Thyrotropin via Cyclic AMP Induces Insulin Receptor Expression and Insulin Co-stimulation of Growth and Amplifies Insulin and Insulin-like Growth Factor Signaling Pathways in Dog Thyroid Epithelial Cells*

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Despite the similarity of their receptors and signal transduction pathways, insulin is regarded as a regulator of glucose, protein, and lipid metabolism, whereas insulin-like growth factors (IGF-I and IGF-II) mainly act as mitogenic hormones. In the dog thyroid primary culture model, the triggering of DNA synthesis by thyrotropin (TSH) through cAMP, or by cAMP-independent factors including epidermal growth factor, hepatocyte growth factor and phorbol esters, requires insulin or IGFs as comitogenic factors. In the present study, in TSH-treated cells, IGF-I receptors and insulin receptors were paradoxically equivalent in their capacity to elicit the comitogenic pathway, which, however, was mediated only by IGF-I receptors in dog thyroid cells stimulated by cAMP-independent mitogens. Moreover, prior cell exposure to TSH or forskolin increased their responsiveness to insulin, IGF-I, and IGF-II, as seen on DNA synthesis and activation of a common insulin/IGF signaling pathway. To understand these observations, binding characteristics and expression of insulin and IGF-I receptors were examined. To analyze IGF-I receptor characteristics, the unexpected interference of a huge presence of IGF-binding proteins at the cell membrane was avoided using labeled Long R3 IGF-I instead of IGF-I. Strikingly, TSH, through cAMP, time-dependently induced insulin binding and insulin receptor mRNA and protein accumulation without any effect on IGF-I receptors. These findings constitute a first example of an induction of insulin receptor gene expression by a cAMP-mediated hormone. In dog thyroid cells, this allows low physiological insulin concentrations to act as a comitogenic factor and might explain in part the enhanced responsiveness to IGFs in response to TSH. This raises the possibility that TSH-insulin interactions may play a role in the regulation of thyroid growth and function in vivo.

Insulin and insulin-like growth factors (IGF-I and IGF-II) are related hormones (50% sequence homology) and bind to highly similar receptors, which consist of two extracellular α subunits (∼130 kDa) and two membrane-spanning β-subunits (∼95 kDa) linked by disulfide bonds (1, 2). Insulin receptors more weakly recognize IGF-I and IGF-II. Conversely, IGF-I receptors preferentially recognize IGF-I but also bind IGF-II and insulin with 10-fold and 100-1000-fold weaker affinity, respectively (1). Upon ligand binding, transautophosphorylation reactions of the receptor β-subunit increase its tyrosine kinase activity toward endogenous substrates (3). Activity of insulin and IGF-I receptors thus converges on the phosphorylation of insulin receptor substrates (IRS-1 (3, 4) and IRS-2 (5)), which then act with the receptor itself as docking proteins for other substrates such as the 85-kDa adaptor subunit of phosphoinositol 3-kinase and Grb-2. Grb-2 in turn recruits the mSOS nucleotide exchange factor, which activates Ras and triggers a kinase cascade culminating in the phosphorylation and activation of MAP kinases (2–4, 6).

Despite the large similarity of insulin and IGF-I receptors and intracellular signaling pathways, the main physiological actions of insulin in vivo and in vitro involve glucose, protein, and lipid metabolism, whereas IGF-I acts as a mitogenic hormone or cytokine. Subtle differences in the biochemistry of the two receptors have been proposed to explain the fundamental difference in the physiological roles of insulin and IGF-I (7). They include some differences in the C-terminal regions of the insulin and IGF-I receptors (8, 9) (of the two tyrosine residues phosphorylated on activation of the insulin receptor, only one is present in the IGF-I receptor; the lacking residue could be important for metabolic signaling but not for mitogenicity (10, 11)), a higher dissociation rate of ligands from the insulin receptor compared with IGF-I receptor (2), or somewhat different substrate specificities of kinase catalytic domains (12). Nevertheless, insulin has been found to signal a mitogenic pathway through its own receptor rather than IGF-I receptors in transfected NIH 3T3 or Chinese hamster ovary cells that overexpress insulin receptors (8, 13–16) or in some cancer cell

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1 The abbreviations used are: IGF, insulin-like growth factor; MAP, mitogen-activated protein; IRS, insulin receptor substrate; Grb-2, growth factor receptor bound 2; TSH, thyrotropin; EGF, epidermal growth factor; BrdUrd, 5-bromodeoxyuridine; HGF, hepatocyte growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; IGFBP, IGF-bind-
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lines such as hepatoma cells (17), breast carcinoma cells (18), and T-cell lymphoma cells (19).

