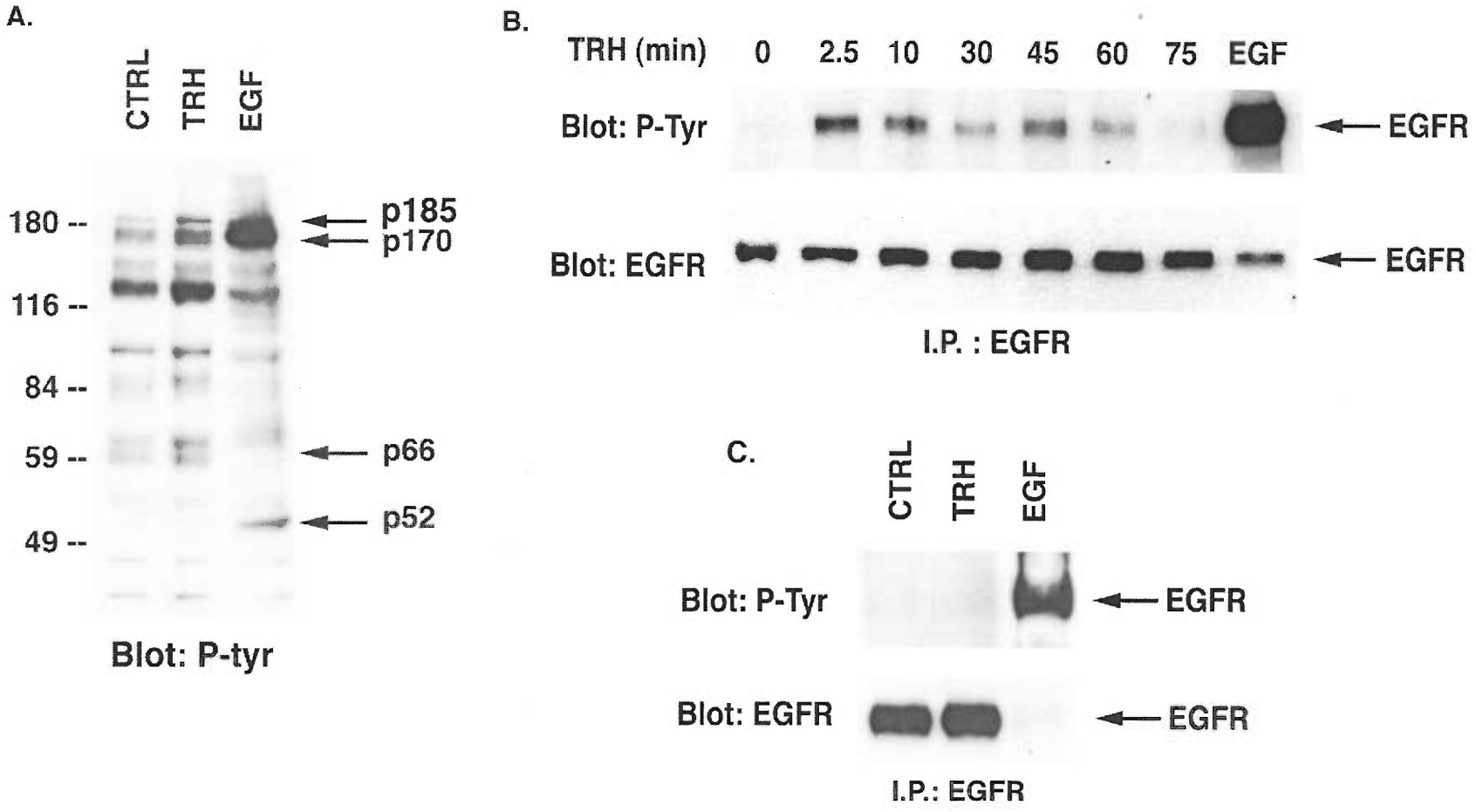
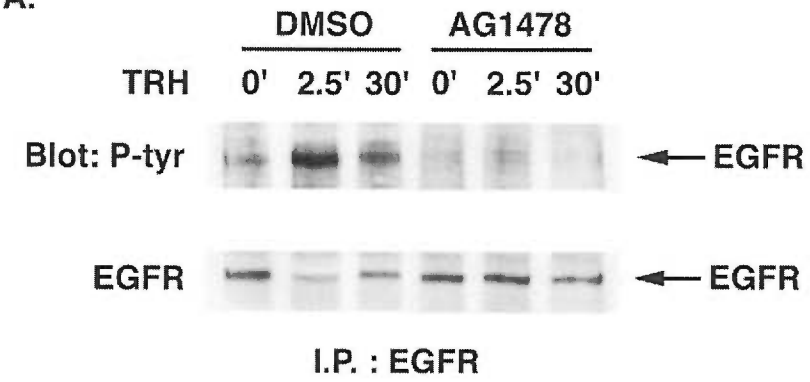

A.
CTRL
TRH
EGF
180 --
116 --
84 --
59 --
49 --
p185
p170
p66
p52
Blot: P-tyr
B.
TRH (min)
0
2.5
10
30
45
60
75
EGF
Blot: P-Tyr
EGFR
Blot: EGFR
EGFR
I.P. : EGFR
C.
CTRL
TRH
EGF
Blot: P-Tyr
EGFR
Blot: EGFR
EGFR
I.P.: EGFR

Figure 3. TRH stimulates the EGF receptor's intrinsic kinase activity.

A. GH3 cells were pretreated with or without 250 nM AG1478 for 20 min, and then challenged with 100 nM TRH. EGF receptor immunoprecipitates were resolved by 8% SDS-PAGE. Tyrosine phosphorylation of the EGF receptor was assayed as described above. B. Analysis of TRH effects on EGF receptor kinase activity. EGF receptor immunoprecipitates from GH3 cell lysates treated with or without TRH were assayed for kinase activity using MBP as a substrate.

A.


DMSO
AG1478
TRH
0'
2.5'
30'
0'
2.5'
30'
Blot: P-tyr
EGFR
EGFR
EGFR
I.P. : EGFR

B.

