Thyroid-stimulating Hormone and Insulin-like Growth Factor-1 Synergize to Elevate 1,2-Diacylglycerol in Rat Thyroid Cells

Stimulation of DNA Synthesis via Interaction between Lipid and Adenylyl Cyclase Signal Transduction Systems

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Abstract

Thyroid-stimulating hormone (TSH) and insulin-like growth factor-1 (IGF-1) synergistically stimulate DNA synthesis in thyroid cells. In this report, a novel mechanism for mediation of this synergistic interaction is described in rat thyroid (FRTL-5) cells. Because phorbol myristate acetate stimulates DNA synthesis, the effects of TSH, IGF-1 and insulin on FRTL-5 cell content of 1,2-diacylglycerol (1,2-DG), the endogenous activator of protein kinase C, were measured. After 6 d, TSH, IGF-1 and insulin caused increases in cellular 1,2-DG (mean±SE) to 180±10%, 540±50%, and 360±40% of control, respectively, whereas TSH plus IGF-1 and TSH plus insulin synergistically increased 1,2-DG to 1,890±310% and 1,690±230%, respectively. In the absence of insulin, the effect of TSH to elevate 1,2-DG exhibited an EC_{50} of ~ 2,000 μU/ml. The synergistic interaction of insulin and TSH was found to increase the potency of TSH by 300-fold (EC_{50} was ~ 7 μU/ml) in addition to increasing the efficacy of TSH. The effect of TSH appeared to be mediated by TSH-stimulated increases in cyclic AMP (cAMP). Forskolin and 8-bromo-cAMP, like TSH, caused modest increases in 1,2-DG and DNA synthesis, whereas forskolin plus insulin and 8-bromo-cAMP plus insulin markedly elevated 1,2-DG content and stimulated DNA synthesis. Under all conditions, increases in 1,2-DG content correlated with stimulation of DNA synthesis. These findings suggest that the synergistic stimulation of DNA synthesis in thyroid cells by TSH, via cAMP, and IGF-1 is mediated by 1,2-DG. Moreover, they implicate a novel interaction between the lipid and adenylyl cyclase signaling systems for the regulation of cell proliferation.

Introduction

The intracellular pathways that mediate the actions of growth factors have been intensively investigated during the last several years (reviewed in references 1–3). Although our understanding of the mediators of growth factor action is still very limited, evidence has accumulated suggesting that 1,2-diacylglycerol (1,2-DG),[1] via activation of protein kinase C (see reference 4 for review), and cyclic 3′:5′-adenosine monophosphate (cAMP) play important roles in the action of several growth factors. Thyrotropin (thyroid-stimulating hormone, TSH) and insulin-like growth factor-1 (IGF-1) or high doses of insulin, which allows for interaction with receptors for IGF-1, are factors that stimulate growth of thyroid cells (5–12). TSH appears to act by stimulating adenylyl cyclase and elevating cAMP (9–11, but 12), whereas the mechanism of action of IGF-1 or high doses of insulin is not known but may involve activation of the IGF-1 receptor tyrosine kinase (13). Most interestingly, and perhaps most relevant under normal physiologic conditions, TSH and IGF-1 have been found to synergistically stimulate DNA synthesis in thyroid cells (5, 6, 8, 9). Because activation of protein kinase C has been implicated as a mediator of mitogenesis in thyroid cells (8, 14, 15), the effects of TSH, IGF-1 and insulin on thyroid cell content of 1,2-DG were measured. These growth factors caused a persistent elevation of 1,2-DG in a cloned line of rat thyroid (FRTL-5) cells. Moreover, there was a profound synergistic effect of TSH plus IGF-1 (or high doses of insulin) on cell 1,2-DG content that could be mimicked by the cAMP analogue, 8-bromo-cAMP (BrcAMP) and by the direct adenylyl cyclase stimulator, forskolin. These data suggest that chronic elevation of 1,2-DG, perhaps by persistently activating protein kinase C, may be the mediator of the synergistic stimulation of TSH, via cAMP, and IGF-1 on proliferation of thyroid cells.