Whether insulin can signal mitogenesis through its own receptor in normal cells containing normal levels of insulin and IGF-I receptors is far less clear (20, 21). The dog thyroid primary culture system is a unique model to reassess the biological role of insulin and IGF ligand-receptor systems. DNA synthesis and proliferation of these cells can be separately triggered either by growth factors including EGF acting through tyrosine kinase-dependent pathways or by TSH acting through cAMP (22, 23). Among other major differences between the two mitogenic pathways (24, 25), we previously noticed that the stimulation of DNA synthesis by EGF depended on high insulin concentrations that could interact with IGF-I receptors, whereas 100-1000-fold lower insulin concentrations were sufficient to support the TSH stimulation of DNA synthesis (23).

In the present study, we characterize the insulin and IGF-I receptors of dog thyrocytes in culture, their expression, and their involvement in the comitogenic effects of insulin and IGFs. Unexpectedly, we found that TSH through cAMP induces the expression of insulin receptors but not of IGF-I receptors. This is associated with an enhanced responsiveness of the cells not only to insulin but also to IGFs and explains the comitogenic effect of low insulin concentrations in the presence of TSH.

EXPERIMENTAL PROCEDURES

Primary Cultures—Dog thyrocytes were cultured in monolayer (2 × 10^5 cells/cm²) in a medium that included Dulbecco’s modified Eagle’s medium, Ham’s F-12 medium, and MCD184 medium (2:1:1, v/v) supplemented by 40 mg/ml ascorbic acid, 500 μg/ml bovine serum albumin (crystallized from Serva, Heidelberg, Germany), and antibiotics (23) (basal medium) and treated as indicated in the figure legends.

DNA Synthesis—Cells in 3-cm Petri dishes were incubated for 24 h before fixation in the presence of BrdUrd. The incorporation of BrdUrd was detected by immunofluorescence, and BrdUrd-labeled nuclei (1000-2000) were counted as described (26).

Radioimmune Assays—IGF-I, des-(1–3)IGF-I, and Long R3 IGF-I were iodinated with mild chloramine-T treatment (27) and separated from free radioactive iodine with Sephadex G-25 (PD-10 column, Pharmacia, Uppsala, Sweden). Specific activities of about 60 μCi/μg were obtained. Cells were cultured for 3 days in basal medium and for 2 additional days with or without TSH (1 milliunit/ml) in 24-well plates (80,000 cells/well). Cells were washed with the binding buffer (20 mM Hepes, 10 mM glucose, 3 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl, 250 mM sucrose, pH 7.6, containing 1 mg/ml bovine serum albumin) and then incubated for 2.5 h at room temperature in 0.2 ml of binding buffer with 80,000–300,000 cpm of labeled ligand and various concentrations of unlabeled competitors. Nonspecific binding was assessed in the presence of 1 μM unlabeled ligand. After incubation, the cells were washed three times with the binding buffer, the cell layer was dissolved in 1 ml NaOH, and the radioactivity was measured in a γ-counter. Nonspecific binding was subtracted from all values.

Western Blotting and Immunofluorescence Analysis of Proteins—Western blotting and immunofluorescence analysis of proteins were done as described previously (26, 28).

Northern Hybridization of Insulin Receptor mRNA—Dog thyrocytes in 100-mm Petri dishes were disrupted in 4 g guanidinium monothiocyanate, and the total RNA (15 μg/lane) was separated as described (29). After Northern blotting transfer, filters were hybridized with the full-size radiolabeled human insulin receptor cDNA excised from a PUC12/HIR clone received from Drs. Lammens and Ulrich. Acidine orange staining of the gels was performed to assess that equal amounts of RNA were loaded in each lane.

Representation of Data—Data points in graphs are the mean values ± range of duplicate determinations. Where not shown, ranges were too small to be graphically represented. All assays were performed at least in triplicate and in general three or four times with similar results.