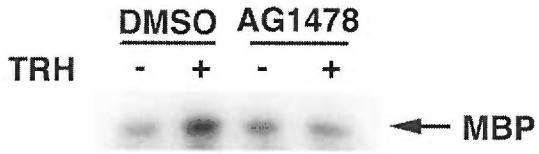
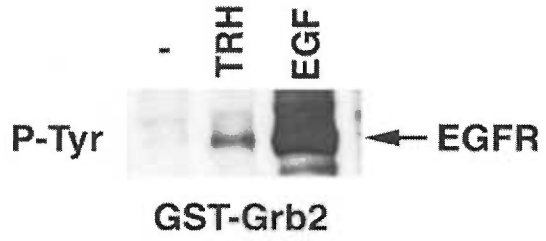

DMSO
AG1478
TRH
-
+
-
+
MBP

Figure. 4 TRH treatment leads to recruitment and phosphorylation of adapter proteins.

A. TRH induce association between the EGF receptor and Grb2. TRH-treated lysates were subjected to *in vitro* association with agarose-conjugated GST-Grb2. The precipitates were separated, transferred and immunoblotted with the anti-P-Tyr antibody. B. TRH-induced tyrosine phosphorylation of Shc is mediated by the EGF receptor. Cells were treated as above. Lysates was immunoprecipitated with 4 μg of anti-Shc antibody, blotted with the anti-P-Tyr antibody and reprobed with the anti-Shc antibody.

A.


-
TRH
EGF
P-Tyr
EGFR
GST-Grb2

B.

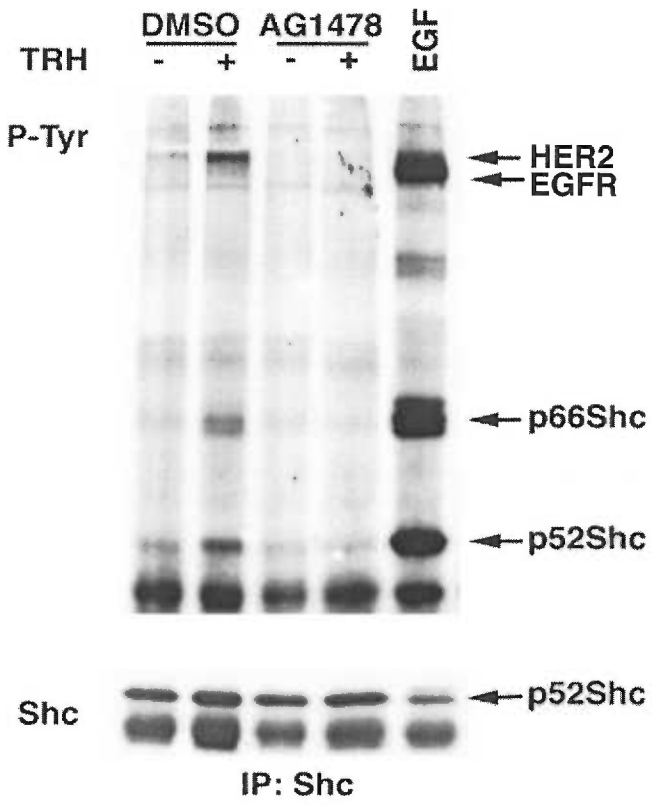
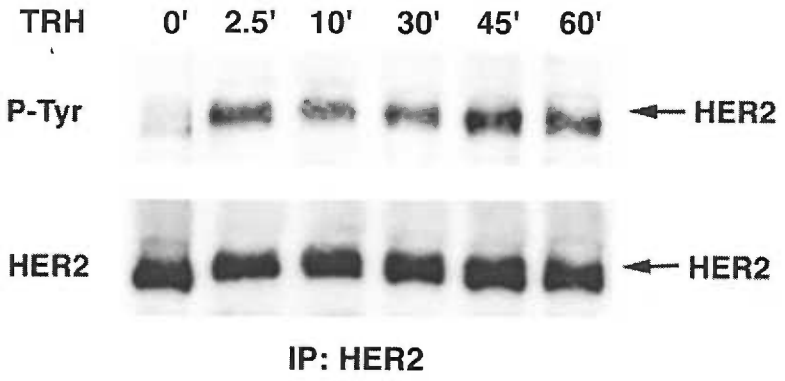

DMSO
AG1478
TRH
- + - +
EGF
P-Tyr
HER2
EGFR
p66Shc
p52Shc
Shc
p52Shc
IP: Shc

Figure 5. TRH-induced tyrosine phosphorylation of HER2 requires EGF receptor activity.

A. GH3 cells were treated with TRH at different time points. Cell lysates were immunoprecipitated (IP) with the anti-HER2 antibody, followed by immunoblotting with the anti-P-tyrosine antibody and reprobing with the anti-HER2 antibody. B. TRH-induced HER2 activation is EGF receptor-dependent. Cells were pretreated with 250 nM AG1478 for 20 min, and then treated with 100 nM TRH or 10 nM EGF. One mg of cell lysates was immunoprecipitated with 4 μg of the anti-HER2 antibody. Immunoprecipitates were resolved by 8% SDS-PAGE, immunoblotting with the anti-P-Tyr antibody and reprobing with the anti-HER2 antibody.

A.


TRH
0'
2.5'
10'
30'
45'
60'
P-Tyr
HER2
HER2
HER2
IP: HER2

B.

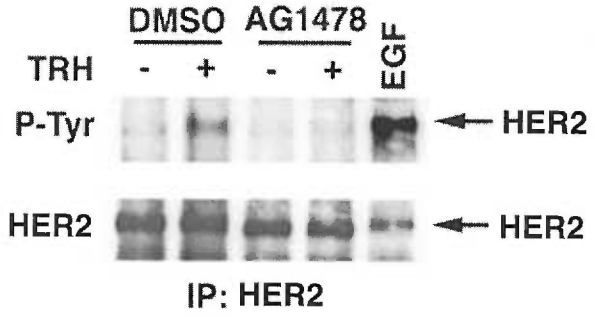

DMSO
AG1478
TRH
-
+
-
+
EGF
P-Tyr
HER2
HER2
HER2
IP: HER2

Figure 6. TRH-induced transactivation of the EGF receptor is not through PP1-sensitive tyrosine kinases.

