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The molecular mechanism of EGF receptor activation in pancreatic β-cells by thyrotropin-releasing hormone

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Luo, LuGuang, Naohiro Yano, and John Z. Q. Luo. The molecular mechanism of EGF receptor activation in pancreatic β-cells by thyrotropin-releasing hormone. *Am J Physiol Endocrinol Metab* 290: E889–E899, 2006; doi:10.1152/ajpendo.00466.2005.—Thyrotropin-releasing hormone (TRH) and its receptor subtype TRH receptor-1 (TRHR1) are found in pancreatic β-cells, and it has been shown that TRH might have potential for autocrine/paracrine regulation through the TRHR1 receptor. In this paper, TRHR1 is studied to find whether it can initiate multiple signal transduction pathways to activate the epidermal growth factor (EGF) receptor in pancreatic β-cells. By initiating TRHR1 G protein-coupled receptor (GPCR) and dissociated αβγ-complex, TRH (200 nM) activates tyrosine residues at Tyr^{845} (a known target for Src) and Tyr^{1068} in the EGF receptor complex of an immortalized mouse β-cell line, βTC-6. Through manipulating the activation of Src, PKC, and heparin-binding EGF-like growth factor (HB-EGF), with corresponding individual inhibitors and activators, multiple signal transduction pathways linking TRH to EGF receptors in βTC-6 cell line have been revealed. The pathways include the activation of Src kinase and the release of HB-EGF as a consequence of matrix metalloproteinase (MMP)-3 activation. Alternatively, TRH inhibited PKC activity by reducing the EGF receptor serine/threonine phosphorylation, thereby enhancing tyrosine phosphorylation. TRH receptor activation of Src may have a central role in mediating the effects of TRH on the EGF receptor. The activation of the EGF receptor by TRH in multiple circumstances may have important implications for pancreatic β-cell biology.

heparin-binding epidermal growth factor-like growth factor; Src; protein kinase C; G protein; signal transduction

TYPE 1 AND TYPE 2 DIABETES both result from an inadequate amount of functioning β-cells, even though type 2 diabetes is characterized by insulin resistance (23). Unveiling the mechanism of growth regulation in β-cells has been an issue of great interest in both the clinical and experimental arenas. Pancreatic β-cells have been characterized as part of the neuronal diathesis, possessing various neuronal derivative peptides and hormones that relate them closely with other neuronal derivatives, sharing a developmental process similar to the cells in the nervous system (19). Thyrotropin-releasing hormone (TRH) (1), first characterized and extensively studied as a hypothalamic factor with a key role in the thyroid axis, has been shown to be synthesized in the pancreas (5, 22) but not strictly limited to the central nervous system (12, 14, 38). Although TRH has been shown to be a neurotrophic factor in protecting neuronal function, aside from regulating thyroid hormone function, the primary role of TRH in pancreas is unclear (29). TRH receptor-1 (TRHR1) was found in the mouse pancreas and HIT-T15 cells (a hamster-immortalized β-cell line). TRHR1 activated by physiological levels of TRH modulates intracellular calcium (40). The TRH knockout mouse model (39) developed hypothyroidism, which could be reversed by thyroid hormone treatment. These animals also developed hyperglycemia; the diabetic state could not be prevented or reversed by treatment with thyroid hormone and was thus attributed to the loss of TRH's effects on β-cells rather than the indirect effects of thyroid deficiency. The impaired insulin secretion in response to glucose from these β-cells could be the cause of hyperglycemia. The lack of morphological changes reported in the pancreas of these mice suggests pancreatic β-cell dysfunction.

TRH has been reported to stimulate TRHR1 and dissociate the G protein-coupled receptor (GPCR) complex, activating protein kinase C (PKC) (6) and mitogen-activated protein kinase (MAPK) (6) in both a PKC-dependent and a PKC-independent manner in neuronal cell lines (34). These effects may involve activation of tyrosine kinase, which leads to the activation of Ras and MAPK cascade. The signaling pathways initiating from G protein-coupled TRH receptor in activating MAPK may overlap with the receptor tyrosine kinases activating the Ras-MAPK cascade (25, 26).

There is evidence that TRH and EGF have overlapping activities (2) that lead to the stimulation of tyrosine phosphorylation of EGF receptors in GH_3 cells, a pituitary cell line (37). TRH-induced EGF receptor phosphorylation led to the recruitment of adapter proteins Grb2 and Shc in GH_3 cells. TRH activates EGF receptors in pancreatic β-cells, which may play a role in β-cell development because EGF receptor expression was found to have a high activation level during the embryonic developmental period in the pancreas (5, 9).

The hypothesis of whether TRH activates EGF receptors in β-cells through multiple pathways is evaluated in this study. Our findings indicate that TRH transactivates EGF receptors through several intra- and extracellular pathways, which are distinct from pituitary-derived cell lines. These diversities might imply the significance and dependability of the biological role of TRH in pancreatic β-cells under varying physiological situations.

EXPERIMENTAL PROCEDURES

Reagents. The mouse βTC-6 and hamster HIT-T15 pancreatic β-cell lines were purchased from American Type Culture Collection (Manassas, VA). Cell culture medium and all supplements were from GIBCO Life Technologies (Gaithersburg, MD). Anti-EGF receptor

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phosphospecific antibodies (pY1068 and pY845) and anti-Src pan- and phosphospecific antibodies were purchased from BioSource International (Camarillo, CA). Anti-EGF receptor antibody sc1005, anti-actin antibody, anti-phosphotyrosine antibody (pY20), anti-phosphothreonine antibody, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG second antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The kit for G protein β-subunit assay and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) were from Calbiochem-Novabiochem (San Diego, CA). Anti-HB-EGF polyclonal antibody was from Oncogene Research Products (7), anti-matrix metalloproteinase (MMP)-3 antibody from Chemicon International (Temecula, CA), and anti-phospho-PKC (1) antibody from Cell Signaling Technology (Beverly, MA). Bisindolylmaleimide (Bis) and 4-α-phorbol 12-myristate 13 acetate (PMA) (7) were from Sigma (St. Louis, MO).

Cell Culture. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum for βTC-6 at 37°C in a humidified 5% CO_2 atmosphere. Cell monolayer (passages 10–35) at 80–90% confluence in cell culture dishes was used for the experiments. The medium was changed to serum-free medium ≤30 h before hormone stimulation (no 15% FBS added).

Western blotting. As reported previously (20), briefly, after hormone treatment with or without various inhibitors, cells were washed once with ice-cold Hanks' balanced salt solution and lysed in lysis buffer containing phosphatase inhibitors (50 mM Tris·HCl, pH 7.5, 0.2% Sigma Phosphatase Inhibitor Cocktail II, 0.1% Nonidet P-40). Cell lysates were shaken on ice for 2 h and centrifuged at 12,000 rpm for 30 min at 4°C. Protein content in the supernatants was determined with a spectrophotometric protein assay kit (Pierce, Rockford, IL). Cell extracts were resolved on polyacrylamide mini gels (typically 50 μg/lane). After transfer to nitrocellulose, membranes were blocked overnight at 4°C in a solution of 5% milk and 0.5% Tween 20 in TBS and incubated with specific antibodies followed by HRP-conjugated second antibody. After a wash, protein bands were identified by enhanced chemiluminescence (Super Signal Pierce) and BioMax MS highly sensitive X-ray film (Eastman Kodak, Rochester, NY).