Materials—The blocking MA-10 insulin receptor monoclonal antibody (18), 31.2 neutralizing IGF monoclonal antibody (31) was shared by Dr. D. Wynford-Thomas (Cardiff, UK), and human insulin receptor cDNA was generously provided by Drs. R. A. Simons (Munich, Germany), DRS. (32) and 1H7 (33) blocking IGF-I receptor monoclonal antibodies were purchased from Oncogene Science (Cambridge, MA) and Pharmingen (San Diego, CA), respectively; the JB1 peptide (34) from Peninsula (St. Helens, U.K.); MAP kinase (ERK2) monoclonal antibody and an anti-human insulin receptor α-subunit polyclonal antibody from Upstate Biotechnology, Inc. (Lake Placid, NY); human insulin receptor β-subunit polyclonal antibody and PV20 anti-phosphotyrosine monoclonal antibody from Transduction Laboratories (Lexington, KY); anti-IGF-I receptor α-subunit (N-20) and anti-c-Fos (fos(4)) polyclonal antibodies from Santa Cruz Biotech (Santa Cruz, CA); bovine insulin from Sigma; human recombinant IGF-I and IGF-II from R & D Systems (Abingdon, UK); Long R3 IGF-I and des(1–3)IGF-I from Genentech (Adelaide, Australia); 125I-labeled insulin (at tyrosine A14), IGF-I and IGF-II from Amersham (Little Chalfont, UK). Other reagents were obtained as they were previously (26).

RESULTS

Comitogenic Effect of Insulin and Insulin-like Growth Factors in the Presence of TSH and Other Growth Factors—When used alone, insulin and insulin-like growth factors, even at high concentrations, had only marginal effects on the DNA synthesis of dog thyrocytes in a serum-free medium. These peptides were required for, or markedly potentiated, the mitogenic effects exerted either by TSH acting through cAMP (23) or by cAMP-independent factors including EGF (23), HGF (35), and TPA (36) (Fig. 1). The synergistic interaction of insulin with EGF, HGF, or TPA was mostly observed at high supraphysiological concentrations of insulin, which might act through low affinity binding to IGF-I receptors (1) (Fig. 1A). The concentration-action curve of the con-mitogenic effect of insulin in the presence of TSH was different. Most of the insulin effect was observed in the physiological nanomolar range with a marked stimulation of DNA synthesis already at 1.6 × 10⁻¹⁰ M (1 ng/ml). In some experiments, a variable supplementary effect of insulin was obtained in the μg/ml range (10⁻⁷ to 10⁻⁶ M).

IGF-I also permitted DNA synthesis stimulation by both TSH and cAMP-independent mitogenic factors (Fig. 1B). IGF-I effects were detected at 5 × 10⁻¹⁰ M (4 ng/ml), but no maximal effect was obtained at 7 × 10⁻⁶ M (500 ng/ml). As compared with insulin (Fig. 1A), less difference was observed in the IGF-I concentration curves in the presence of TSH or the other mitogenic factors (Fig. 1B).

IGF-II was a less potent comitogenic factor than IGF-I (Fig. 1C). Like insulin, but somewhat at variance with IGF-I, its permissive effect on DNA synthesis required lower concentrations in the presence of TSH than in the presence of EGF (Fig. 1C).

Identification of Receptors Involved in the Comitogenic Effects of Insulin and IGF-I in the Presence of TSH—JB1 is a 12-amino acid peptide analogue of residues 61–69 of human IGF-I, which efficiently blocks IGF-I receptor autophosphorylation and effects on DNA synthesis (34). It competitively inhibited the effects of IGF-I (by 40–80% in different experiments), but not the effects of a low insulin concentration, on DNA synthesis in the presence of TSH in dog thyrocytes (Fig. 2A). JB1 also lowered the effect of high insulin concentrations until the JB1-resistant effect of low insulin concentrations (Fig. 2A). Similar effects were obtained using αIR-3 and 1H7 monoclonal antibodies that completely block IGF-I binding and effects in other systems (32, 33) (data not shown). These results suggest that IGF-I, but not low insulin concentrations, acted at least in part through IGF-I receptors and that IGF-I receptors mediated the additional stimulatory effect of high supraphysiological concentrations of insulin obtained in some experiments.