Serum-starved GH3 cells were pretreated with 5 μM PP1 for 20 min and then left untreated or treated with 100nM TRH for 2.5 or 30 min. Tyrosine phosphorylation of the EGF receptor was analyzed by immunoprecipitating with the anti-EGF receptor antibody (from Dr.Earp), and then immunoblotting with the anti-P-tyrosine antibody. The expression level of the EGF receptor was analyzed by reprobing with the anti-EGF receptor antibody.

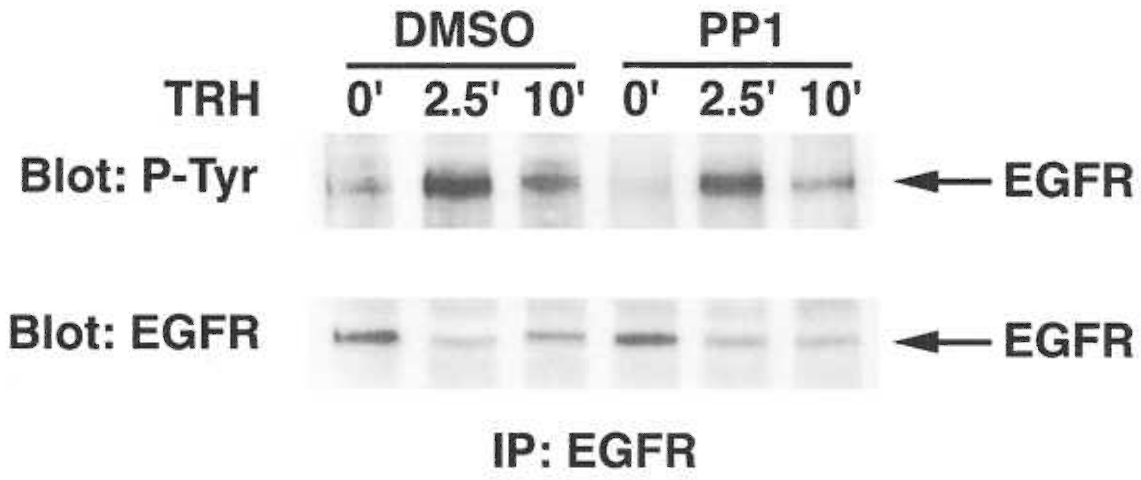
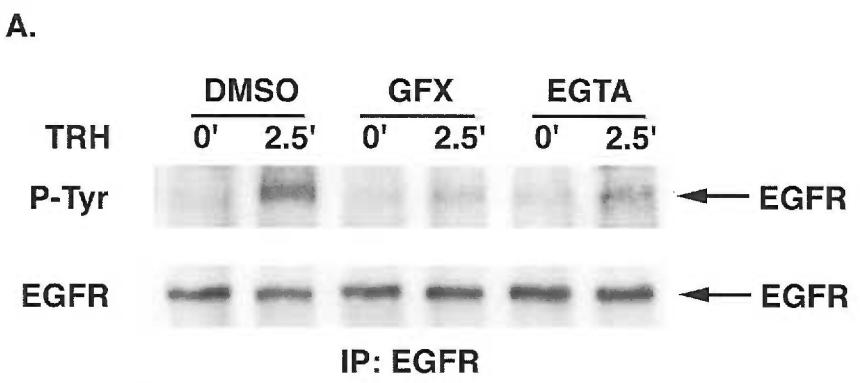
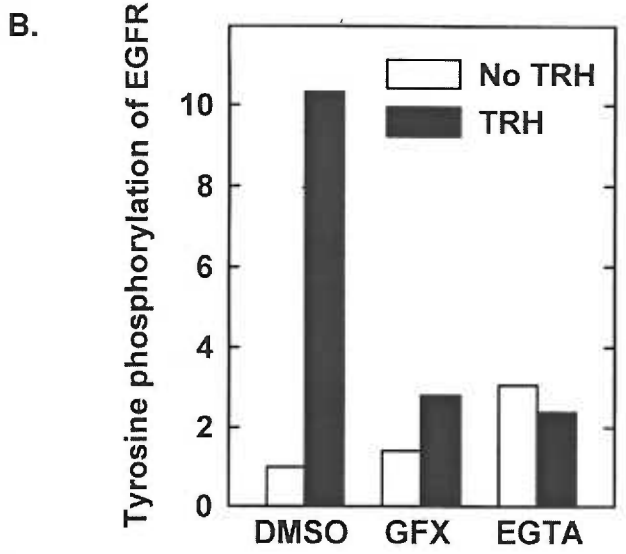
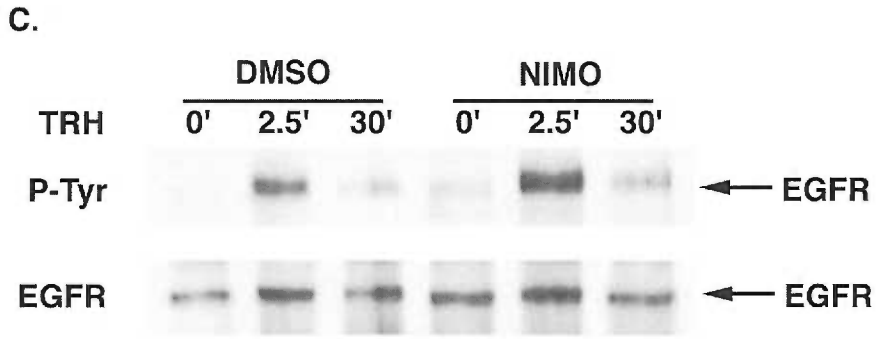

DMSO
PP1
TRH
0'
2.5'
10'
0'
2.5'
10'
Blot: P-Tyr
EGFR
Blot: EGFR
EGFR
IP: EGFR

Figure 7. TRH-induced transactivation of the EGF receptor is through PKC- and Ca^{2+}-dependent pathway.