RT-PCR and direct sequencing. TRIzol reagent (Invitrogen, Carlsbad, CA) was used to prepare total RNA from cultured βTC-6 cells, and then cDNA was made using M-MLV reverse transcriptase (Promega, Madison, WI). As described previously (41), cDNA (20 μl) was produced from total RNA (3 μl), and cDNA (1.25 μl) was subjected to 30 cycles of PCR using the following primers: β-actin sense, 5′-TGA TGA CGC AGA TAA TGT TTG-3′; β-actin antisense, ATG ATG GAG TTG AGG TGG TC-3′; TRHR1 sense, 5′-GCC CTC GAG TAC CAG GTG GTT ACC ATC-3′; TRHR1 antisense, 5′-AGT AGT AGT TCC TGG AGA TCT TGT AGC CA-3′. cDNA prepared from rat pituitary was served as positive control. Each cycle consisted of 94°C for 1 min, 70°C for 2 min, and 72°C for 2 min. The PCR product was run on 2% agarose gel stained with ethidium bromide and then photographed. The band was ex-

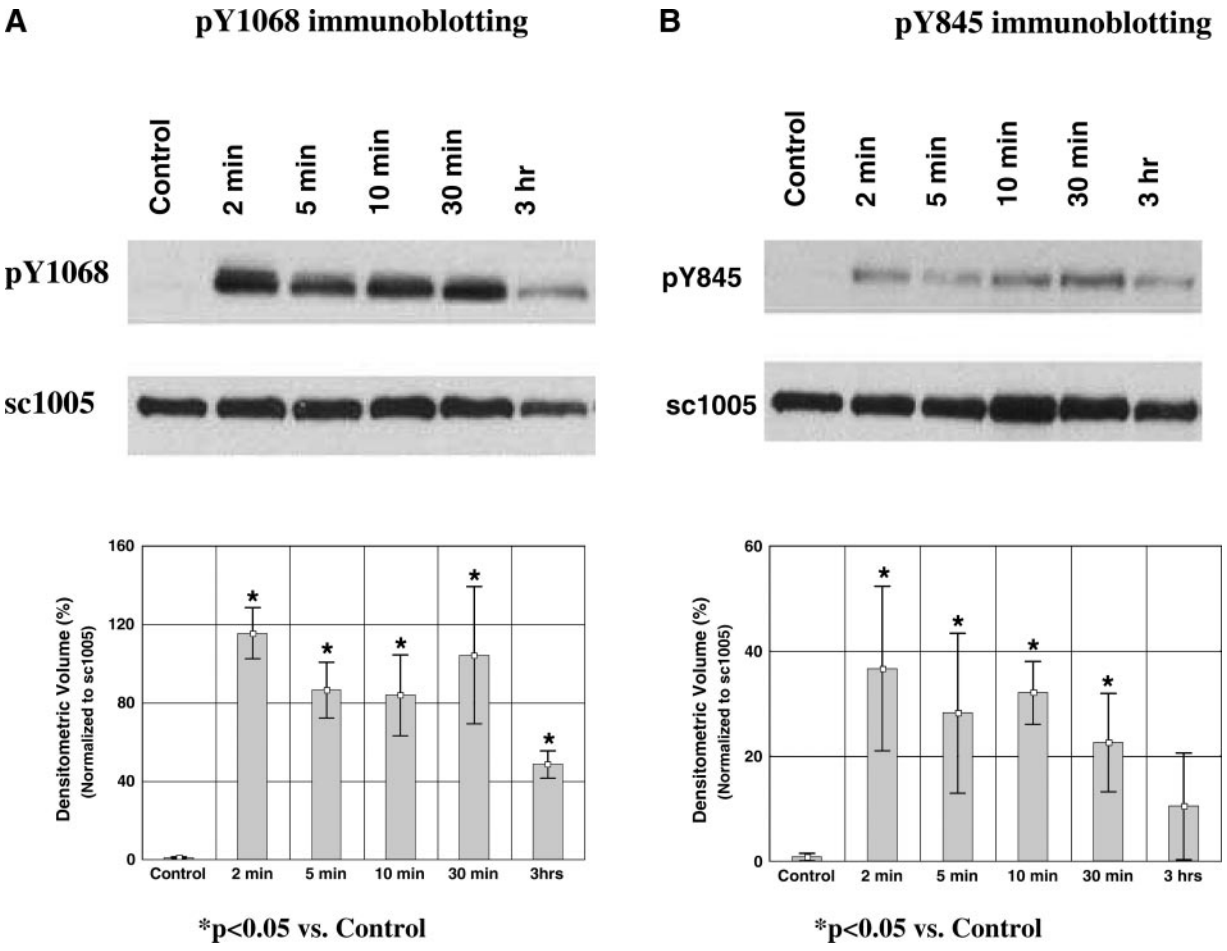

Fig. 1. Time course of epidermal growth factor (EGF) on EGF receptor phosphorylation. βTC-6 cells were incubated with 20 nM of EGF for various time periods. Maximum EGF receptor tyrosine phosphorylation appeared within 2 min and lasted ≥30 min at the pY1068 phosphorylation site and at the pY845 phosphorylation site. Graphs represent ratio of the densities of phosphorylated-receptor bands, with total receptor determined by the sc1005 antibody (expressed as %, means ± SE from 3 repeated experiments).

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tracted and reconstituted in Tris-EDTA buffer for direct sequencing by the Taq DNA polymerase cycle sequence method as described before (42). Briefly, the sense primer that was used for RT-PCR was used as a primer for the sequencing. Each sequencing cycle consisted of 15 s at 94°C, 45 s at 64°C, and 60 s at 70°C. After 30 cycles of the reaction, alkaline-heat denatured samples were loaded onto the sequencing gel. Analysis was performed using automated DNA sequencer (model ABI PRISM 377; PerkinElmer, Foster City, CA) in the Brown University Automated DNA Sequencing Facility.

Immunoprecipitation. Cell lysates were mixed with agarose-conjugated antibody and incubated overnight at 4°C with gentle rotation. The lysate-antibody mixture was washed three times with radioimmunoprecipitation assay buffer (20 mM Tris·HCl, pH 7.4, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate). Pellets were dissolved in 50 μl 2× SDS protein sample buffer that was cleared by centrifugation, run on polyacrylamide gels of appropriate concentration, transferred to nitrocellulose, and analyzed by the Western blotting procedure described above.

Image analysis. Densitometry analysis was performed using Scion Image for Windows (Scion, Frederick, MD).

Statistical evaluations. All data are presented as means ± SE and were analyzed by analysis of variance followed by Student's *t*-test unless otherwise indicated. Sigma Plot (SPSS, Chicago, IL) was used to draw charts. All data represent the results of three independent experiments unless otherwise indicated.