αIR-3, 1H7, and JB1 (Fig. 2A) also inhibited the weak stimulation of DNA synthesis by TSH alone, i.e. without adminis-
tration of IGF-I or insulin. This inhibition suggests that this TSH effect depends on the activation of IGF-I receptors through an autocrine mechanism. Indeed, the weak stimulation by TSH in the absence of exogenous insulin or IGF was also partially inhibited by Sm 1.2 (31), a monoclonal antibody specifically neutralizing IGF-I and IGF-II but not insulin (not shown).

The monoclonal antibody MA-10 competitively inhibits insulin binding and insulin responses mediated by insulin receptors but not by IGF-I receptors (18). It recognizes the dog insulin receptor (30). As shown in Fig. 2B, MA-10 completely blocked the mitogenic effect of a low insulin concentration but marginally affected the stimulation by IGF-I or a high supraphysiological concentration of insulin. The mitogenic effect of low insulin concentrations, which was observed only in the presence of TSH (Fig. 1A), thus occurred via insulin receptors.

Cell Pretreatment with TSH Enhances the Effect of both IGF-I and Insulin on DNA Synthesis—Upon mitogenic stimulation, quiescent dog thyrocytes do not enter DNA synthesis (S phase) before completion of a prereplicative lag phase of about 16–20 h (23). As shown in Fig. 3A, the stimulation of DNA synthesis required the simultaneous presence of TSH and IGF-I. Thus, when TSH was added 24 h after IGF-I, or when IGF-I was administered 24 h after TSH, DNA synthesis fol-

Fig. 1. Concentration-response curves of the comitogenic effect of insulin (A), IGF-I (B), and IGF-II (C), in the presence of various mitogenic factors. The cells were cultured for 4 days in the basal medium without insulin. They were then stimulated for 48 h with TSH (1 milliunit/ml) (■), EGF (25 ng/ml) (▲), HGF (40 ng/ml) (●), TPA (10 ng/ml) (▲), or none of these (○), together with various concentrations of insulin, IGF-I, or IGF-II. BrdUrd was added for the last 24 h. The fraction of BrdUrd-labeled nuclei was determined.

Fig. 2. Inhibition of the comitogenic effects of insulin and IGFs by specifically blocking IGF-I or insulin receptors. The cells were cultured for 4 days in the basal medium. They were then stimulated for 48 h by TSH (1 milliunit/ml) (■), or TSH in the presence of 2.5 × 10⁻⁹ M insulin (▲), 8 × 10⁻⁷ M insulin (●), or 2.5 × 10⁻⁹ M IGF-I (▲) with or without (○) various concentrations of JB1 IGF-I receptor antagonist peptide (A), or MA-10 insulin receptor blocking antibody (B). BrdUrd was added for the last 24 h. The fraction of BrdUrd-labeled nuclei was determined.

Fig. 3. A, kinetics of the synergy of TSH (1 milliunit/ml) and IGF-I (1.3 × 10⁻⁸ M) on DNA synthesis. Cells were seeded and cultured for 4 days in basal medium without insulin (○). At day 4 (0 h), they were incubated with BrdUrd and the following additions: 1.3 × 10⁻⁸ M IGF-I added at 0 h; 1 milliunit/ml TSH added at 0 h or 24 h (■); IGF-I and TSH added together at 24 h, TSH added at 24 h to cells incubated since 0 h with IGF-I, or IGF-I added at 24 h to cells incubated since 0 h with TSH (■, as indicated in the figure). The cumulative index of BrdUrd-labeled nuclei was determined at the indicated times. Note the highest proportion of labeled nuclei in cells exposed to TSH prior to IGF-I. B, synergy of TSH (1 milliunit/ml) and insulin (2.5 × 10⁻⁹ M) on DNA synthesis. Cells were stimulated exactly as in A but with insulin instead of IGF-I. The cumulative index of BrdUrd-labeled nuclei was determined at 72 h. Notice the highest proportion of labeled nuclei in cells exposed to TSH prior to insulin.

allowed with a similar 16–20-h lag phase the time when TSH and IGF-I were present together, regardless of which factor was added first. Nevertheless, when TSH was administered 24 h before IGF-I, a higher rate of DNA synthesis was obtained, compared with the response to the simultaneous addition of IGF-I and TSH or to the addition of TSH 24 h after IGF-I (Fig. 3A). The synergistic cooperation on DNA synthesis stimulation of TSH and a low insulin concentration was similarly more potent when TSH was added 24 h before insulin (Fig. 3B). A 24-h TSH pretreatment could thus enhance cell responsiveness to both insulin and IGF-I.

