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EPIDERMAL GROWTH FACTOR (EGF) PRODUCES INOSITOL PHOSPHATES AND INCREASES CYTOPLASMIC FREE CALCIUM IN CULTURED PORCINE THYROID CELLS

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The initial signal for thyroid cell proliferation is unknown. This is the first report to show that epidermal growth factor (EGF) produces inositol phosphates and increases cytoplasmic free calcium ($[Ca^{2+}]i$) in the thyroid gland. In cultured porcine thyroid cells, 10 nM EGF produces a breakdown of phosphatidylinositol and stimulates inositol phosphate production. Ten nM EGF increases $[Ca^{2+}]i$, measured using fura-2, a fluorescent Ca^{2+} indicator; the EGF-induced $[Ca^{2+}]i$ response occurs immediately, reaches a maximum within several seconds, and then slowly declines. EGF stimulates production of inositol phosphates, which seem to increase $[Ca^{2+}]i$. Inositol phosphate production and an increase in $[Ca^{2+}]i$ after EGF-stimulation may function as an initial signal for thyroid cell proliferation.

Epidermal growth factor (EGF) stimulates proliferation of porcine thyroid cells in primary culture (1). However, the mechanism of this is unknown. Growth stimulation of cells triggers a cascade of biochemical and physiological changes in the cells, eventually leading to replicate DNA synthesis. In the search for putative intracellular mitogenic signals, much attention has been focused on the earliest detectable events following the stimulation by growth factors. The initial consequences of growth factor-stimulation include tyrosine-specific protein phosphorylations (2-5), inositol lipid breakdown (6,7), and an increase in cytoplasmic free calcium ($[Ca^{2+}]i$) (8-10). Some growth factors cause inositol lipid breakdown and produce inositol phosphates, which increase $[Ca^{2+}]i$ (11). However, as yet, no direct evidence for a role of inositol phosphates or $[Ca^{2+}]i$ in growth factor action has been reported in thyroid cells. A phosphatidylinositol breakdown is associated with an increase in $[Ca^{2+}]i$, and inositol triphosphate produces an increase in $[Ca^{2+}]i$ (11). Thus it is of interest to know whether EGF causes inositol phosphate production

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and $[Ca^{2+}]i$ increase in thyroid cells.

We studied the effects of EGF on inositol phosphate production and $[Ca^{2+}]i$ increase in cultured porcine thyroid cells. We used the intracellularly trapped fluorescent Ca^{2+} indicator, fura-2 (12), to monitor continuously the effect of EGF on $[Ca^{2+}]i$. We report here that EGF produces inositol phosphates and induces an increase in $[Ca^{2+}]i$, which represents the earliest cellular response to EGF thus far described in the thyroid gland.

MATERIALS AND METHODS

Thyroid cell culture: Thyroid cells were obtained from porcine thyroid glands as described previously (13). Freshly isolated cells were suspended ($3x10^6$ cells/ml) in Eagle's minimum essential medium (EMEM) or inositol-free EMEM supplemented with 1% fetal-calf 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.

Inositol phosphates measurement: Inositol phosphate production was studied as reported by Berridge et al. (14). The cells were cultured in an inositol-free medium for 16 h, when 5 µCi/ml myo-[2-^{3}H]inositol (New England Nuclear, Boston, MA) 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 3 times with PBSG, suspended in PBSG (10^6 cells/0.5 ml) containing 10 mM LiCl [to inhibit the hydrolysis of inositol 1-phosphate (15)], and preincubated for 15 min; 10 nM EGF or buffer was then added, to study the effects of EGF on inositol phosphates. Incubations were terminated by the addition of 3 ml $CHCl_3/CH_3OH/13$ N HCl (200:100:1, vol/vol) and 100 µl 100 mM EDTA. The tubes were vortex-mixed for 30 sec and subsequently centrifuged to separate the two phases. The upper aqueous phase was removed, neutralized with 200 µl 1 M NaOH, mixed with 2.5 ml H_2O, and applied to a column containing 1 ml Dowex AGIX8 (200-400, formate form). The free inositol and inositol phosphates were eluted in a stepwise manner as described by Berridge et al. (14). The radioactivity of each fraction was counted following the addition of Aquasol scintillant.