Methods

Materials. Coon's modified Ham's F-12 medium, calf serum, nonessential amino acids and glutamine were from Gibco Laboratories, Grand Island, NY. Fatty acid free bovine serum albumin, Hepes, insulin, transferrin, hydrocortisone, somatostatin and glycyl-L-histidyl-L-lysine acetate and BrcAMP were from Sigma Chemical Co., St. Louis, MO. Forskolin was from Calbiochem-Behring, La Jolla, CA. Bovine TSH was from Armour Pharmaceuticals (Milwaukee, WI). Diacylglycerol kinase was from Lipidex. Cardiolipin was from Avanti Polar Lipids. [^{3}H]Thymidine (86 Ci/mmol) and [^{32}P]ATP (3,000 Ci/mmol) were from New England Nuclear, Boston, MA. Organic sol-

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1. *Abbreviations used in this paper:* BrcAMP, 8-bromo-cAMP; 1,2-DG, 1,2-diacylglycerol; IGF-1, insulin-like growth factor-1.

vents were HPLC grade from Fisher Scientific, Pittsburgh, PA. Silica G chromatography plates were from Whatman Inc., Clifton, NJ.

Cells. FRTL-5 cells were kindly provided by Dr. Leonard D. Kohn of the National Institutes of Health. Cells were grown in the usual complex medium (6H) consisting of Coon's modified Ham's F-12 medium supplemented with 5% calf serum, nonessential amino acids, glutamine, and six hormones or growth factors—TSH (1,000 μU/ml), insulin (1.7×10^{-6} M), transferrin (5 μg/ml), hydrocortisone (10^{-8} M), somatostatin (10 ng/ml) and glycyl-L-histidyl-L-lysine acetate (10 ng/ml) (16). In preparation for experiments, cells were washed twice with HBSS containing calcium and magnesium and refed with "basal medium" consisting of Coon's modified Ham's F-12 medium, 1 mM nonessential amino acids, 0.03% glutamine, 0.4% BSA, and 10 mM Hepes, pH 7.5. Cells were maintained in basal medium for 7 d before the initiation of experiments and were refed every 3–4 d with the appropriate medium.

Studies of DNA synthesis. Cells were grown to a subconfluent level in 6H medium, washed twice with balanced salt solution, and refed with basal medium. After 7 d, cells received basal medium alone or basal medium supplemented with IGF-1, insulin or TSH. After 3 d, cells were refed with basal medium containing the indicated factors and 1 μCi/ml [^{3}H]thymidine. After an additional 3 d, cells were harvested by washing twice with HBSS without Ca^{2+} or Mg^{2+} followed by addition of trypsin/collagenase and EGTA solutions. For the studies of insulin dose response, total incubation with insulin was for 3 d. Cell suspensions were obtained from which aliquots were taken for cell count by hemocytometer and for measurement of DNA synthesis as [^{3}H]thymidine incorporation. [^{3}H]Thymidine incorporation was measured by precipitation with 5% TCA, solubilization with 1 N NaOH, and beta scintillation counting. DNA content of the NaOH extract was measured by the Burton method (17).

Measurement of 1,2-DG. For experiments in which 1,2-DG content was measured, basal cells were refed with either basal medium alone or basal medium containing the indicated factors for 6 d. Cells were extracted with chloroform/methanol (1:1) to which ¼ vol of 10 mM EDTA was added to break phase, and the lower phases were washed twice with preequilibrated upper phase. A measured volume of the lower phase extracts were evaporated under nitrogen and assayed for 1,2-DG by a radioenzymatic assay that uses bacterial 1,2-DG kinase and gamma-[^{32}P]ATP to phosphorylate endogenous 1,2-DG and convert it quantitatively to [^{32}P]phosphatidic acid (18). Phosphatidic acid was isolated by TLC on silica G plates; the plates were developed using chloroform/methanol/glacial acetic acid/water (25:15:4:2) (19). [^{32}P]Phosphatidic acid spots were visualized by autoradiography and identified by comparison to standards and from known R_f values. 1,2-DG content is calculated from the ^{32}P-radioactivity in phosphatidic acid and the specific activity of the gamma-[^{32}P]ATP. Measurement of inorganic phosphorus content was also performed on aliquots of the lower phase cell extract (19).