Effects of TSH on Binding Characteristics of Insulin-like
IGF-II competed with 125I-IGF-I as well as at all to IGFBPs (37, 38). Therefore, we tested the binding of 125I-des(1–3)IGF-I characterizing dog thyroid IGF-I receptors without interference with IGFBPs. The binding capacities of 125I-des(1–3)IGF-I (not shown) and 125I-Long R3 IGF-I (Fig. 4, A and B) were 8- and 32-fold lower, respectively, than 125I-IGF-I binding capacity. The binding characteristics of 125I-Long R3 IGF-I (better than those of 125I-des(1–3)IGF-I, which likely still binds significantly to IGFBPs) were typical of IGF-I receptors. 125I-Long R3 IGF-I was well displaced by unlabeled IGF-I and Long R3 IGF-I (ED50 ∼1.5 × 10−9 M), better than by IGF-II (ED50 ∼2 × 10−8 M) and insulin (ED50 ∼2 × 10−7 M). Scatchard analysis provided an estimate of approximately 5 × 104 IGF-I receptors/cell. The binding characteristics of Long R3 IGF-I were not affected by a 48-h pretreatment of cells with TSH (Fig. 4B).

The binding of 125I-insulin on control unstimulated dog thyroid cells was extremely weak and did not allow an evaluation of its characteristics (Fig. 4C). A 48-h pretreatment of cells with TSH markedly enhanced 125I-insulin binding (Fig. 4D). Characteristics of competition with unlabeled insulin (ED50 ∼1.5 × 10−9 M), IGF-II (ED50 ∼4 × 10−8 M), and IGF-I (ED50 ∼1.5 × 10−7 M) were typical of insulin receptors (1). The curvilinear Scatchard plot provided an estimate of approximately 6 × 104 high affinity insulin binding sites/cell in cells stimulated by TSH for 2 days. These experiments thus suggest that TSH increases the amount of insulin receptors, but not that of IGF-I receptors.

TSH Induces Insulin Receptor Expression—Insulin and IGF-I receptor contents of dog thyroid cells were examined on Western blots using antibodies against α and β subunits of these receptors (Fig. 5). The β subunit of insulin receptor was almost undetectable in control unstimulated cells, but it appeared 16 h after TSH administration and gradually accumulated thereafter. The effect of TSH was mimicked by the adenylate cyclase activator forskolin, which suggests that it is mediated by cAMP (Fig. 5). TSH and forskolin also increased the accumulation of the α subunit of insulin receptor as detected using the polyclonal antibody from Upstate Biotechnology, Inc. (not shown). Upon elimination of forskolin, intracellular cAMP concentrations rapidly returned to basal levels (39). After washing out of forskolin, insulin receptor presence remained elevated for at least 6 h. Then it progressively disappeared, and by 24 h it had returned to barely detectable control levels. In contrast, α and β subunits of IGF-I receptors were detected in control unstimulated dog thyroid cells, and their content did not vary after the addition of TSH or forskolin (Fig. 5).

The accumulation of insulin receptors in response to forskolin was associated with a similar increase of insulin receptor mRNA content. The effect of forskolin was weaker in cells continuously cultured in the presence of 8.3 × 10−7 M insulin. Insulin receptor mRNA accumulation was not stimulated by EGF or TPA (data not shown).

FIG. 5. Western blotting analysis of insulin receptor and IGF-I receptor expression. Dog thyrocytes were cultured in basal conditions for 4 days and then with TSH (1 milliunit/ml) for 1–48 h or with forskolin (FK) (10−5 M), or they remained in basal conditions (cont.). Cells incubated with forskolin for 24 h were rinsed and reincubated for 1–24 h with forskolin (FK (24 h) → FK), or without forskolin (FK (24 h) → cont.). Detection was done using polyclonal antibodies against insulin receptor (ins.R) β-subunit (Transduction Laboratories), IGF-I receptor α-subunit (Santa Cruz Biotech), and the IGFR1–2 monoclonal antibody against IGF-I receptor (IGF-IR) β-subunit from K. Siddle.

