Insulin-like growth factor I (IGF-I) stimulates thyroid cell proliferation. Using primary cultured porcine thyroid cells, we studied the intracellular pathways that mediate the action of IGF-I on thyroid cell proliferation. IGF-I stimulates inositol phosphate accumulation, a rise in cytoplasmic free calcium ([Ca\(^{2+}\)]\(_i\)), and cell proliferation. Exposure to IGF-I results in a time- and dose-dependent accumulation of inositol monophosphate, inositol bisphosphate, and inositol trisphosphate. IGF-I also increases [Ca\(^{2+}\)]\(_i\), measured using fura-2, a fluorescent Ca\(^{2+}\) indicator; the IGF-I-induced [Ca\(^{2+}\)]\(_i\) response occurs immediately, reaches a maximum within 1 min, and then slowly declines. IGF-I stimulates thyroid cell proliferation, stimulates thymidine incorporation, and increases cell numbers. The IGF-I-induced inositol phosphate accumulation and [Ca\(^{2+}\)]\(_i\), response parallel thyroid cell proliferation in a dose-dependent manner; the maximal response is observed at a concentration of 100 ng/ml IGF-I, with half-maximal stimulation at approximately 10 ng/ml. Inositol phosphate accumulation and [Ca\(^{2+}\)]\(_i\), response after IGF-I stimulation may function as intracellular messengers for thyroid cell proliferation. This report may constitute the first demonstration of IGF-I-stimulated inositol phosphate accumulation and [Ca\(^{2+}\)]\(_i\), response in the cells.

Insulin-like growth factor I (IGF-I), a growth hormone-dependent serum growth factor, stimulates proliferation of porcine thyroid cells in primary culture (1). However, we do not know much about the intracellular pathways that mediate the action of IGF-I on thyroid cell proliferation. The intracellular pathways that mediate the action of growth factors have been intensively investigated during the last few years (2-4). Although our understanding of the mediators of growth factor action is still very limited, calcium plays important roles in the action of growth factors (3). In porcine thyroid cells, epidermal growth factor (EGF), which stimulates thyroid cell proliferation, increases cytoplasmic free calcium ([Ca\(^{2+}\)]\(_i\)), indicating that [Ca\(^{2+}\)]\(_i\) is a possible mediator of thyroid cell proliferation (5). Because an increase in [Ca\(^{2+}\)]\(_i\) has been implicated as a mediator of mitogenesis in thyroid cells, the effect of IGF-I on [Ca\(^{2+}\)]\(_i\), was studied. We show that IGF-I causes an increase in [Ca\(^{2+}\)]\(_i\) in cultured porcine thyroid cells.

As a further step, we studied the mechanism of this IGF-I-stimulated increase in [Ca\(^{2+}\)]\(_i\). Some growth factors cause inositol lipid breakdown and produce inositol phosphates, which increase [Ca\(^{2+}\)]\(_i\) (4). EGF was reported to stimulate inositol phosphate accumulation in thyroid cells (5). Thus, it is of interest to know whether IGF-I causes inositol phosphate accumulation in these cells.

We report here that IGF-I leads to an accumulation of inositol phosphates and induces an increase in [Ca\(^{2+}\)]\(_i\). IGF-I also stimulates thyroid cell proliferation. These data suggest that inositol phosphate accumulation and [Ca\(^{2+}\)]\(_i\), response may function as intracellular messengers for IGF-I-stimulated thyroid cell proliferation.

**EXPERIMENTAL PROCEDURES**

Thyroid Cell Culture—Thyroid cells were obtained from porcine thyroid glands as described previously (6). Freshly isolated cells were suspended (3 \times 10^5 cells/ml) in Eagle's minimum essential medium (EMEM) or inositol-free EMEM supplemented with 0.5% human IGF-I-deficient serum and antibiotics (penicillin, 200 units/ml; streptomycin, 50 μg/ml). Cells were cultured as a suspension at 37°C in a 95% air:5% CO\(_2\) water-saturated atmosphere (7).

**Inositol Phosphate Accumulation**—The cells were cultured in an inositol-free EMEM for 16 h, when 5 μl/ml of myo-[2-\(^3\)H]inositol was added. Then the cells were incubated in the presence of \(^3\)H]inositol for 24 h, after which the experiments were conducted.

The labeled cells were washed three times with phosphate-buffered saline (6), resuspended in phosphate-buffered saline glucose (10 cells/ml), and preincubated for 15 min; IGF-I or buffer was then added to study the effects of IGF-I on inositol phosphate accumulation. Incubations were terminated by the addition of 3 ml of CHCl\(_3\)-CH\(_2\)-OH:13 N HCl (200:100:1, v/v) and 100 μl of 100 mM EDTA. The tubes were vortex-mixed for 30 s and subsequently centrifuged to separate the two phases. The upper aqueous phase was removed, neutralized with 200 μl of 1 M NaOH, mixed with 2.5 ml of H2O2, and applied to a column containing 1 ml of Dowex AG 1-X8 (200-400, formate form). The free inositol and inositol phosphates were eluted in a sieving manner as described by Berridge et al. (8). Free \(^3\)H]inositol was washed through with water. Inositol phosphates were eluted with increasing concentrations of ammonium formate in 0.1 M formic acid:inositol monophosphate (IP\(_1\)), 0.2 M ammonium formate; inositol bisphosphate (IP\(_2\)), 0.4 M ammonium formate; inositol trisphosphate (IP\(_3\)), 1 M ammonium formate. Fractions (8 ml) were collected and counted for radioactivity in Aquasol scintillant. Previous studies have indicated that isomers of inositol phosphates are formed (9), but no attempts were made to characterize the different isomers, because the purpose of the present study was to study whether IGF-I could couple to inositol metabolism.