Statistical analysis. Analyses were made by t test; P values < 0.05 were considered significant.

Results and Discussion

To investigate the mechanism(s) of stimulation of DNA synthesis in FRTL-5 thyroid cells by TSH, IGF-1 and insulin, we have established serum-, hormone-, and growth factor-free culture conditions ("basal medium") that supports FRTL-5 cell viability and responsiveness to mitogenic factors. FRTL-5 cells incubated in basal medium for 7 d ("basal cells") can resume growth at a rate indistinguishable from control cells when reexposed to complete 6H medium and can be maintained in basal medium for up to 3 wk (data not shown). Because (*a*) phorbol ester-induced activation of protein kinase C has been reported to be mitogenic in thyroid cells (8, 14, 15), (*b*) phorbol myristate acetate stimulated DNA synthesis by

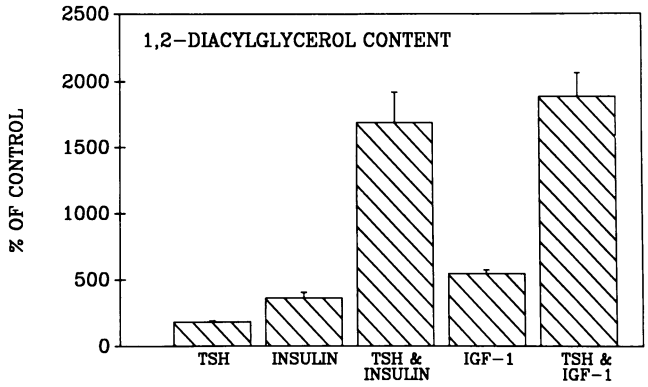

Figure 1. Effects of TSH, IGF-1 and insulin on 1,2-DG content in FRTL-5 cells. Cells were maintained in basal medium for 7 d before initiation of experiments. 1,2-DG content was measured in cells incubated in basal medium without (control) or with 30,000 μU/ml TSH, 1.3×10^{-9} M IGF-1, 1.7×10^{-5} M insulin, TSH plus insulin, or TSH plus IGF-1 for 6 d. 1,2-DG content of control cells was 180±30 pmol/10^6 cells. Data are presented as percent control (mean±SE) of triplicate determinations from six experiments for all conditions except IGF-1. Data for IGF-1 (mean±SD) are triplicate determinations in a single confirmatory experiment.

threefold in basal FRTL-5 cells (data not shown), and (*c*) transformed fibroblasts have persistently elevated cellular 1,2-DG content (20), we measured the 1,2-DG content of basal cells and of basal cells stimulated by TSH, IGF-1, and insulin. We utilized a recently developed radioenzymatic method that did not require metabolic labeling of cells and allowed for direct determination of 1,2-DG mass (18). In a series of six experiments (Fig. 1), a maximally effective dose of TSH alone stimulated a twofold increase in 1,2-DG levels. IGF-1 (1.3×10^{-9} M) and high doses of insulin (1.7×10^{-5} M) caused similar four- to fivefold increases in 1,2-DG content. More importantly, TSH plus insulin and TSH plus IGF-1 stimulated synergistic 18-fold elevations of cellular 1,2-DG content. These elevations are specific for 1,2-DG and can be accounted for only in small part by a general increase in cell lipid content. When the data were normalized for total cell phospholipids, the molar content of 1,2-DG in control cells was 0.62±0.2% (mean±SE) of total phospholipids. The increases in 1,2-DG caused by TSH, insulin, and IGF-1, expressed as percent of total phospholipids were: TSH, 150±10%; insulin, 290±40%; IGF-1, 410±50%; TSH plus insulin, 880±110%; TSH plus IGF-1, 790±30%.