**Growth Factors and Insulin**—The following experiments were performed to understand how TSH could allow insulin to act as a comitogenic factor through its own high affinity receptors and enhance the comitogenic responsiveness to both insulin and IGF-I receptors in dog thyroid cells. We first tried to characterize the IGF-I receptors of dog thyroid cells using 125I-IGF-I and unlabeled ligands in competition-inhibition studies in intact cells. Unexpectedly, the binding of 125I-IGF-I was extremely high (in the absence of competing ligand, bound/free ratios ranged from 35 to 70% for 80,000 cpm 125I-insulin with or without the indicated concentrations of various unlabeled peptides. Nonspecific binding was 2000 cpm/well for 125I-insulin (not shown) and 250 cpm/well for 125I-Long R3 IGF-I (Fig. 4, B and D). Cells incubated with forskolin for 24 h were rinsed and reincubated for 2.5 h at room temperature with either 250,000 cpm 125I-Long R3 IGF-I or with 80,000 cpm 125I-insulin with or without the indicated concentrations of various unlabeled peptides. Nonspecific binding was 2000 cpm/well for 125I-IGF-I and 250 cpm/well for 125I-insulin (not shown). Upon elimination of forskolin, intracellular cAMP concentrations rapidly returned to basal levels (39). After washing out of forskolin, insulin receptor presence remained elevated for at least 6 h. Then it progressively disappeared, and by 24 h it had returned to barely detectable control levels. In contrast, α and β subunits of IGF-I receptors were detected in control unstimulated dog thyroid cells, and their content did not vary after the addition of TSH or forskolin (Fig. 5).

The accumulation of insulin receptors in response to forskolin was associated with a similar increase of insulin receptor mRNA content. The effect of forskolin was weaker in cells continuously cultured in the presence of 8.3 × 10−7 M insulin. Insulin receptor mRNA accumulation was not stimulated by EGF or TPA (data not shown).
dog thyrocytes were cultured for 2 days in basal conditions. This included the tyrosine phosphorylation of the 95-kDa b-subunit, IRS-like proteins, and p42 MAP kinase using the PY20 monoclonal antibody and the p42 and p44 MAP kinases using Posada’s 1913.2 polyclonal antibody. Positions of IRS-like proteins and insulin/IGF-I receptor β-subunits were indicated. On the MAP kinase Western blot, the proportion of the phosphorylated (upper band in the doublet) versus the nonphosphorylated form of MAP kinases can be appreciated. Dog thyrocytes were cultured for 2 days in basal conditions and then for 2 days with or without TSH (1 milliunit/ml). Cells were stimulated or not (C) with insulin (8 × 10⁻⁷ m) for 2, 5, and 15 min.

**TSH Pretreatment Potentiates the Insulin/IGF-I Signaling Pathway**—In control cells, high concentrations of insulin (8 × 10⁻⁷ m) had only a weak stimulatory effect on the phosphorylation of proteins on tyrosine residues (Fig. 6). The phosphotyrosine content of the 185-kDa band (likely corresponding to an IRS protein according to molecular weight) was maximum 5 min after insulin addition and thus preceded the MAP kinase phosphorylation (maximum at 15 min), which was detected on MAP kinase Western blots by its migration shift (Fig. 6). In agreement with previous studies, TSH did not stimulate tyrosine phosphorylation of proteins including MAP kinases (28, 40). However, a 48-h pretreatment of cells with TSH (Fig. 6) or forskolin (not shown) strongly amplified the response to insulin. This included the tyrosine phosphorylation of the 95-kDa β subunit of the receptor of insulin and/or IGF-I and of 175–185-kDa proteins. Interestingly, the tyrosine phosphorylation of the 175-kDa band was more transient than that of the 185-kDa band and was detected only in TSH pretreated cells (Fig. 6). The phosphorylation of both p42 and p44 MAP kinases in response to insulin was also markedly amplified and accelerated by the TSH pretreatment (Fig. 6). The kinetics of the amplification effect of TSH or forskolin on insulin-dependent tyrosine phosphorylation paralleled the kinetics of insulin receptor expression. The potentiation was weakly detected 16 h but not 1 or 6 h after the TSH addition and further increased 24 and 48 h afterwards. It was reversed 24 but not 6 h after washing out forskolin (data not shown).