$[Ca^{2+}]i$ measurement by fura-2: Isolated thyroid cells were cultured in EMEM for 40 h and then the cells were loaded with fura-2, as described before (12). Fura-2 AM (1 µM; final concentration) was added to the EMEM buffered with 10 mM HEPES (pH 7.4). The cells were incubated for 45 min in the culture conditions. Under these conditions, fura-2 AM permeates the cells, is hydrolyzed to fura-2, and binds cytoplasmic free calcium. To remove the unincorporated probe, the cells were centrifuged for 3 min at 500xg, and washed in PBSG-HEPES solution, consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.45 mM $CaCl_2$, 0.49 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES (12). The cells were washed twice and resuspended in the same buffer (10^6 cells/ml). The cell suspension was transferred to a thermostatic quartz cuvette (37 °C), for measurement of calcium concentrations ($[Ca^{2+}]i$) in a Hitachi F-4000 fluorometer (Hitachi, Tokyo). The cells were stirred continuously. After reaching a steady state, EGF was added to the cuvette using a syringe. Fluorescence was recorded with excitation and emission wavelengths of 340 and 505 nm respectively. Fluorescence was corrected for cell autoflurescence at 380 nm. The cells were subsequently lysed by adding Triton X-100 (0.3 %; final concentration) to obtain the signal of the calcium-saturated dye (Fmax). The signal (Fmin) from the Ca^{2+} free form of the dye was recorded by adding EGTA at pH 8.2 to a final concentration of 10 mM. The $[Ca^{2+}]i$ corresponding to an intracellular fura-2 fluorescence (F) was calculated by the equation: $[Ca^{2+}]i = Kd (F-Fmin/Fmax-F)$, using Kd = 224 mM according to Grynkiewicz et al. (12).

Materials etc.: Murine epidermal growth factor (EGF) was obtained from Collaborative Research (Waltman, MA, U.S.A.); Dowex AGIX8 from Sigma Chemical Co. (St. Louis, MO, U.S.A.); fura-2 AM from Molecular Probes, Inc. (Eugene, OR, U.S.A.); fetal-calf serum from Flow Laboratories (Irvine, Scotland, U.K.). All other chemicals were of the highest purity available commercially. Experiments were conducted at least 4 times. Typical data and the final concentration of EGF are shown in the text and figures. Student's t-test was used for statistical evaluations.

RESULTS

EGF-stimulated inositol phosphate production. The porcine thyroid cells were prelabeled with ^{3}H-inositol. Ten nM EGF stimulated inositol phosphate (IP), inositol biphosphate (IP_2) and inositol triphosphate (IP_3) production (Figure 1), which increased gradually up to 15 min. Incubation of cells without EGF did not increase the formation of inositol phosphates during this 15 min incubation.

EGF-induced increases in $[Ca^{2+}]i$. The cells were loaded with fura-2. Addition of 10 nM EGF resulted in a prompt increase in $[Ca^{2+}]i$ (Fig. 2). The EGF-induced