Serum-starved GH3 cells were pretreated with 1 μM GF109203X (A), 500 nM nimodipine (C) or TMB-8 (D) for 20 min or 2 mM EGTA (A) for 5 min. Cells were stimulated with 100nM TRH for 2.5 min, 30 min. Tyrosine phosphorylation of the EGF receptor was analyzed as described above. (B) quantitative results are the mean of two separate experiments of (A).


A.
DMSO
GFX
EGTA
TRH
0'
2.5'
0'
2.5'
0'
2.5'
P-Tyr
EGFR
EGFR
EGFR
IP: EGFR


B.
Tyrosine phosphorylation of EGFR
No TRH
TRH
0
2
4
6
8
10
DMSO
GFX
EGTA


C.
DMSO
NIMO
TRH
0'
2.5'
30'
0'
2.5'
30'
P-Tyr
EGFR
EGFR
EGFR

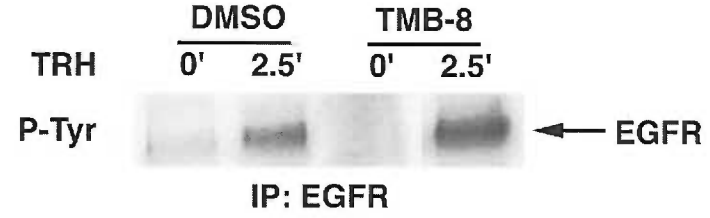
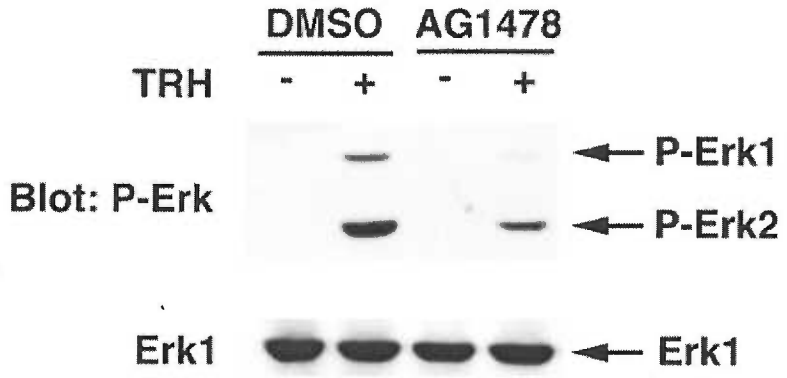

D.
DMSO
TMB-8
TRH
0'
2.5'
0'
2.5'
P-Tyr
EGFR
IP: EGFR

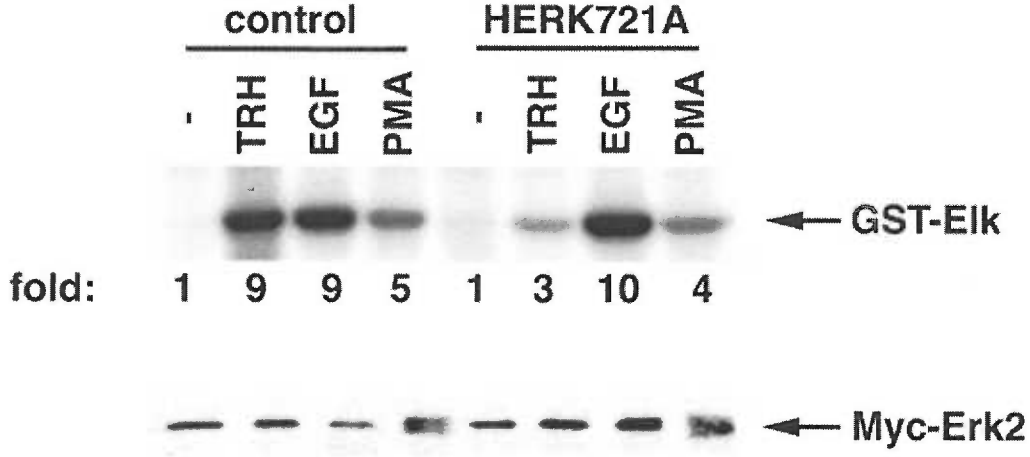
Figure 8. EGF receptor activity is required for TRH-induced activation of MAPK.

A. Inhibition of the EGF receptor reduces TRH-induced Erks activation. Erks activation was analyzed by immunoblotting with the anti-P-Erks antibody. The expression level of Erks was analyzed by reprobing the membrane with anti-Erk1 antibody. B. EGF receptor kinase activity is required for TRH-induced Erk activation. The Myc-Erk2 expresson vector was transfected into GH3 cells with or without the kinase-defective mutant of the EGF receptor (HERK721A). Cells were treated with TRH (100nM) or EGF (10nM). Myc immunoprecipitates were assayed for Erk activation. The expression level of Myc-Erk2 was analyzed by immunoblotting Myc immunoprecipitates with anti-Erk2 antibody. C. the EGF receptor mutant inhibits EGF-induced Myc-Erk2 activation when the ligand concentration is low (100pg/ml).

A.

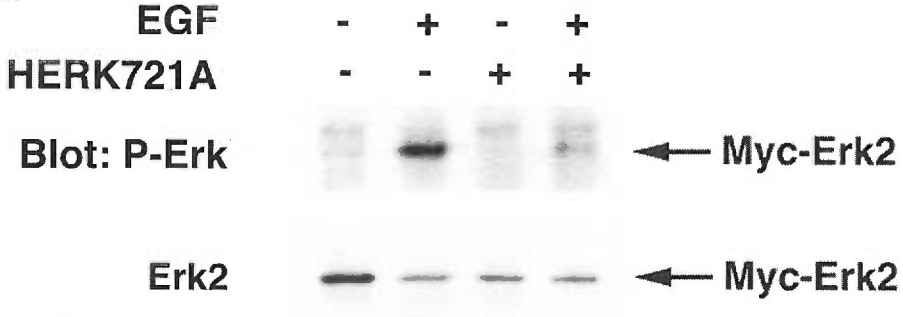


B.