To begin to study whether the increase in 1,2-DG content could be a mechanism for stimulation of DNA synthesis, the effects of TSH, IGF-1 and insulin on DNA synthesis were measured and compared to their effects to cause elevation of 1,2-DG. After 6 d, TSH (30,000 μU/ml) caused a modest stimulation of [^{3}H]thymidine incorporation to 600±40% of control (mean±SE). IGF-1 (1.3×10^{-9} M) and insulin (1.7×10^{-5} M) resulted in a more marked stimulation of DNA synthesis to 2,500±100 and 3,800±720% of control, respectively. When TSH plus IGF-1 and TSH plus insulin were added to basal cells increases in [^{3}H]thymidine incorporation to 4,400±150 and 5,300±910% of control were observed. Fig. 2 illustrates the dose-dependent effect of insulin to cause elevation of 1,2-DG and to stimulate DNA synthesis. After 6 d, insulin caused elevations of 1,2-DG content of basal cells in a dose-dependent

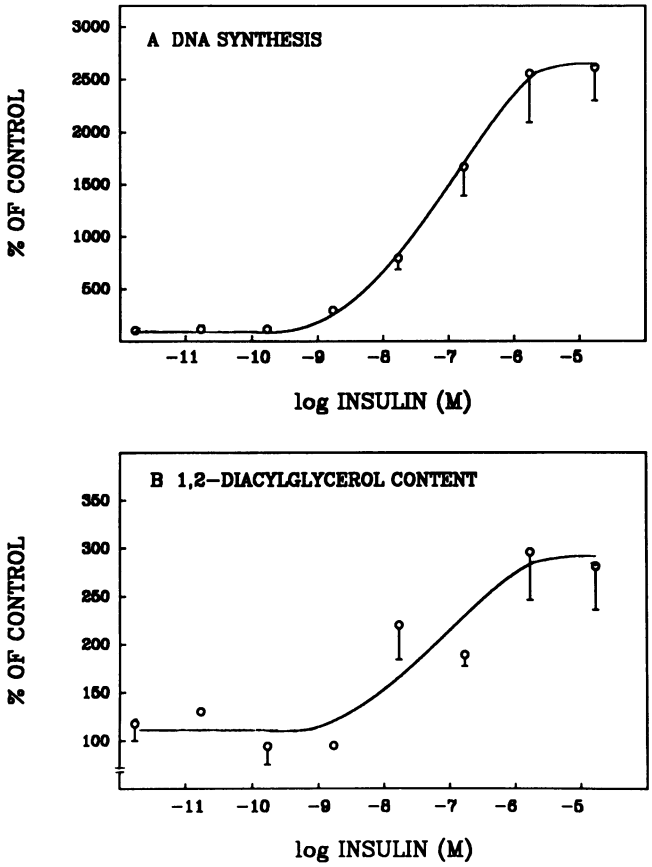

Figure 2. Effects of insulin on DNA synthesis and 1,2-DG content in FRTL-5 cells. Cells were maintained in basal medium for 7 d before initiation of experiments. (*A*) DNA synthesis, as [^{3}H]thymidine incorporation, was measured in cells incubated in basal medium without (control) or with the indicated concentrations of insulin for 3 d. [^{3}H]Thymidine incorporation in control cells was 610±110 dpm/μg DNA. Data are presented as percentage of control (mean±SE) of triplicate determinations from two experiments. When error bars are absent the SE fell within the symbol. (*B*) 1,2-DG content was measured in cells incubated in basal medium without (control) or with the indicated concentrations of insulin for 6 d. 1,2-DG content was normalized to lipid phosphorus. Data points are mean±SE of duplicate determinations from one to four experiments.

manner that was similar to that for stimulation of DNA synthesis; half-maximal stimulation of both responses occurred with ~ 5×10^{-8} M insulin. This dose-dependence is consistent with insulin acting primarily via the IGF-1 receptor. Consistent with this idea, IGF-1 and insulin stimulated similar increases in 1,2-DG content and DNA synthesis, and similarly synergized with TSH to further enhance both of these effects. However, as reported in other cell types (21), insulin may interact with both IGF-1 and insulin receptors to cause some of the effects observed herein.

