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CYTOPLASMIC pH IN THE ACTION OF EPIDERMAL GROWTH FACTOR (EGF) IN CULTURED PORCINE THYROID CELLS

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We demonstrate measurement of cytoplasmic pH (pHi), using 2',7'-bis(2-carboxyethyl)-5 (and 6-) carboxyfluorescein (BCECF), an internalized fluorescent pHi indicator, in thyroid cells. Using cultured porcine thyroid cells, we studied the effects of epidermal growth factor (EGF) on pHi and [^{3}H] thymidine incorporation; 10 nM EGF alkalinizes thyroid cells and stimulates thymidine incorporation. The results indicate that Na^+/H^+ exchange or cell alkalinization may function as a transmembrane signal transducer in the action of EGF in the thyroid cells.

The mechanisms by which growth factors stimulate metabolism and cell proliferation are largely unknown. Recent evidence suggests that mitogens rapidly activate Na^+/H^+ exchange in the plasma membrane and alkalinize the cells, implicating cytoplasmic pH (pHi) as a potential "messenger" for cell growth (1-5). Recently, 2',7'-bis(2-carboxyethyl)-5 (and 6-) carboxyfluorescein (BCECF) (6), an internalized fluorescent pHi indicator, has been used to examine the pHi-regulating mechanisms. Epidermal growth factor (EGF) is known to stimulate thyroid cell proliferation (7-9). However, the mechanism of this is unknown. Using cultured porcine thyroid cells, we studied the effects of EGF on pHi and thymidine incorporation. We intended to show that Na^+/H^+ exchange or cell alkalinization might function as a transmembrane signal transducer for thyroid cell growth.

MATERIALS AND METHODS

Thyroid cell culture: Thyroid cells were obtained from porcine thyroid glands as described previously (10). Freshly isolated cells were suspended ($3x10^6$ cells/ml) in Eagle's minimum essential medium (MEM) supplemented with 0.5%

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fetal-calf serum and antibiotics (penicillin, 200 units/ml; streptomycin, 50 ug/ml). Cells were cultured as a suspension at 37°C in a 95% air: 5% CO_2 water-saturated atmosphere (11).

Cytoplasmic pH (pHi): Cytoplasmic pH (pHi) was measured with the tetraacetoxymethyl ester (BCECF-AM) of 2',7'-bis(2-carboxyethyl)-5 (and 6-) carboxyfluorescein (BCECF) (6). Porcine thyroid cells, cultured for 16 h, were washed twice in a standard HEPES-Na^+ solution containing 140 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and buffered with 20 mM HEPES, pH 7.2. The cells were resuspended in the same buffer containing 3 μM BCECF-AM and incubated for 30 min at 37°C. BCECF-AM was converted to the impermeant BCECF by cytoplasmic esterases (6). After loading, the cells were washed twice in HEPES-Na^+ solution to remove extracellular dye and were resuspended at $3x10^6$ cells/ml. The BCECF-loaded cells were put into a thermostatic cuvette in a Hitachi F-4000 fluorometer (Hitachi, Tokyo), and the cell suspension was continuously stirred with a magnetic microbar. The pHi-dependent emission intensity was continuously recorded (excitation wavelength 500 nm; emission wavelength 530 nm; slits 5.5/11-nm bandpass). Calibration of cytoplasmic dye fluorescence as a function of pHi was obtained from H^+ equilibration methods using the K^+/H^+ ionophore nigericin, which sets $[H^+]i/[H^+]o$ equal to $[K^+]i/[K^+]o$ as previously described (12). Briefly, calibration was obtained by incubation of BCECF-loaded cells in K^+ medium (140 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose) buffered with 20 mM HEPES, and containing nigericin (0.7 μg/ml). The pHi-dependent emission intensity was then measured.

Incorporation of [^{3}H] thymidine: Isolated thyroid cells were cultured for 16 h and then EGF and 0.1 μCi of [^{3}H] thymidine were added to measure the effect of EGF on thymidine incorporation. After incubation, the cells were collected and washed three times with 10% trichloroacetic acid (TCA) and dissolved in 0.3 N NaOH. Then their radioactivities were measured in a liquid scintillation counter.

Materials etc.: Murine epidermal growth factor (EGF) was obtained from Collaborative Research (Waltman, MA, U.S.A.); nigericin and HEPES from Sigma (St. Louis, MO, U.S.A.); fetal calf serum and Eagle's MEM from Flow Laboratories (Irvine, Scotland, U.K.); [^{3}H] thymidine (2 Ci/mmol) from New England Nuclear Corp. (Boston, MA, U.S.A.); BCECF-AM from Molecular Probes, Inc. (Junction City, OR, U.S.A.). BCECF-AM was dissolved in dimethyl sulfoxide and stored at -20°C. 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 the figure. Data were statistically analyzed with analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

RESULTS

EGF-stimulated thyroid cell alkalinization and thymidine incorporation.