I.P. : anti-Myc antibody

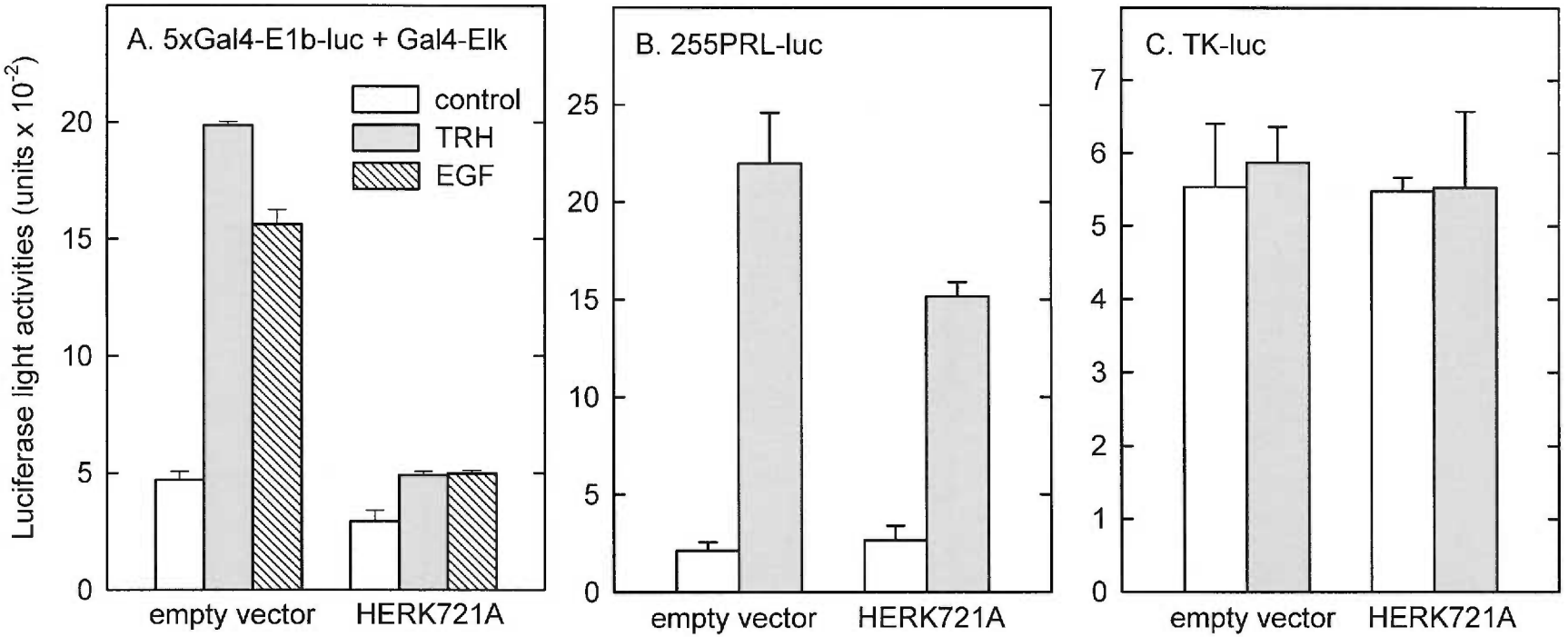
C.



I.P. : anti-Myc antibody

Figure 9. EGF receptor kinase activity is required for mediating transcriptional events induced by TRH.

GH3 cells were transfected with empty vector or expression vector for the kinase-defective mutant of the EGF receptor (HERK721A) and pRL reporter gene, which expresses *Renilla* luciferase. Forty-eight hrs after transfection, the cells were treated with TRH (100nM) or EGF (10 ng/ml) and then collected for luciferase assays 6 hr later. A. Gal4 promoter activity assay. B. Prolactin transcription assay. C. Thymidine kinase (TK) promoter activity assay. Firefly luciferase values are the average ± standard error of three separate transfections, which have been corrected for transfection efficiency.


A. 5xGal4-E1b-luc + Gal4-Elk
control
TRH
EGF
Luciferase light activities (units x 10-2)
0
5
10
15
20
empty vector
HERK721A
B. 255PRL-luc
0
5
10
15
20
25
empty vector
HERK721A
C. TK-luc
0
1
2
3
4
5
6
7
empty vector
HERK721A

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The data presented in this thesis provide insight into different signal transduction pathways activated by TRH (Fig. 1). These studies also provide information concerning how different signaling pathways interact to regulate cell-specific responses. Our data suggest that the MAPK pathway is required for full activation of TRH-induced prolactin transcription. We demonstrate a requirement for PKC in TRH-induced prolactin transcription. In addition, we also identify the MAPK pathway as one of the signaling pathways that permit Ca^{2+} influx to regulate prolactin transcription. Finally, we provide evidence that EGF receptor transactivation plays a role in TRH-induced MAPK activation.

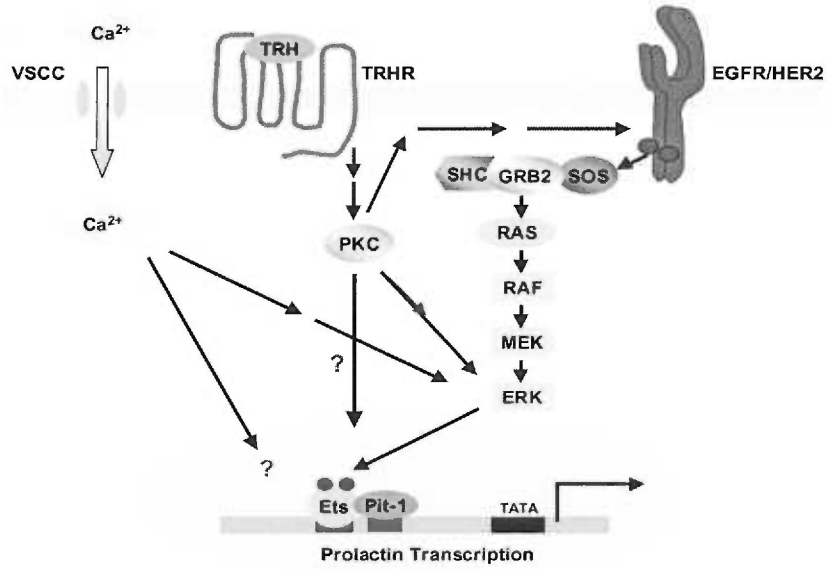


Figure 1. A model for TRH-induced signaling pathways based on the studies in this thesis