Fig. 3 illustrates dose-dependent elevation of 1,2-DG content and stimulation of DNA synthesis caused by TSH in the absence or presence of 1.7×10^{-5} M insulin. In the absence of insulin, TSH caused a modest elevation of 1,2-DG and a moderate stimulation of DNA synthesis. These effects occurred at concentrations of TSH that were above the physiologic range, EC_{50} of ~ 2,000 μU/ml. In contrast, in the presence of insulin, there was a marked increase in the potency as well as the efficacy of TSH. The marked elevation of 1,2-DG content and stimulation of DNA synthesis were attained with TSH concentrations that were within the physiologic range, EC_{50} of ~ 7 uU/ml. There was an additional small increase in 1,2-DG content at high doses of TSH in the presence of insulin that corresponds to the elevation of 1,2-DG caused by high concentrations of TSH alone. The observed increase in potency of TSH in the presence of high doses of insulin was most likely not caused by changes in receptors for TSH because IGF-1 alone has no effect on TSH binding to FRTL-5 cells and IGF-1 enhances the effect of TSH to decrease TSH binding (22). Hence, the synergism of the interaction of TSH and IGF-1 (or high doses of insulin) not only allows for increased efficacy of TSH at maximum concentrations but, more importantly, permits a marked increase in the potency of TSH such that the responses occur at concentrations that are within the physiologic range.

Finally, since TSH acutely stimulates adenylyl cyclase in FRTL-5 cells and cAMP appears to mediate, at least in part, the effects of TSH to stimulate DNA synthesis (9–11), it was

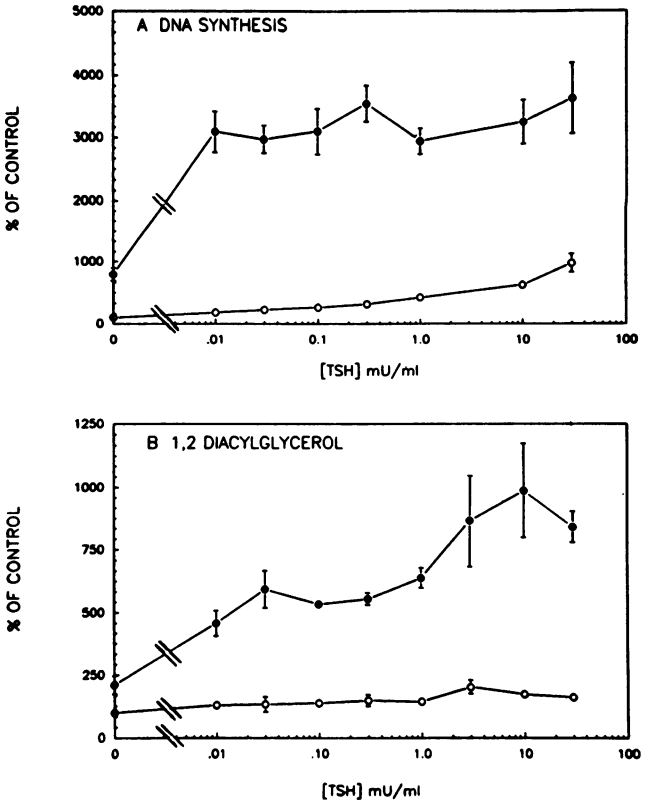

Figure 3. Effects of TSH, in the presence or absence of insulin, on DNA synthesis and 1,2-DG content in FRTL-5 cells. Cells were maintained in basal medium for 7 d before initiation of experiments. DNA synthesis, as [^{3}H]thymidine incorporation, and 1,2-DG content was measured in cells incubated in basal medium without (control) or with 1.7×10^{-5} M insulin and the indicated concentrations of TSH (denoted in mU/ml) for 6 d. (*A*) [^{3}H]Thymidine incorporation in control cells was 280±50 dpm/μg DNA. Data are presented as percent of control (mean±SD) of triplicate determinations from one experiment that is representative of four experiments. When error bars are absent the SE fell within the symbol. (*B*) 1,2-DG content was normalized to lipid phosphorus. Data points are mean±SE of triplicate determinations from two or three experiments.