RESULTS

Time course of two EGF receptor phosphorylation residues in response to EGF and TRH. In an initial series of experiments, the effects of TRH at a concentration expected to yield the maximum TRH receptor binding (18) on EGF receptor tyrosine phosphorylation was studied in three different cultured β-cell lines HIT-T15, βTC-6, and INS-1, derived from rats (a gift from Dr. Claes Wollheim, Centre Médical Universitaire, Geneva, Switzerland). Before this study, all three cell lines were shown to tolerate incubation in serum-free medium for 30 h without vacuolization or other morphological changes that were evident by phase-contrast microscopy (data not shown). After incubation in serum-free medium for 16 h, EGF receptor tyrosine phosphorylation was increased after the addition of TRH for 10 min (determined by immunoprecipitation with EGF receptor antibody and sequential immunoblotting with phosphorylation antibodies). Identification of the EGF receptor was performed by stripping and reblotting the membranes with EGF receptor antibody (6). The 175-kD band stimulated by TRH was confirmed as the EGF receptor. Although TRH increased EGF receptor tyrosine phosphorylation in each β-cell line, it was weaker than achieved with EGF itself. The greatest

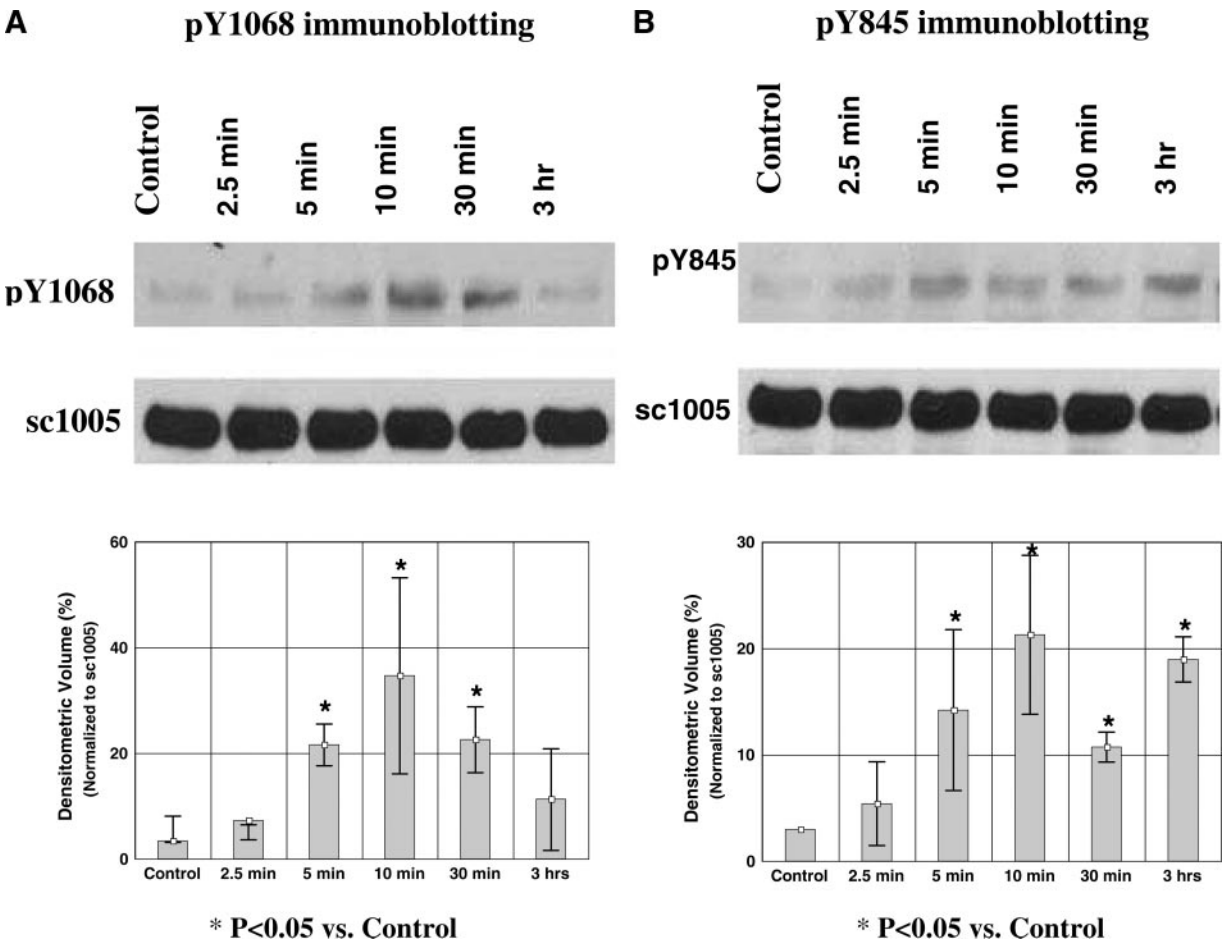

Fig. 2. Time course of thyrotropin-releasing hormone (TRH)-stimulated EGF receptor phosphorylation. βTC-6 cells were incubated with 200 nM of TRH for various time periods. Maximum EGF receptor tyrosine phosphorylation occurred at 10 min and lasted ≥30 min at the pY1068 phosphorylation site. The pY845 phosphorylated site appeared to reach a maximum point at 5 min and sustained longer. Graphs represent ratio of the densities of phosphor-receptor bands, with total receptor determined by the sc1005 antibody (expressed as %, means ± SE from 3 repeated experiments).

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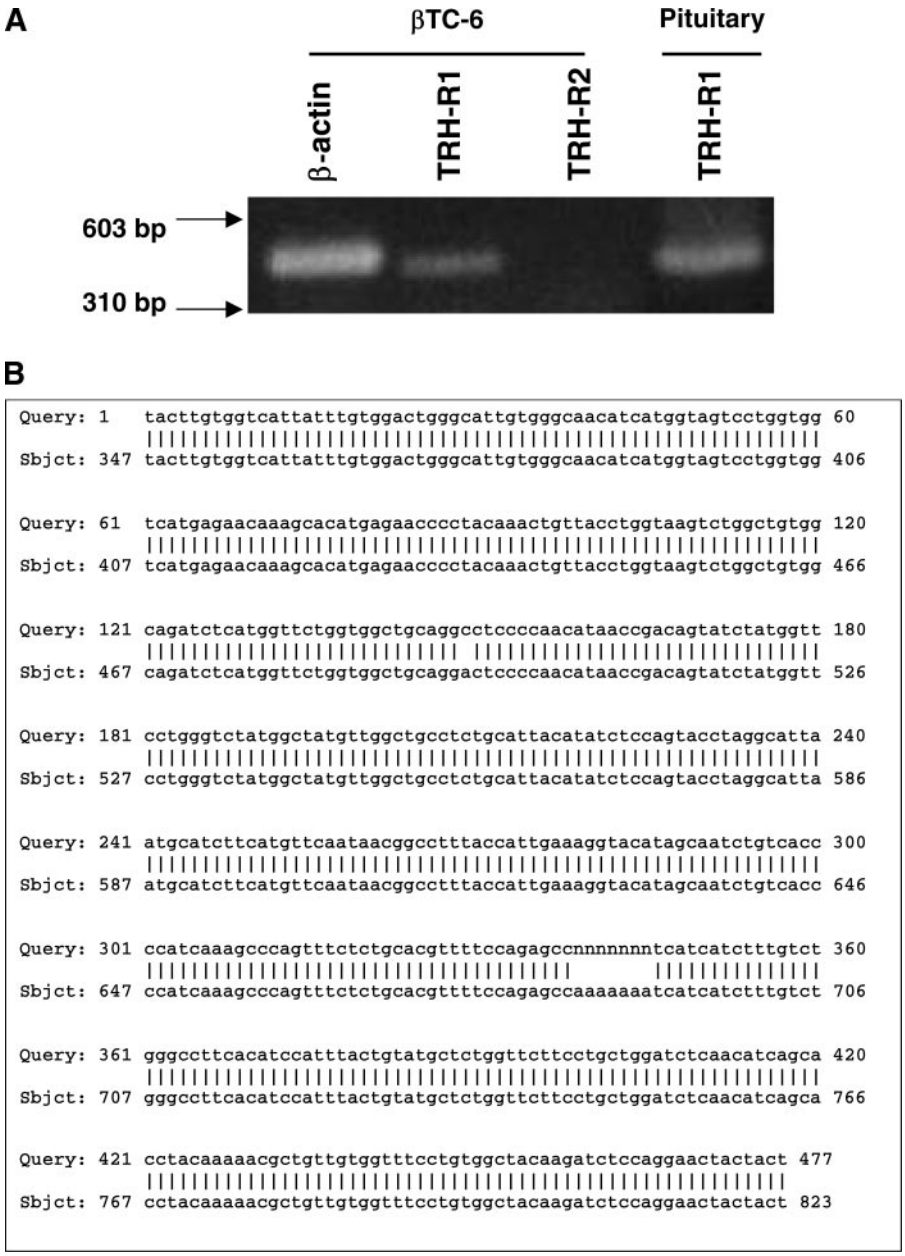