\([Ca\(^{2+}\)]\(_i\), Measurement by Fura-2—Isolated thyroid cells were cul-

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1. The abbreviations used are: IGF-I, insulin-like growth factor I; EGF, epidermal growth factor; [Ca\(^{2+}\)]\(_i\), cytoplasmic free calcium; EMEM, Eagle's minimum essential medium; IP\(_1\), inositol monophosphate; IP\(_2\), inositol bisphosphate; IP\(_3\), inositol trisphosphate; EGTA, ethylene bis(oxyethylenenitrilo)tetracetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
IGF-I Increases IP₃, Ca²⁺, and Growth in Thyroid Cells

Phosphate (IP₃) (Fig. 1, A)

Inositol Phosphate Accumulation—Addition of 100 ng/ml IGF-I to the cells, prelabeled with [³H]inositol, resulted in rapid increases in inositol bisphosphate (IP₂) and inositol trisphosphate (IP₃) (Fig. 1, A, B, D, and E). Within 30 s of IGF-I exposure, IP₂ and IP₃ concentrations began to rise and reached peak levels within 2–3 min in the absence (Fig. 1, A and B) or presence of 10 mM LiCl (Fig. 1, D and E). In the presence of LiCl, the IP₂ and IP₃ levels were much higher both under the basal and IGF-I-stimulated conditions (note the differences in ordinate scales). The ranges of maximal increases in IP₂ and IP₃ levels provoked by IGF-I to control values were both 2.0-fold in the absence of LiCl and 2.4- and 2.6-fold, respectively, in the presence of LiCl. The peak levels of radiolabeled IP₂ and IP₃ in the presence of LiCl were twice as high as in its absence under the basal and IGF-I-stimulated conditions.

One hundred ng/ml IGF-I increased inositol monophosphate (IP₁) (Fig. 1, C and F). In the absence of LiCl, IP₁ levels reached a maximum at about 5 min and remained nearly constant at approximately twice the basal levels. In contrast, IP₁ continued to accumulate in the presence of LiCl. In all cases, IP₁ levels were much higher in the presence of LiCl.

The dose dependence of IGF-I on inositol phosphate accumulation in the presence of 10 mM LiCl is shown in Fig. 2.
IGF-I Increases IP<sub>3</sub>, Ca<sup>2+</sup>, and Growth in Thyroid Cells

**FIG. 3.** IGF-I-induced increase in cytoplasmic free calcium concentration ([Ca<sup>2+</sup>]). Fura-2-loaded cells were stimulated by 100 ng/ml IGF-I at the point indicated by an arrow.

**FIG. 4.** Dose dependence of IGF-I on peak response of cytoplasmic free calcium concentration ([Ca<sup>2+</sup>]). Fura-2-loaded cells were incubated with increasing doses of IGF-I. The peak [Ca<sup>2+</sup>] response to IGF-I stimulation are shown; 1 ng/ml IGF-I increased the peak [Ca<sup>2+</sup>] response significantly. Each point is the mean ± S.E. of four to six determinations.

The maximal inositol phosphate accumulation was observed at a concentration of 100 ng/ml IGF-I, with half-maximal stimulation at approximately 10 ng/ml.

Cytoplasmic Free Calcium ([Ca<sup>2+</sup>])—In 20 different preparations, the cytoplasmic free calcium ([Ca<sup>2+</sup>]) concentration was 120 ± 22 nM. Addition of 100 ng/ml IGF-I resulted in a prompt increase in [Ca<sup>2+</sup>], (Fig. 3). The IGF-I-induced [Ca<sup>2+</sup>] response occurred immediately with no detectable lag, reached a maximum within 1 min, and then slowly declined.

The dose-response characteristics using the peak increase in [Ca<sup>2+</sup>], following IGF-I administration are shown in Fig. 4. A small but clear increase in [Ca<sup>2+</sup>], was induced by IGF-I at 1 ng/ml. The maximal increase in [Ca<sup>2+</sup>], was observed at a concentration of 100 ng/ml IGF-I, with half-maximal stimulation at approximately 10 ng/ml.