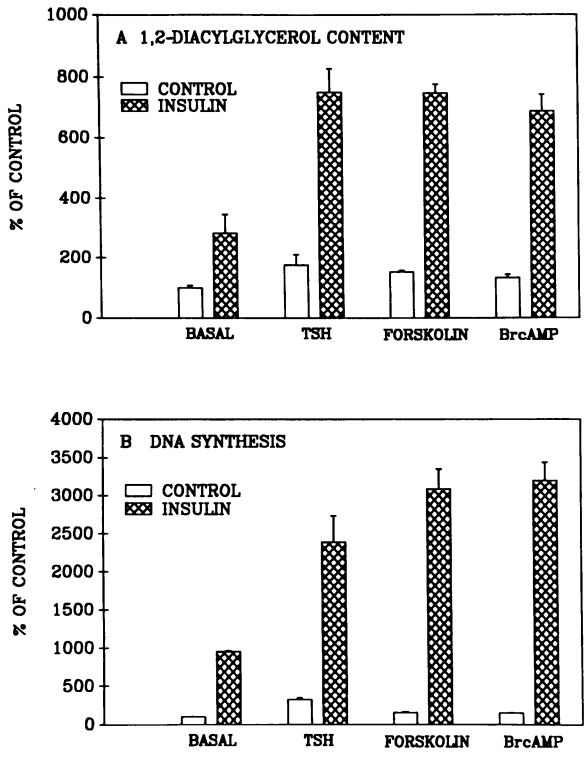

Figure 4. Effects of TSH, forskolin, BrcAMP, and insulin on 1,2-DG content and DNA synthesis in FRTL-5 cells. After 7 d in basal medium, cells were incubated in basal medium or basal medium plus 30,000 μU/ml TSH, 10 μM forskolin, or 1 mM BrcAMP without (control) or with 1.7×10^{-5} M insulin for 6 d. (*A*) 1,2-DG content of basal cells was 90±25 pmol/10^6 cells (mean±SE). Data are presented as percentage of control of triplicate determinations in two experiments. (*B*) DNA synthesis, as [^{3}H]thymidine incorporation, was 3,500±940 dpm/10^6 cells in basal cells. Data are presented as percentage of control of triplicate determinations in two experiments.

possible that cAMP was a proximal mediator of the TSH-induced increase in 1,2-DG. Fig. 4 compares the effects of TSH, forskolin, a direct stimulator of adenylyl cyclase, and BrcAMP, a cAMP analogue, alone or in combination with insulin, on cellular 1,2-DG content and DNA synthesis. TSH, forskolin, or BrcAMP alone all caused similar small increases in 1,2-DG content. When each factor was combined with insulin a synergistic increase was observed to levels that were not different from one another. In this series of experiments, insulin plus TSH, insulin plus forskolin, or insulin plus BrcAMP caused an approximately eightfold increase in 1,2-DG content. TSH, forskolin or BrcAMP alone all significantly stimulated DNA synthesis, however, TSH was approximately twice as effective as either forskolin or BrcAMP. In this series of experiments, insulin alone caused a 9.5-fold increase in DNA synthesis; this is smaller than that found in the experiments shown in Fig. 2. The cause of these variable fold stimulations in DNA synthesis is not known but does not affect comparisons within individual experiments. When insulin was combined with TSH, forskolin or BrcAMP a clearly synergistic increase in DNA synthesis was observed. As with cellular 1,2-DG content, there was no difference in stimulation of DNA synthesis caused by insulin plus TSH, insulin plus forskolin, or insulin plus BrcAMP. The reason for the enhanced effect of TSH alone to stimulate DNA synthesis as compared to maximal concentrations of forskolin or BrcAMP alone, an observation made in several series of experiments, is presently not clear. TSH may be more effective than forskolin or BrcAMP in chronically elevating intracellular cAMP concentrations. If this were correct, it suggests that less than maximal elevation of intracellular cAMP is sufficient to synergize with insulin to yield substantial elevations of cellular 1,2-DG content and stimulation of DNA synthesis. This could account, at least in part, for the effects of TSH within the physiologic concentration range. Alternatively, TSH may generate another mediator(s) in addition to cAMP and 1,2-DG that is responsible for the further increment in DNA synthesis. The involvement of another proximal mediator generated by high doses of TSH may be responsible for the increases in 1,2-DG which occur with TSH alone at supraphysiologic doses and for the additional increment in 1,2-DG observed with high doses of TSH in the presence of insulin (Fig. 3).