Fig. 3. TRH receptor-1 (TRHR1) gene expression in βTC-6 cells. cDNA was prepared from βTC cells, and RT-PCR was performed using TRHR1 and -R2 gene-specific primer pairs. *A*: gene was amplified from βTC-6 cell cDNA and rat pituitary cDNA as positive control (*lane 4*). Results indicate that only TRHR1 receptor is expressed in βTC-6 cells (*lane 2*), whereas no TRHR2 receptor expression is detected (*lane 3*). *B*: nucleotide sequence of the PCR product showed 98% (469/477) homology to mouse TRHR1 sequence (Sbjct) that was deposited in the National Center for Biotechnology Information database (NM 013696).

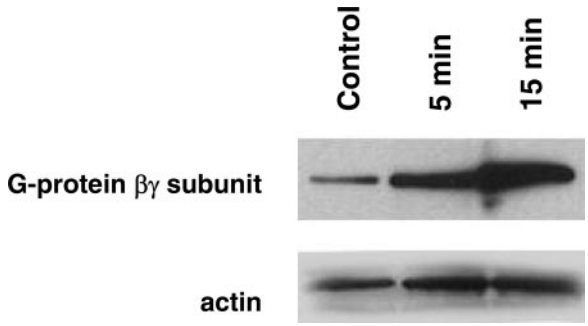

Fig. 4. Effect of TRH on G protein βγ-subunit. βTC-6 cells were treated with 200 nM of TRH for 5 or 15 min. TRH stimulates G protein βγ-subunit's dissociation from the αβγ complex at 5 and 15 min (*top*) and actin as loading standard for blotting (*bottom*).

EGF receptor phosphorylation in response to TRH was the βTC-6 cell line, which was generated from three individual experiments (data not shown). Thus the βTC-6 cell line was selected in this study.

Because there are various domains of EGF receptor phosphorylation in response to different stimulus, the use of the specific antibody that corresponds to EGF receptor phosphorylation sites would be a useful tool in pinpointing the specific domain of the EGF receptor in response to TRH. We are able to show that TRH activates phosphorylation of the EGF receptor cytoplasmic domain Tyr^{1068} at 10 min in βTC-6, HIT-T15, and INS-1 cells using anti-pY1068 antibody (26).

Two tyrosine residues (pY1068 and pY845) of the EGF receptor that responded to EGF and TRH were evaluated by immunoblotting. This was performed using corresponding

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phosphorylated specific antibodies for various times. The results indicate that maximum EGF receptor tyrosine phosphorylation occurred within 2 min and lasted until at least 30 min for the Tyr^{1068} (Fig. 1*A*) and the Tyr^{845} (Fig. 1*B*). For Tyr^{1068}, the maximum EGF receptor tyrosine phosphorylation stimulated by TRH occurred at 10 min and lasted until 30 min (Fig. 2*A*). For Tyr^{845}, although phosphorylation was weaker than for Tyr^{1068}, it reached a maximum at 5 min and lasted longer (Fig. 2*B*). These results show that there exists a delay for the EGF receptor in response to TRH, as well as a weaker signal, compared with the response to EGF.

TRHR1 gene expression in βTC-6 cells. The delay in the EGF receptor phosphorylation response to TRH compared with EGF indicates that there are several different mechanisms between TRH and EGF for EGF receptor activation. It is unclear whether TRH phosphorylates the EGF receptor through TRHR1 (21) or -R2 (37). TRHR1 has been found in the mouse pancreas, but no references were reported as to