**Thyroid Cell Proliferation**—The effects of IGF-I on thyroid cell proliferation are shown in Figs. 5 and 6. One hundred ng/ml IGF-I produced a gradual increase in [3H]thymidine incorporation (Figs. 5A and 6A). A significant effect was observed at 6 h (Fig. 5A). IGF-I increased [3H]thymidine incorporation in a dose-dependent manner (Fig. 5B); the minimal effective dose was 1 ng/ml, and the maximal response was observed at a concentration of 100 ng/ml IGF-I, with half-maximal stimulation at approximately 10 ng/ml. The maximal stimulation of thymidine incorporation by IGF-I was 6 times the basal level. To determine that thymidine incorporation represented the mitogenic effect of IGF-I, the numbers of thyroid cells were counted (Figs. 5C and 6B). Forty-eight-h exposure to IGF-I increased the numbers of thyroid cells in a dose-dependent manner; the maximal response was observed at a concentration of 100 ng/ml IGF-I, with half-maximal stimulation at approximately 10 ng/ml. One hundred ng/ml IGF-I produced a gradual increase in cell numbers (Fig. 6B); 200% increase was obtained after a 3-day exposure to 100 ng/ml IGF-I.

**DISCUSSION**

Little is known about the intracellular pathways that mediate the action of IGF-I on thyroid cell proliferation. Using primary cultured porcine thyroid cells, we clearly demonstrated IGF-I-stimulated inositol phosphate accumulation and [Ca<sup>2+</sup>] response. IGF-I also stimulates thyroid cell proliferation. Inositol phosphate accumulation and [Ca<sup>2+</sup>] response may function as intracellular messengers for proliferation. This report may constitute the first demonstration of IGF-I-stimulated inositol phosphate accumulation and [Ca<sup>2+</sup>] response in the cells.

As has been reported for primary cultured thyroid cells (1) and a continuous function line of cultured rat thyroid cells (FRTL-5) (13, 14), IGF-I stimulated primary cultured porcine thyroid cell proliferation. We demonstrated that IGF-I increased [Ca<sup>2+</sup>]. As Ca<sup>2+</sup> is implicated as a messenger for cell proliferation (3), our findings suggest that Ca<sup>2+</sup> may be a messenger in the action of IGF-I on thyroid cell proliferation.
IGF-I Increases IP₃, Ca²⁺, and Growth in Thyroid Cells

EGF has been reported to increase [Ca²⁺], in porcine thyroid cells (5). These results indicate that [Ca²⁺] plays a messenger for thyroid cell proliferation.

By which route does IGF-I trigger the release of intracellular Ca²⁺? A striking feature of Ca²⁺-mobilizing hormones is that they provoke a rapid breakdown of inositol phospholipids in their target cells, and, indeed, the growth factors are no exception (4). In particular, the very rapid accumulation of inositol trisphosphate (IP₃), occurring without a measurable lag period, appears to constitute the key signal for internal Ca²⁺ release and thus underlies the [Ca²⁺] response. Here, we show that IGF-I stimulates IP₃, IP₂, and IP₃ accumulation, which increase [Ca²⁺] (15).

The IGF-I-induced inositol phosphate accumulation and [Ca²⁺] response parallel thyroid cell proliferation in a dose-dependent manner. Thus, IGF-I-stimulated thyroid cell proliferation may be mediated by inositol phosphate accumulation and [Ca²⁺] response. A similar conclusion has been reached for the messengers of EGF- and platelet-derived growth factor-stimulated fibroblast and EGF-stimulated epidermoid carcinoma cell proliferation (16–18). Recently, Berridge (4) reviewed inositol lipids and cell proliferation and summarized growth stimuli which were known to stimulate the hydrolysis of inositol lipids. Metcalfe et al. (3) also reviewed calcium and cell proliferation. However, IGF-I was not included in their reviews. The new finding that IGF-I induces inositol phosphate accumulation and [Ca²⁺] response in the cells will provide further information on the intracellular pathways of IGF-I-stimulated cellular events.

A major question, to which we have no answer as yet, is what is the physiological role of [Ca²⁺] in the action of IGF-I on cell proliferation? Further studies are needed to establish whether [Ca²⁺] plays a functional role in the initiation of proliferative response. A tumor promoter, 12-O-tetradecanoylphorbol-13-acetate, an activator of protein kinase C, has been reported to stimulate porcine thyroid cell proliferation in primary culture (11), indicating that thyroid cell proliferation is stimulated through activation of protein kinase C. An increase in [Ca²⁺], may augment this activation.

In thyroid cells, epinephrine, thyroid-stimulating hormone, and carbachol have been reported to stimulate inositol phosphate production and increase [Ca²⁺]. (19–23). IGF-I has been reported to elevate 1,2-diacylglycerol in FRTL-5 cells (24), suggesting that IGF-I-stimulated DNA synthesis might be associated with phosphatidylinositol hydrolysis. However, IGF-I-stimulated inositol phosphate accumulation and [Ca²⁺] response have not been reported, although EGF-stimulated ones have been (5).

IGF-I has been isolated from human plasma and plays an important role in cell growth. Patients with acromegaly always have elevated levels of IGF-I, a growth hormone-dependent serum growth factor, and they usually have goiter. Our findings are compatible with the view that IGF-I may be involved in their goiter formation. IGF-I stimulates thyroid cell proliferation possibly through inositol phosphate accumulation and [Ca²⁺] response.

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