While these studies support a role for MAPK in mediating TRH effects, it also seems likely that other pathways may participate in mediating responses to TRH. Disruption of Ets factor binding sites in the prolactin promoter was found to block the transcriptional response to the constitutively active MEK mutant but only partially inhibit the response to TRH. This differential effect of the Ets site mutations suggests that pathways other than the MAPK pathway, such as intracellular Ca^{2+} and the Ca^{2+}/calmodulin-dependent kinase II (CaMKII)-mediated pathways (Fig. 1 and see below), may play a role in mediating TRH effects on the prolactin gene.

CaMKII has been implicated in mediating TRH-induced prolactin transcription [46]. Expression of a constitutively active CaMKII mutant does not activate MAPK in GH3 cells and a CaMKII inhibitor has very little effect on TRH-induced MAPK activation (Y. -H. Wang, unpublished results). Thus, CaMKII probably is not upstream of MAPK. Although the constitutively active CaMKII mutant can activate a reporter gene containing the 2 kb of 5' flanking sequence of the prolactin promoter [46], it cannot activate a construct containing only 0.6 kb of 5' flanking sequence (0.6PRL-Luc). As TRH as well as the constitutively active MEK mutant can activate the 0.6PRL-Luc construct, it appears that CaMKII responsiveness of the prolactin promoter may be complex, involving multiple DNA elements and the CAMKII pathway alone is not sufficient to activate the proximal promoter of the prolactin gene [45, 46]. It is possible that CAMKII

acts in concert with the MAPK pathway to mediate TRH effect on the prolactin promoter.

PKC may also function as a MAPK-independent pathway that plays a role in mediating TRH effects. As demonstrated previously [13] and reported in this thesis, PKC plays a role in TRH-stimulated MAPK activation. The PKC inhibitor, GF109203X, partially blocked TRH-induced MAPK activation. Due to the partial blocking effects of GF109203X, it is not clear if all of the PKC effects are mediated by MAPK. One way to approach this question may be to test for PKC responsiveness of a prolactin reporter gene in which the Ets sites have been disrupted. As disruption of multiple Ets site in the proximal region of the prolactin promoter eliminated most of the ability of MAPK to activate transcription, this construct should provide a sensitive indicator for MAPK-independent responsiveness. Thus, this construct could be tested for responsiveness to PMA treatment or an expression vector for constitutively active PKC.

The findings presented in this thesis have also provided new insights into TRH-induced signaling events that occur at the plasma membrane. TRH treatment stimulates tyrosine phosphorylation of the EGFR and HER2. Importantly, tyrosine phosphorylation of HER2 and activation of downstream events appears to depend on activation of the EGF receptor. Consistent with its role in TRH-induced MAPK activation, TRH-induced EGF receptor phosphorylation has a similar time-course as that of MAPK activation. Both events initiate and reach maximum at similar time points and persist for more than an hour. We did find that MAPK activation appears to attenuate at earlier

time points than that of receptor phosphorylation. As the time course of these events was not compared within the same experiment, it is not possible to make a strong conclusion about this possible difference. Additional quantitative experiments would be needed to determine if this is a consistent, repeatable observation. If, in fact, the two events uncouple from each other at later time points, it may be caused by a feedback mechanism. Previous studies have shown that activation of the MAPK pathway has a feedback effect on receptor tyrosine kinase-mediated Ras activation [218]. MEK-dependent phosphorylation of Sos causes dissociation of the Sos-Grb2 complex, leading to reduction of the GTP-bound form of Ras [218]. This feedback mechanism may account for the uncoupling of MAPK activation from receptor tyrosine phosphorylation. Since activated RTK will be inactivated by tyrosine phosphatases, and this process is determined by the delicate balance between the RTK activity and tyrosine phosphatase activity, this may explain the slower down-regulation of the EGF receptor phosphorylation. Nonetheless, our studies suggest that, besides PKC and Ca^{2+}, the TRH receptor can also act through RTK.

The molecular mechanisms that underlie the EGF receptor transactivation by TRH are unclear. Receptor dimerization is generally considered to be the primary signaling event for ligand-induced receptor activation. Little, however, is known about the precise molecular details of this process. An exception is in the case of human growth hormone (hGH) binding to its receptor. hGH is a monomer that is able to bind to two receptor molecules simultaneously [231]. Detailed structural and functional studies showed that the hGH molecule uses two

different sites to bind to receptor molecules sequentially. Whereas the first binding reaction is primarily diffusion-controlled, the second is enhanced by the formation of direct receptor-receptor contact areas [232, 233]. Growth factors such as PDGF are a covalently linked dimer; each protomer binds to a single receptor molecule, leading to crosslinking of the two receptors [234]. The two known forms of PDGF (A and B types) are able to form AA, BB homodimers and AB heterodimers, which bind specifically to various dimeric forms of α and β PDGF receptor isoforms [235, 236]. The insulin receptor, on the other hand,