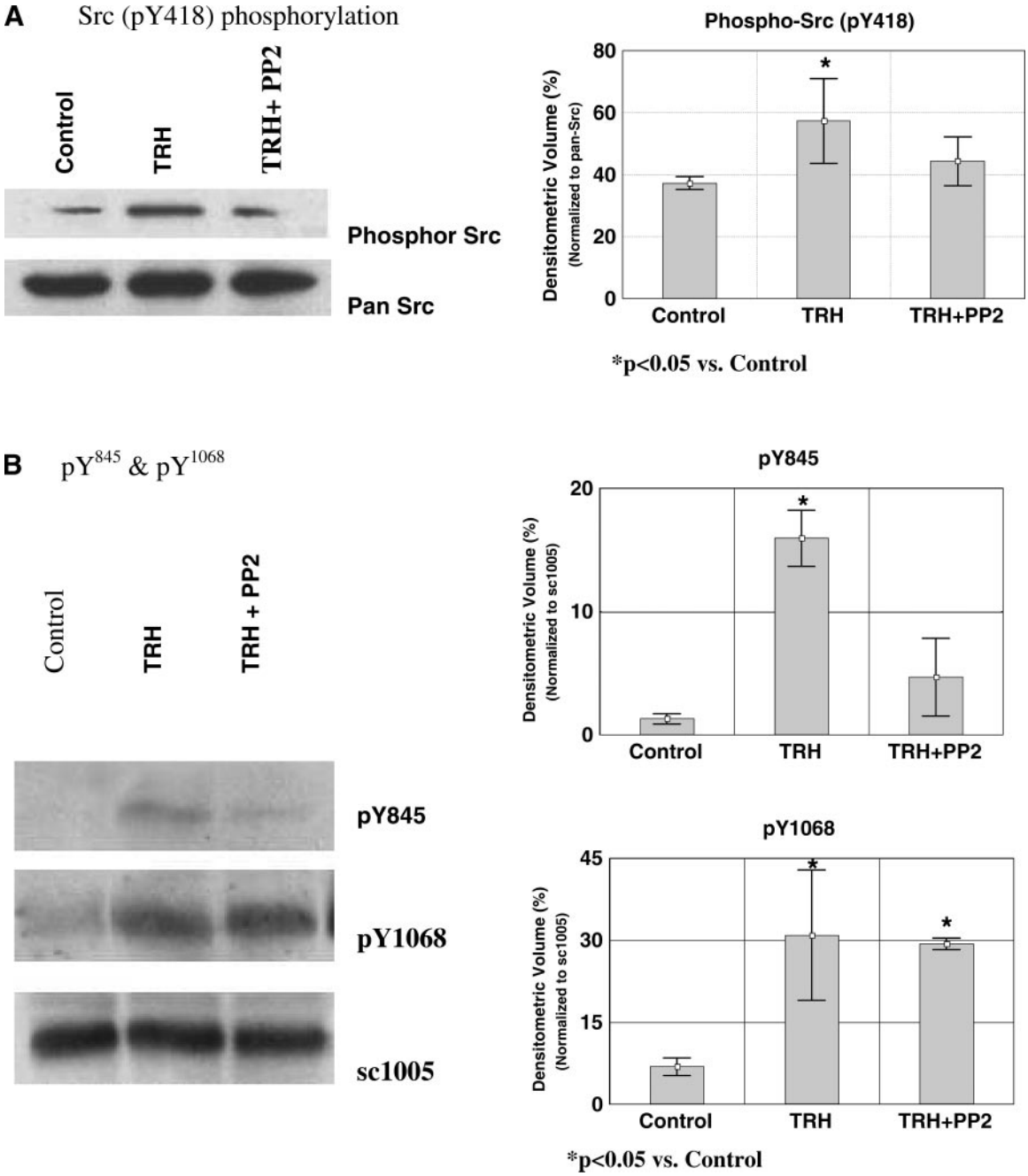

Fig. 5. Effects of 4-amino-5-(4-chlorophenyl)-7(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) on EGF receptor phosphorylation. *A*: βTC-6 cells were treated with 200 nM TRH with or without the Src inhibitor PP2 at 50 μM. *B*: PP2 inhibited TRH activation of Src. PP2 blocked TRH-stimulated phosphorylation of Tyr^{845} (*top*) but had no effect on Tyr^{1068} phosphorylation (*middle*). Membrane was stripped and reprobed with anti-EGF receptor antibody (sc1005; *bottom*). Graphs represent ratio of the densities of phosphor-receptor bands, with total EGF receptor tyrosine phosphorylation determined by the sc1005 antibody (expressed as %, means ± SE from 3 repeated experiments).

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whether the TRH receptor is present in mouse β-cells. To verify the expression of the TRH receptor in βTC cells, we prepared cDNA from the βTC cell line and performed RT-PCR by using specific primer pairs of TRHR1 and -R2 genes. As shown in Fig. 3*A*, only TRHR1 showed one single distinct band of expected size (507 bp by RT-PCR) in βTC-6 cells as well as in rat pituitary tissue. No expression of TRHR2 was detected. The band detected from PCR was a direct sequence, showing 98% homology (469/477) to the mouse TRHR1 sequence from the National Center for Biotechnology Information database (NM 013696) (Fig. 3*B*).

TRH enhanced G protein βγ subunit dissociation. The TRH receptor is a G protein-coupled binding receptor (2). TRH may activate the EGF receptor phosphorylation through its own receptor, a G-protein complex αβγ activation. A rabbit anti-G protein β-subunit internal sequence (127–139) was used to detect the βγ subunit dissociated from the TRH-activated αβγ-complex. The antibody can detect the βγ subunit in concentrations as low as 3.125 ng/ml. The results indicate that TRH stimulates dissociation of βγ subunits from the αβγ complex in 5–15 min (Fig. 4). This result is consistent with the TRH activation of its own GPCRs in βTC-6 cells (32).

TRH-induced EGF receptor phosphorylation by Src activation. Tyr^{845} in the EGF receptor is a known Src phosphorylation target (31). TRH should activate Src phosphorylation if