The influence of a 24-h TSH pretreatment on the stimulation of tyrosine phosphorylation of proteins by IGF-I, IGF-II, and insulin has been investigated (Fig. 7). In control cells, a weak stimulation of tyrosine phosphorylation of the insulin/IGF-I receptor β subunit, IRS-like proteins, and p42 MAP kinase was detected only in response to 8 × 10⁻⁷ m insulin or 1.3 × 10⁻⁸ m IGF-I. The cell pretreatment by TSH amplified not only the effects of insulin at both low and high concentrations as expected but also the effects of 1.3 × 10⁻⁸ m IGF-I or IGF-II (Fig. 7). Nevertheless, the effects of 2.5 × 10⁻⁸ m IGF-I were less enhanced than the effects of the same concentration of insulin, especially for MAP kinase phosphorylation (Fig. 7). The amplification effect of cAMP specifically concerned the insulin/IGF-I signaling pathway, since it poorly affected the stimulation of tyrosine phosphorylation by EGF (not shown).

Upon activation, MAP kinases are translocated to the nucleus, where they phosphorylate transcription factors including p62CRE, which mediates the activation of c-fos promoter by some growth factors (41). Double immunofluorescence labeling of p42 MAP kinase and c-Fos (26) showed that insulin, even at high concentrations (8 × 10⁻⁷ m), only modestly stimulated MAP kinase nuclear translocation and c-Fos appearance in a relative minority of control dog thyrocytes (Fig. 8, B and B’). Both insulin responses were more intense and general after a 48-h forskolin pretreatment (Fig. 8, C and C’).

**DISCUSSION**

In dog thyroid cell primary cultures (22, 23), as in other in vitro thyroid cell culture systems (42–44), the stimulation of cell proliferation by TSH or growth factors requires comitogenic factors. As illustrated here, the dependence on such permissive factors can be fulfilled by the administration of IGF-I, IGF-II, or insulin. Even the weak variable stimulation of DNA synthesis by TSH in the absence of exogenous insulin or IGFs (23) was partly dependent on autocrine production of IGF-I or IGF-II, as demonstrated in this and other thyroid cell culture systems (45, 46). Again, as in other systems (1, 20, 43) the comitogenic action of either low IGF-I concentrations or high supraphysiological concentrations of insulin was at least in part mediated by IGF-I receptors in dog thyrocytes. The originality of these cells is that, in the presence of TSH, but not in the presence of cAMP-independent mitogenic factors including EGF, HGF, and TPA, insulin receptors beside IGF-I receptors also can mediate the comitogenic signaling cascade. Indeed, in the presence of TSH, the permissive effect of insulin on DNA synthesis was exerted mainly at low physiological concentrations, dependent on insulin receptors (since it was completely inhibited by the MA-10 blocking antibody), and was completely independent of IGF-I receptors (since it was not affected by the blockade of IGF-I receptors). Therefore, in TSH-treated dog thyroid cells, IGF-I receptors and insulin receptors are equivalent in their capacity to mediate a comitogenic pathway, which, however, can be elicited only by IGF-I receptors when the same cells are stimulated by cAMP-independent growth factors.

We explain this apparent paradox by our present observation that TSH, through cAMP, induces the expression of insulin receptors but not of IGF-I receptors. In control unstimulated cells, insulin binding, insulin receptor protein (as assessed by Western blotting), and mRNA (as assessed by Northern blotting) were at the limit of detection, at variance with IGF-I receptors (which, however, can be elicited only by IGF-I receptors when the same cells are stimulated by cAMP-independent growth factors).

Insulin receptor gene expression has been studied in various systems, and its regulation has been found to be especially important for directing insulin to specific target tissues (47–49). Insulin receptors are expressed ubiquitously, but the classic insulin target tissues contain higher levels of insulin receptor protein, leading to increased insulin sensitivity. Insulin receptors are induced in vitro and in vivo during muscular, pancreatic, and adipose differentiation (47–52). In addition, in various tissues and cell types, insulin receptor expression is under positive regulation by glucocorticoid hormones and negative regulation by insulin (47–59). Until now, hormones acting through cAMP have never been found to stimulate insulin receptor expression or binding capacity (47). On the contrary, forskolin was reported to inhibit insulin receptor mRNA accu-
The relative levels of expression rather than intrinsic structural differences (8–11) might be a major factor determining the propensity of insulin or IGF-I receptors for signaling mitogenesis.