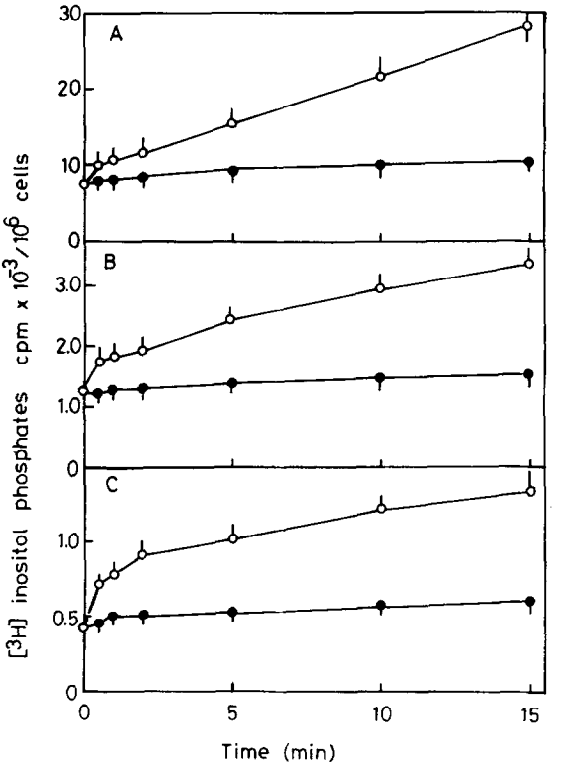

Fig. 1. EGF-induced production of inositol phosphates in porcine thyroid cells. The cells, prelabeled with ^{3}H-inositol, were incubated with (O-O) or without (●-●) 10 nM EGF. EGF stimulated production of inositol phosphate (A), inositol biphosphate (B) and inositol triphosphate (C). Results shown are the mean±SE of four sets of experiments using different cell preparations.

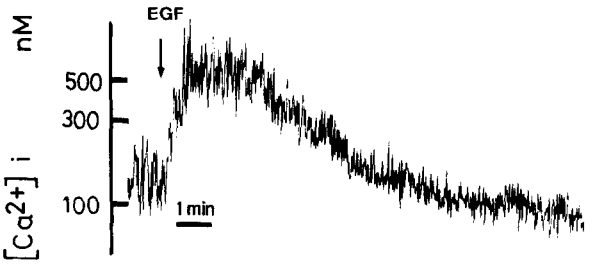

Fig. 2. EGF-induced increase in cytoplasmic free calcium concentration ($[Ca^{2+}]i$). Fura-2-loaded cells were stimulated by 10 nM EGF as indicated by an arrow.

$[Ca^{2+}]i$ response occurred immediately with no detectable lag, reached a maximum within several seconds, and then slowly declined.

DISCUSSION

EGF stimulates inositol phosphate production and induces an increase in $[Ca^{2+}]i$ in cultured porcine thyroid cells. The present study represents the first demonstration of inositol phosphate (IP), inositol biphosphate (IP_2) and inositol triphosphate (IP_3) production and an increase in $[Ca^{2+}]i$ after EGF-stimulation in the thyroid cells.

EGF has been reported to stimulate proliferation of porcine thyroid cells in primary culture (1). As Ca^{2+} is well known as a regulator of numerous cellular functions, our findings suggest that Ca^{2+} is a primary messenger in the action of EGF on thyroid cell proliferation. A similar conclusion has been reached for the action of growth factors in human fibroblasts (8), in Swiss 3T3 fibroblasts (16) and in human A431 epidermoid carcinoma cells (10).

By which route does EGF trigger the release of intracellular Ca^{2+}? A striking feature of Ca^{2+}-mobilizing hormones is that they provoke a rapid breakdown of inositolphospholipids in their target cells (17,18), and, indeed, the growth factors are no exception (6,7,19). In particular, the very rapid formation of inositol triphosphate (IP_3), occurring without a measurable lag period, appears to constitute the key signal for internal Ca^{2+} release (20) and thus underlies the $[Ca^{2+}]i$ responses. Here, we show that EGF stimulates IP_1, IP_2 and IP_3 production, which increases $[Ca^{2+}]i$.

EGF induces an increase in $[Ca^{2+}]i$, which may stimulate thyroid cell proliferation. A major question, to which we have no answer as yet, is what is the physiological role of $[Ca^{2+}]i$ in the action of EGF on cell proliferation? Further studies are needed to establish whether $[Ca^{2+}]i$ plays a functional role in the initiation of a proliferative response.

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