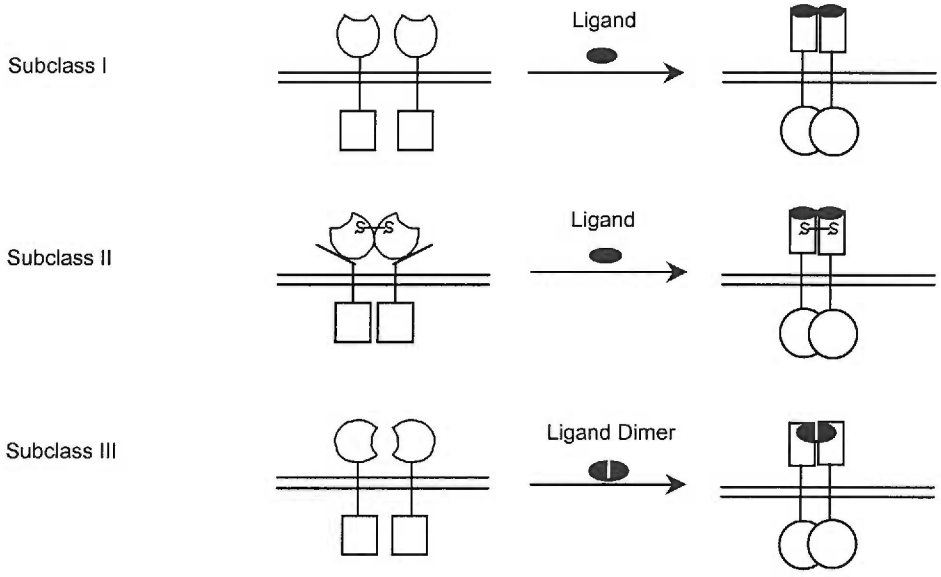


Figure 2. Models of receptor subclass-specific variations of the mechanism of activation by dimerization [100].

Receptor activation may occur by binding of monomeric ligands resulting in a conformational change of the extracellular domain and dimer formation (subclass I), by interaction of the ligand with a disulfide-stablized receptor dimer and subsequent intracomplex conformation change (subclass II), or by mediation of dimer formation through a dimeric ligand (subclass III) [100].

is present on the cell surface as a disulfide-bridged homodimer of α and β insulin receptor subunits [100]. Insulin appears to activate its surface receptor by inducing an allosteric transition within a preexisting dimeric structure [100]. Although growth factor-induced receptor dimerization was first demonstrated for the EGF receptor, it is still unclear how monomeric EGF induces receptor dimerization. One model suggests that the ligand induces a conformational change and this exposes a cryptic receptor dimerization site, leading to dimerization [237]. The second model assumes that EGF-like ligands are bivalent, therefore their mechanism of action may be similar to that of the growth hormone [238]. Recent studies provide evidence for the second model. Biophysical studies suggest that EGF receptor dimerization requires the participation of two molecules of monomeric EGF (in a 2:2 dimer), and involves the dimerization of a stable intermediate 1:1 EGF-EGFR complex [239]. Analysis of HER2-HER3 heterodimers using biophysical, biochemical and immunological approaches indicates that an NDF-like ligand binds with a high affinity site to the HER3 receptor and recruits the HER2 receptor molecule into the complex with a second low affinity binding site [240]. These studies provide a context for understanding how the different EGF-like ligands act to induce heterodimerization of the ErbB family of receptor tyrosine kinases.

Receptor dimerization is essential for stimulation of the intrinsic catalytic activity and for autophosphorylation of growth factor receptors. The mechanism by which receptor tyrosine kinase dimerization stimulates catalytic activity is not yet fully understood. Activation of receptor tyrosine kinases involves

phosphorylation of one or more tyrosines within the activation loop in the catalytic domain. One possible mechanism is that a cytoplasmic dimer stabilizes a more active conformation of the kinase's active site, which enables transphosphorylation of the activation loop [219]. An alternative mechanism is that receptor dimerization increases the local concentration of the kinase domain leading to more efficient transphosphorylation of tyrosine residues within the activation loop of two neighboring kinase domains. The kinase domains of the receptor pair may associate only transiently, the two kinase domains acting as substrate and enzyme for one another [220]. This model explains why insulin receptors stay active after removing insulin from the medium [221].

Although the mechanisms by which TRH activates the EGF receptor are still unclear, our studies may provide some clues. TRH receptor activation can lead to activation of PKC and an increase in intracellular Ca^{2+} concentration. Application of the PKC inhibitor GF109203X and the extracellular Ca^{2+} chelator EGTA substantially attenuates tyrosine phosphorylation of the EGF receptor. However, neither Ca^{2+} release from intracellular stores nor Ca^{2+} influx is required for the EGF receptor phosphorylation, suggesting that EGTA doesn't affect the receptor phosphorylation through inhibition of Ca^{2+} influx but through changes in available extracellular Ca^{2+} concentration. The effects of PKC and Ca^{2+} on tyrosine phosphorylation of the EGF receptor have been studied in several cell lines. Ca^{2+} influx induced by membrane depolarization [148] or by bradykinin [149] is sufficient to induce tyrosine phosphorylation of the EGF receptor in PC12 cells. In rat vascular smooth muscle cells, however, angiotensin II-induced EGF

receptor transactivation is only affected by intracellular Ca^{2+} mobilization [150]. In 293 cells stably transfected with the m1 mAChR, carbachol-induced EGF receptor transactivation is solely PKC-dependent, and does not require a Ca^{2+} signal [151]. In contrast, PKC suppresses angiotensin II-induced tyrosine phosphorylation of the EGF receptor in GN4 rat liver epithelial cells [152]. These observations suggest that cell-specific mechanisms modify PKC and Ca^{2+} signaling to activate the EGF receptor.

TRH-induced tyrosine phosphorylation of the EGF receptor requires PKC activation and is sensitive to changes in the extracellular Ca^{2+} concentration. These findings suggest that the mechanisms by which TRH activates the EGF receptor, could involve a transmembrane protein that can coordinate signals from both sides of the membrane. One of the candidates could be integrins, which belong to a family of cell surface receptors that mediate cell attachment to the extracellular matrix [222]. Various intracellular molecules such as PKC, which can modify the intracellular domains of integrins, induce a conformational change that propagates to the ligand-binding site and results in increased affinity for the ligand. This process is called 'inside-out' signaling [223], which might possibly explain the requirement for PKC activation in TRH-induced EGF receptor phosphorylation. Binding of ligands to the extracellular domain of an integrin results in a conformational change that might be propagated across the plasma membrane to expose key sites within integrin cytoplasmic domains, leading to downstream events. This process is called 'outside-in signaling' [224]. Divalent cations are essential for ligand binding and integrin activation [225], which might

explain the sensitivity of the EGF receptor phosphorylation to changes in extracellular Ca^{2+} concentration.