Because the measurements made were of total cellular 1,2-DG, it was possible that the observed elevations included pools of 1,2-DG that did not serve a regulatory role to activate protein kinase C. For example, the increases may have occurred in pools that are intermediates in de novo lipid synthesis. However, three findings are consistent with the hypothesis that the elevated 1,2-DG content is functionally related to the enhanced DNA synthesis. First, the increases in cellular 1,2-DG content correlated with increases in DNA synthesis in a highly significant manner ($r = 0.73$, $P < 0.001$). Second, the synergistic stimulation of DNA synthesis observed at 18 h was preceded by an increase in 1,2-DG content at 9 h. Third, pretreatment of these cells with phorbol esters, which we have found to down-regulate protein kinase C activity (unpublished observation) causes marked inhibition of the synergistic stimulation of DNA synthesis by TSH plus IGF-1.

The mechanism by which TSH, via cAMP, and IGF-1 interact to synergistically increase 1,2-DG content of thyroid cells is not known. It has been suggested that an extracellular signal acting via cAMP could rapidly stimulate de novo lipid synthesis (23). Also, other investigators (24, 25) have reported that TSH acutely stimulates phospholipase C-mediated hydrolysis of inositol lipids in FRTL-5 cells and it was possible that 1,2-DG could have been formed by this mechanism. Recent experiments in our laboratory, however, indicate that the synergistic elevation of 1,2-DG is not a rapid process because no increase in 1,2-DG was found at early time points and up until 6 h of exposure to TSH plus insulin (data not shown). Moreover, the stimulation by TSH of inositol lipid hydrolysis reported by others (24, 25) occurred only with concentrations of TSH that were many fold higher than needed to synergistically stimulate DNA synthesis and elevate 1,2-DG content in FRTL-5 cells. We have not observed any acute effect of TSH on inositol lipid hydrolysis with concentrations up to 30,000 uU/ml. Hence, rapid stimulation of de novo lipid synthesis or of hydrolysis of inositol lipids does not explain the increase in 1,2-DG mass reported here. It is possible that a delayed phospholipase C-mediated hydrolysis of inositol lipids or of another lipid, for example, phosphatidylcholine may be involved. It seems more likely, however, that these persistent effects of TSH and IGF-1 are mediated at the transcriptional level to induce enzymes of lipid synthesis, such as glycerol 3-phosphate acyltransferase or phosphatidic acid phosphohydrolase, and thereby stimulate formation of 1,2-DG.

Our studies suggest that protein kinase C activation resulting from the synergistic interaction of TSH, via cAMP, and IGF-1 to elevate cellular 1,2-DG levels, is an important modulator of DNA synthesis in thyroid cells. Our finding that two growth factors can interact to elevate cellular 1,2-DG levels suggests a novel site of interaction between the lipid and adenylyl cyclase signaling systems for the regulation of cell growth.

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