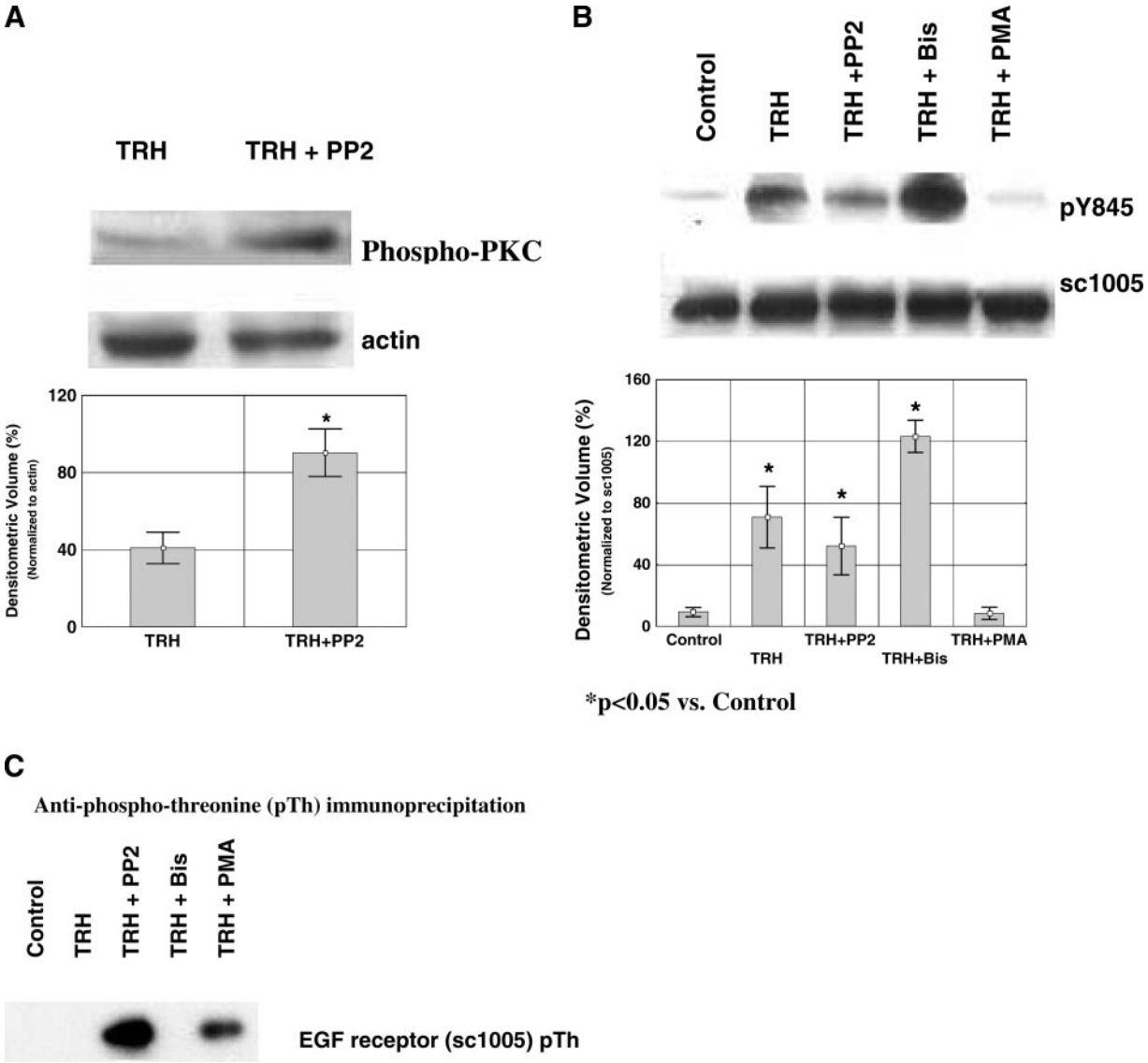

Fig. 6. Effect of PKC activity on EGF receptor phosphorylation. *A*: protein samples from βTC-6 cells were immunoblotted with anti-phosphorylated PKC antibody after treatment with 200 nM TRH, with or without PP2 (50 μM; *top*). Graphs represent ratio of the densities of phosphorylated-PKC bands against those of actin (expressed as %, means ± SD from 3 repeated experiments; *bottom*). *B*: combination of PP2 (50 μM), bisindolylmalemide (Bis; 10 μM), or 4-α-pholbol 12-myristate 13 acetate (PMA; 10 ng/ml) with 200 nM TRH was used to treat βTC-6 cells, and EGF receptor pY485 phosphorylation was detected by immunoblotting with anti-pY845 antibody (*top*). Graphs represent ratio of the densities of phosphor-receptor bands, with total EGF receptor tyrosine phosphorylation determined by the sc1005 antibody (expressed as %, means ± SD from 3 repeated experiments; *bottom*). *C*: in addition, the use of anti-EGF receptor (sc1005) immunoprecipitation and blotting with anti-phosphorylated threonine antibody indicated that both PP2 and PMA enhance threonine phosphorylation of EGF receptor (1).

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Src is involved in the TRH activation of EGF receptor phosphorylation. The stimulation and inhibition at Tyr^{845} by TRH and PP2, an Src family kinase inhibitor, would provide evidence supporting Src as a mediator in the TRH activation of EGF receptor phosphorylation. As expected, TRH stimulates Src phosphorylation (Fig. 5*A*) as well as EGF receptor Tyr^{845} phosphorylation (Fig. 5*B*, *top right*) in βTC-6 cells. PP2 blocked TRH-stimulated phosphorylation of Src (Fig. 5*A*) and Tyr^{845} (Fig. 5*B*, *top*). However, no effects of PP2 on TRH-stimulated Tyr^{1068} phosphorylation (Fig. 5*B*, *bottom right*) were observed.

Activation of Src-PKC pathway is involved in the TRH mediation of EGF receptor phosphorylation. On the basis of data shown in Fig. 5, the Src inhibitor PP2 (50 μM) reduced the effect of TRH on EGF receptor Tyr^{845}. The reduced effect at Tyr^{1068} (Fig. 5*B*) suggests that multiple mechanisms are involved. We suspect there is a PKC mechanism involved between TRH and EGF receptor activation. Suppression of Src by PP2 significantly enhances TRH during PKC phosphorylation (Fig. 6*A*), which indicates the Src inhibition of PKC activation, presenting evidence for PKC involvement. We propose that Src-PKC activation plays an important role in EGF receptor phosphorylation. When combining PP2 (50 μM), Bis (10 μM, a PKC inhibitor), and PMA (10ng/ml, a PKC stimulator) with TRH (18) in βTC-6 cell lines, PP2 suppressed TRH-induced EGF receptor phosphorylation at Tyr^{845}, Bis enhanced TRH activation of EGF receptor tyrosine phosphorylation, and PMA (10 ng/ml) inhibited EGF receptor phosphorylation (Fig. 6*B*). Furthermore, by using anti-phosphorylated threonine antibody in an immunoprecipitation study, both PP2 and PMA were enhanced, but Bis significantly inhibited threonine phosphorylation of the EGF receptor (Fig. 6*C*). This indicates that there is Src-PKC activating threonine phosphorylation, which indirectly inhibits EGF receptor tyrosine phosphorylation in the TRH regulation of the EGF receptor.

The role of HB-EGF in TRH activation of EGF receptor. HB-EGF binds to the cell surface heparin sulphate proteoglycan matrix and is proteolytically converted to a soluble form that can bind to the EGF receptor (30). Several intrinsic proteinases cleave the cell surface binding of HB-EGF, including MMP-3 (32, 35). HB-EGF activation induced by GPCR signal transduction (13) suggests the possibility that HB-EGF may be involved in the TRH cross talk with the EGF receptor. We explored this issue by using a specific anti-HB-EGF antibody to block the release of soluble HB-EGF by TRH in culture medium. The antibody dramatically suppressed the activity of TRH on EGF receptor phosphorylation (32). Meanwhile, TRH increased the amount of MMP-3, which may cleave the outer cell membrane portion of HB-EGF off into the culture medium (Fig. 7). The inhibition of Src by PP2 significantly neutralized this effect (Fig. 8).

DISCUSSION

In this paper, it has been demonstrated that TRH induced EGF receptor tyrosine phosphorylation by activating intracellular signal transductions in pancreatic β-cell lines (Fig. 1–2). The concentrations of TRH (200 nM) and EGF (20 nM) were selected according to our preliminary dose response studies (data not shown) and those of other investigators (17). The dosages of TRH and EGF used in this project and the cells' response to the stimulation were considered physiological. Multiple steps of intervening processes involved in EGF receptor phosphorylation in response to TRH resulting in the delayed and prolonged EGF receptor activation suggest the unique biological role of TRH regulation of EGF receptor function in β-cells (Fig. 3).

TRHR1 expression in β-cell lines (βTC-6 cells derived from mice; Fig. 3) and in the rat pancreas (21) are consistent with previous reports (40). A high-affinity (K_d) value for TRHR1