Some G protein-coupled receptors, including bombesin, vasopressin, endothelin and bradykinin receptors, induce a rapid increase in tyrosine phosphorylation and activation of focal adhesion kinase (FAK), a tyrosine kinase that mediates signaling from integrins, in Swiss 3T3 cells [226]. Peptides containing the motif Arg-Gly-Asp, which block integrin dimerization by mimicking integrin ligands, inhibit carbachol-induced tyrosine phosphorylation of FAK in human embryonic kidney cells [227]. These observations suggest that some G protein-coupled receptors can induce integrin activation. TRH can also induce FAK tyrosine phosphorylation in GH_3 cells (Y. -H. Wang unpublished data). This raises the possibility that addition of TRH in GH_3 cells may induce integrin activation.

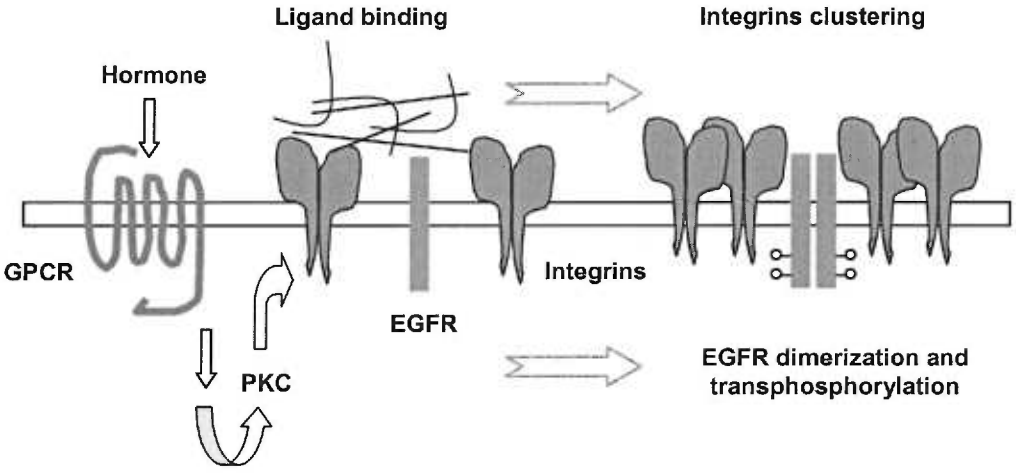


Figure 3. A schematic diagram shows that intracellular signals generated by GPCR induce integrin clustering and EGF receptor activation

Several studies have shown that integrin stimulation induces ligand-independent tyrosine phosphorylation of the PDGF [228] or EGF receptor [229]. Culturing of human primary skin fibroblasts AG1518 [228] and N6 [229] and human endothelial cells ECV304 [229] on matrix proteins in serum-free medium stimulates tyrosine phosphorylation of the PDGF receptors [228] or EGF receptor [229] in the apparent absence of ligand. The subsequent tyrosine phosphorylation of Shc and activation of MAPK appears to be required for anchorage-dependent cell survival [229]. The molecular mechanisms underlying these events remain to be defined. Integrin activation is accompanied by integrin clustering (Fig. 3), which may change the spatial distribution of EGF receptors and increase local concentration of the kinase domains of the EGF receptors, leading to transphosphorylation of the tyrosine residues in the activation loop and receptor activation [220]. Moreover, integrin clustering might also lead to dimerization of receptor tyrosine phosphatases. In contrast with RTKs, dimerization between protein tyrosine phosphatases inhibits their activity [230]. Therefore, integrin association might also protect the receptors from dephosphorylation by protein tyrosine phosphatases, maintaining high receptor activity.

EGF receptor activation is required for full activation of TRH-induced transcriptional responses. Expression of the kinase-defective EGF receptor mutant has a similar effect as that of the dominant-negative MEK mutant or MEK inhibitor treatment on Gal4-Elk activation, suggesting that the EGF receptor

pathway mediates the majority of TRH-induced MAPK activation. However, expression of these two mutants affects prolactin transcription quite differently. Expression of the dominant-negative MEK mutant substantially reduces activation of the prolactin promoter by TRH, whereas expression of the EGF receptor mutant reduces prolactin promoter activation by 30 to 40 percent. One should be cautious about interpreting these apparent differences, as the results were not obtained in the same experiment. If these apparent differences in fact represent genuine differences it may reflect the fact that Ets-1 but not Elk-1 has been implicated in prolactin transcription [37]. Even though both transcriptional factors are regulated by MAPK, there are significant differences in their structure [84-88]. Thus analysis of Gal4-Elk1 activity may not accurately reflect Ets activation and activation of the prolactin promoter. Alternatively, the EGF receptor pathway is likely not the only pathway that transmits TRH signaling to the prolactin promoter. In previous chapters, we have shown that Ca^{2+} influx affects TRH-induced MAPK activation even though it doesn't affect TRH-induced EGF receptor phosphorylation. These results suggest that an EGF receptor-independent pathway exist. Therefore, when EGF receptor activation is blocked by expression of the kinase-inactive EGF receptor mutant, MAPK can still be activated by TRH. The different results from expression of the two kinase-defective mutants might result from synergistic interactions between the MAPK-independent pathway and the residual EGF receptor-independent MAPK-dependent pathway on the prolactin promoter as we discussed earlier in this chapter.

The data presented in this thesis have shown that MAPK plays a crucial role in TRH-induced prolactin transcription. TRH induces MAPK activation not only through PKC and extracellular Ca^{2+} but also through the receptor tyrosine kinase pathway. These studies suggest that individual signals, such as TRH, are not transduced in a linear pathway (Fig. 1). Instead, TRH can activate several intracellular pathways, and signal molecules such as the EGF receptor, PKC, MAPK and Ets family transcriptional factors link these pathways together to form a large and interdependent signaling network. These signal molecules actually act as molecular switches, which help cells to coordinate signals from their environment.

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