As the likely consequence of increased insulin receptor expression, the pretreatment of dog thyrocytes by TSH or forskolin induced the insulin responsiveness of a signaling pathway that includes the tyrosine phosphorylation of the insulin receptor and IRS-like proteins, the phosphorylation of p42 and p44 MAP kinases, their nuclear translocation, and the appearance of the c-Fos protein. Interestingly, the sensitivity of this pathway to IGF-I and IGF-II (and high supraphysiological insulin concentrations that also interact with IGF-I receptor) was also enhanced by TSH as well as the comitogenic sensitivity to IGF-I and IGF-II. Transfection of NIH 3T3 with insulin receptors also increases their responsiveness to IGF-I (13, 14). In TSH-treated dog thyrocytes, our characterization of IGF-I and insulin receptors showed that IGF-II has only a little preference for IGF-I receptors. It might thus act on insulin receptors as well, which might explain why IGF-II, like insulin, was comitogenic at lower concentrations in the presence of TSH and was relatively insensitive to antibodies blocking IGF-I receptor. At high concentrations (>10^−8 M), IGF-I also significantly competed for insulin receptor binding. In the presence of TSH, it could thus act in part through insulin receptors, which would explain its partial resistance to IGF-I receptor blockade (not shown). Nevertheless, TSH also somewhat enhanced the sensitivity to lower IGF-I concentrations that did not significantly compete for insulin receptor binding. Conceivably, the induction of insulin receptor expression could also increase the presence of insulin/IGF-I receptor hybrids that preferentially bind IGF-I (58), but we failed to detect an increase of IGF-I binding capacity in response to TSH. Despite the good correlation of the kinetics of the stimulatory effects of TSH and forskolin on insulin and IGF responsiveness and on insulin receptor expression, additional mechanisms should be envisaged to explain the increased sensitivity to IGF-I. In FRTL5 rat thyroid cell line, prior TSH exposure also amplifies various responses to IGF-I (43, 59, 60), including the tyrosine phosphorylation of a 175-kDa IRS-like protein (61, 62), without increasing IGF-I binding (63). The mechanism(s) are unclear and controversial (61, 62).

cAMP as a second messenger for mitogenic hormones can positively control cell proliferation both by direct and indirect mechanisms (25). This is especially well illustrated in the present study. TSH, through cAMP but independently of MAP kinases (28), c-Jun (29) and c-Fos (26), directly controls dog thyroid cell cycle progression, including a late retardation point just before DNA synthesis initiation (39). In addition, as shown here, it indirectly stimulates the MAP kinase-dependent insulin/IGF-I comitogenic pathway, at least in part by inducing insulin receptors. It is noteworthy that this TSH effect on insulin receptors is delayed and relatively stable after removal of the cAMP stimulus. Therefore, it probably does not mediate the direct effect of cAMP on G0-S prereplicative phase progression, which continuously requires an especially labile cAMP-dependent intermediary (39). In Balb-C 3T3 fibroblasts, platelet-derived growth factor stimulates proliferation at least in
part by inducing IGF-I receptors, thus increasing cell competence to progress into the cell cycle in response to IGF-I (56). In dog thyroid cells, cAMP thus acts both as a progression factor and as a competence factor increasing responsiveness to insulin and IGF-I, which then cooperate with cAMP as progression factors. By a distinct mechanism that does not involve a potentiation of MAP kinase activation, cAMP also increases the competence of dog thyroid cells to respond to EGF (26). TSH and cAMP can thus very potently promote thyroid cell proliferation by separately stimulating multiple pathways that cooperate at various levels.

In dog thyroid cells, insulin at low concentrations moderately stimulates the expression of some thyroid-differentiated functions, such as thyroglobulin mRNA accumulation (64). The induction of insulin receptors by TSH and cAMP might thus be a factor in the delayed induction of thyroid transcription by TSH. As observed in several tissues (47–52) insulin receptor expression might be associated with differentiation expression, which in thyroid is mainly supported by TSH (24). The present observations might be related to the impairment of thyroid growth and function in response to TSH in diabetic rats and mice, which is restored upon insulin administration (65–67). In vivo, a direct synergy of TSH and circulating insulin, besides locally produced IGF-I (68), might be an interesting possibility that deserves further studies.

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