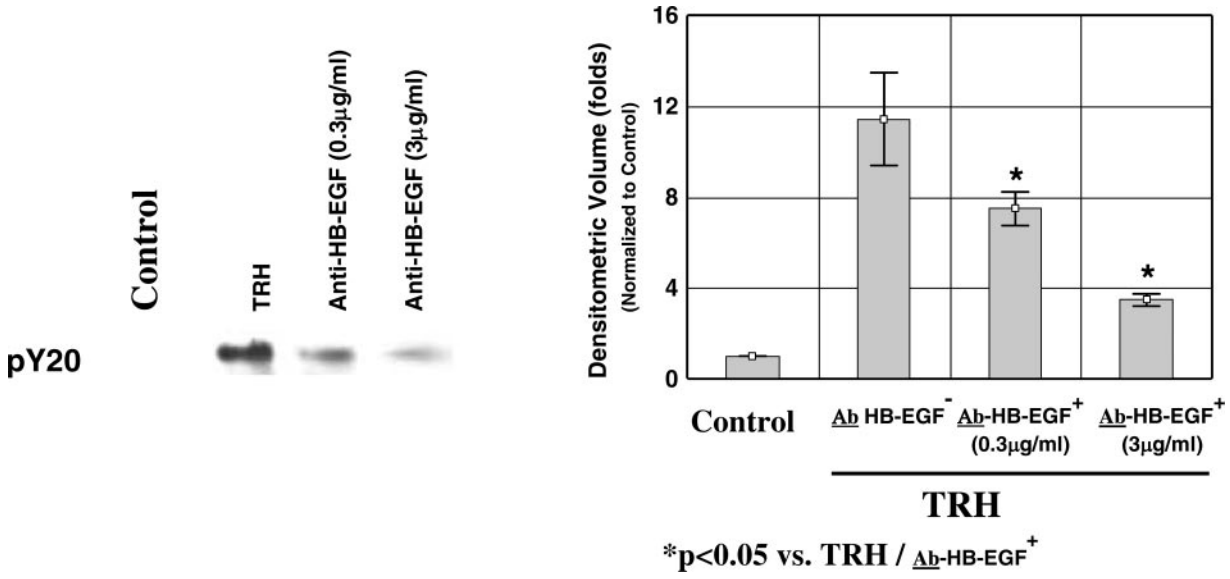

Fig. 7. Effects of anti-heparin-binding EGF-like growth factor (HB-EGF) antibody on EGF receptor phosphorylation. βTC-6 cells were treated with 200 nM TRH, with or without anti-HB-EGF antibody (0.3 or 3.0 μg/ml). EGF receptor tyrosine phosphorylation was assessed by sequential immunoprecipitation and immunoblotting. Graph represents densitometric analysis of immunoblotting signals (means ± SE from 3 repeated experiments).

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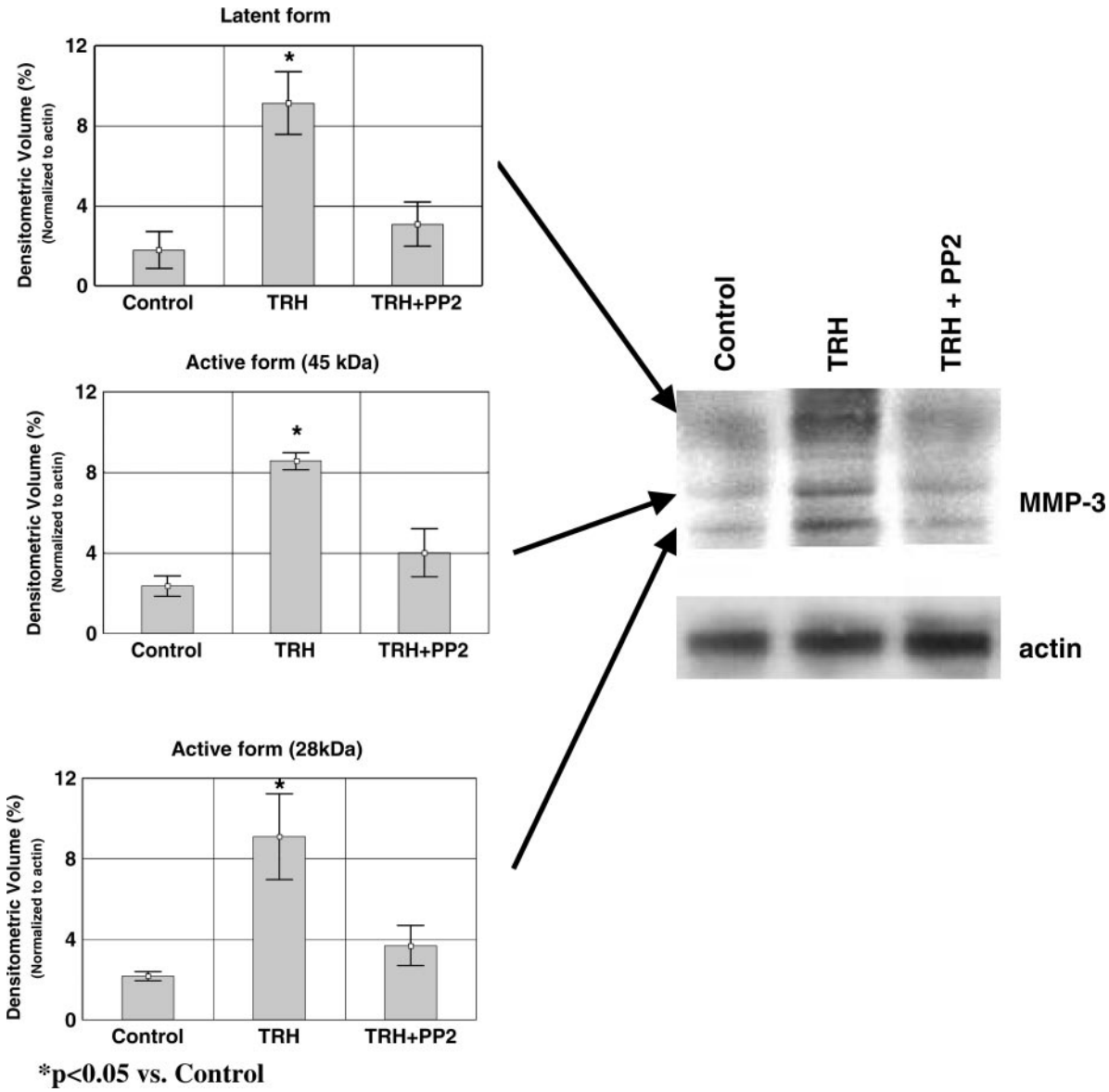

Fig. 8. Effect of TRH and PP2 on expressions matrix metalloproteinase 3 (MMP-3). βTC-6 cells were incubated with 200 nM TRH, with or without PP2 at 50 μM. TRH enhanced expressions of both latent and active forms of MMP-3, but PP2 erased these effects. Graph represents densitometric analysis of immunoblotting signals (means ± SE from 3 repeated experiments).

(determined by radioreceptor assay with H^3-Met-TRH) (40) indicated that TRHR1 has a significant function in pancreatic β-cells and may be involved in the mechanisms related to paracrine and/or autocrine effects. TRH is not able to bind to the EGF receptor directly. TRH initiated EGF receptor phosphorylation, which may be through the TRHR1 receptor paracrine effect in β-cells. A higher-affinity MT-TRH (TRH analog) induced stronger EGF receptor phosphorylation than native TRH (21), which fits the notion that the TRH stimulation of EGF phosphorylation depends on TRH receptor-activation. TRH binds to its receptor, causing dissociation of the α- and βγ-G protein subunit and thus triggering signal cascades after GPCR activation (Fig. 4) (2).

Cytoplasmic tyrosine kinases may play a role as downstream components of GPCR's pathways (2, 10, 11). Recent studies have indicated that several types of GPCR activation and inhibitive regulation of the Src family kinase (2) suggest that the Src family plays a critical role in GPCR growth factor receptor's transactivation (15).

Many growth factors that signal via tyrosine phosphorylation mechanisms such as EGF affect islet development and maturation (33). Transient upregulation of both EGF and EGF receptors has been observed during islet proliferation and regeneration (3). In pancreatic β-cells, our studies show that there are multiple pathways involved in TRH activation of EGF receptor phosphorylation distinctly from pituitary cells (37). A series of our own experiments using the Src inhibitor PP2 verified the involvement of the tyrosine kinase in TRH-induced EGF receptor tyrosine phosphorylation. PP2 partially inhibits TRH-induced Src activation (Fig. 5*A*), indicating

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that Src is a critical link between TRH and EGF receptors in β-cells. This is similar to Src in vasopressin-mediated mitogenic signaling in intestinal epithelial cells (8). However, Src does not completely inhibit the effects of TRH on the EGF receptor, which suggests that there are other factors involved.

PKC has been found to activate TRH in the neuronal system, which regulates pituitary function (27, 34, 36). Expression of various PKC subtypes in pancreatic β-cells is involved in multiple functions from insulin secretion to cell differentiation (6, 17). However, the role of PKC in TRH stimulation of EGF receptor in β-cells is unclear. We found that PMA (a stimulator of PKC) suppresses TRH-induced EGF receptor tyrosine phosphorylation (Fig. 6*B*). The participation of PKC in TRH activation of EGF receptor phosphorylation is linked to Src activation, wheras the inhibition of Src enhances the reaction of PKC. Therefore, we proposed that Src can activate EGF receptor phosphorylation indirectly by suppressing PKC (Fig. 6*B*), which is known to initiate a serine/threonine phosphorylation, thus inhibiting the phosphorylation of the tyrosine residue in the EGF receptor (8). As expected, both PP2 and PMA induce threonine phosphorylation in the EGF receptor (Fig. 6*C*), consequently inhibiting tyrosine phosphorylation. Integrating all these findings, we propose that there is an Src-PKC pathway in the TRH activation of EGF receptor phosphorylation.

GPCR-EGF receptor transactivation through HB-EGF has been reported (4, 16, 28, 30). HB-EGF, a member of the EGF family, has the ability to bind to cell surface heparin sulphate proteoglycans; this prevents the immediate release of the growth factor and increases the local growth factor concentration within the cellular microenvironment (24). Previous reports (13, 30) implicate the involvement of HB-EGF in EGF receptor transactivation in GPCR's stimulation and HB-EGF-cleaving metalloproteinase, which is rapidly induced by GPCR-ligand interaction. HB-EGF binds to the cell surface heparin sulphate proteoglycan matrix and is proteolytically released into a soluble form that can bind to the EGF receptor (30). Several intrinsic proteinases cleave the cell surface binding of HB-EGF, including MMP-3 (32, 35). In this study, TRH-induced tyrosine phosphorylation of the EGF receptor is partially inhibited by neutralizing HB-EGF through the use of a specific antibody (Fig. 7). The stimulation of TRH in MMP-3 activation in βTC-6 cells was suppressed significantly by PP2 (Fig. 8), suggesting that TRH activation of the proteinase for MMP-3 may be through activation of Src. This mechanism may represent an alternate pathway for GPCR-EGF receptor transactivation. The use of immortalized cell lines in this study overcomes the replicative senescence of primary β-cell culture. However, the biological characteristics of immortalized cell lines may differ from normal β-cells. Therefore, caution should be used when extrapolating our results to normal β-cells.

In summary, the TRHR1 (but not the TRHR2) receptor found in β-cells supports receptor cross talk mechanisms for TRH-stimulated EGF receptor activation. TRH binds to its receptor, activating multiple pathways including *1*) GPCR-dissociated αβγ-complex, *2*) activation of the Src kinase resulting in phosphorylation of EGF receptor Tyr^{845}, *3*) release of HB-EGF as a consequence of MMP-3 activation, and *4*) inhibition of PKC activity resulting in reducing inhibitory EGF receptor serine/threonine phosphorylation. Because the Src inhibitor PP2 blocks TRH-induced MMP-3 activation and PKC inhibition, TRH receptor activation of Src may play a central role in mediating the effects of TRH on the EGF receptor (Fig. 9). The TRH activation of EGF receptor phosphorylation indicates that TRH could regulate pancreatic β-cell development and maturation by mediating phosphorylation of the EGF receptor, leading to a model involving the peptide regulation of development and function in the pancreas.

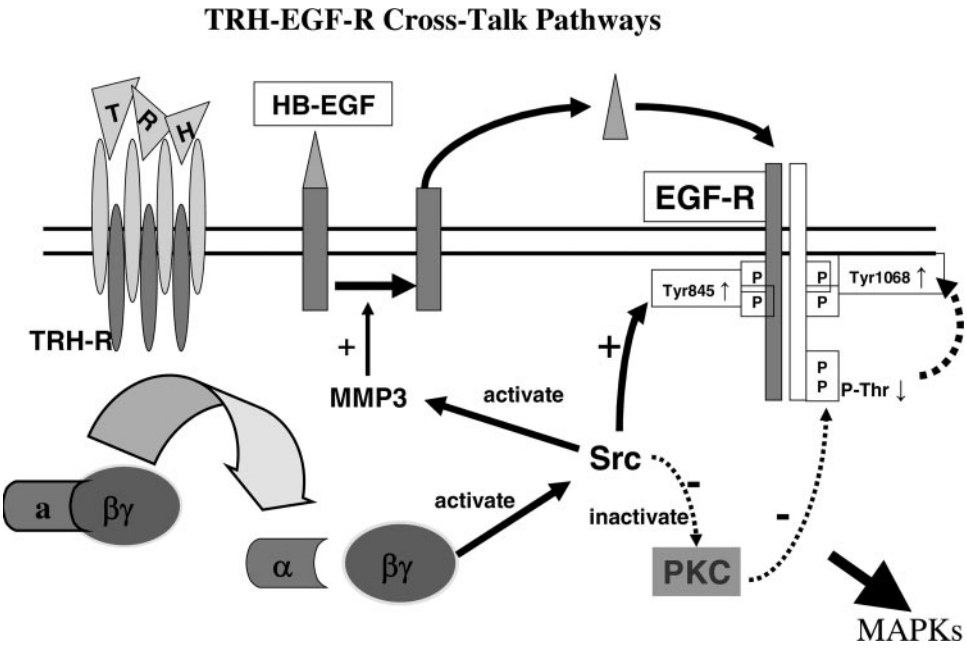

Fig. 9. Scheme summarizes mechanism of TRH cross talk with EGF receptor in pancreatic β-cells. TRH binds to its receptor and dissociates G protein-coupled receptor (GPCR) αβγ complex into α- and βγ-units. The βγ-unit activation of the Src kinase directly results in phosphorylation of EGF receptor Tyr^{845}. In addition, Src indirectly stimulates Tyr^{845} phosphorylation by activation of MMP-3 to release HB-EGF. Meanwhile, activation of Src kinase inhibition of PKC results in the reduction of serine/threonine phosphorylation, which blocks off the inhibition of serine/threonine phosphorylation on tyrosine phosphorylation and indirectly activates Tyr^{1068} phosphorylation in EGF receptor. TRH activation of EGF receptor phosphorylation results in the activation of cellular signal pathways such as MAPKs. Activation of Src may have a central role in mediating the effects of TRH on EGF receptor. (R, receptor; solid arrow, activation; dotted arrow, suppression).

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