The cells were loaded with BCECF and then pHi was recorded continuously (Fig. 1). The resting pHi of porcine thyroid cells in HEPES-Na^+ solution at 37°C was 7.22 ± 0.04 (n=8). Addition of 10 nM EGF to these cells induced intracellular alkalinization, reaching a pH of 7.39 ± 0.06 (n=8) in 3 min.

Ten nM EGF produced a gradual increase in [^{3}H] thymidine incorporation (Fig. 2). A significant effect was observed at 6 h.

DISCUSSION

We have demonstrated measurement of intracellular pHi, using BCECF, an internalized fluorescent pHi indicator, in thyroid cells. EGF stimulates cell

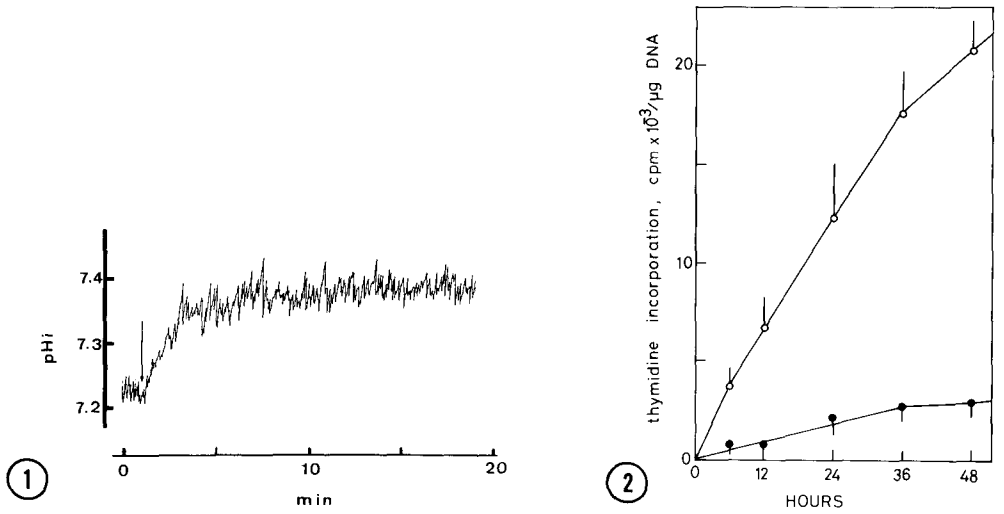

Fig. 1. EGF-induced alkalinization in cytoplasmic pH (pHi). BCECF-loaded cells were stimulated by 10 nM EGF as indicated by an arrow.

Fig. 2. EGF-induced [^{3}H] thymidine incorporation. The cells were incubated with (O-O) or without (●-●) 10 nM EGF. EGF stimulated thymidine incorporation significantly at 6 h ($P<0.01$). Each point is the mean±SE of 8-12 determinations.

alkalinization and proliferation, indicating that Na^+/H^+ exchange or cell alkalinization may function as a transmembrane signal transducer in the action of EGF in the thyroid cells.

EGF has been reported to stimulate thyroid cell proliferation (7-9). A central problem that remains to be fully elucidated is the cascade of early events stimulated by EGF. Stimulation with EGF induces an ordered sequence of biochemical events leading to cell division. How does the plasma membrane transfer the environmental information to the cell in order to elicit the proliferation response? The first step in the action of EGF is the binding to the EGF receptor at the cell surface. The EGF-receptor complex is coupled to membrane effector systems that generate internal signals. The initial signals trigger complex cellular responses and initiate DNA synthesis. One of the earliest responses to EGF stimulation is Na^+/H^+ exchange or cell alkalinization (1-5). In thyroid cells, EGF stimulates cell alkalinization and proliferation, indicating that stimulation of Na^+/H^+ exchange or alkalinization may play an important role in thyroid cell proliferation. A variety of mitogens have been reported to induce cytoplasmic alkalinization (1-5). Thus stimulation of the

Na^+/H^+ exchange appears to be a ubiquitous phenomenon in the proliferation process. However, this is the first report to demonstrate that EGF induces cytoplasmic alkalinization in thyroid cells.

A very important consequence of the activation of Na^+/H^+ exchange by mitogens is alkalinization of the cytoplasm. A significant insight into our understanding of this phenomenon has come from the use of a fluorescence pH indicator, BCECF (6). Changes in cytoplasmic pH can be conveniently monitored by recording the pH-dependent emission. Here we show that we can use BCECF to measure thyroid cell pHi.

EGF induces cytoplasmic alkalinization, which may stimulate thyroid cell proliferation. A major question, to which we have no answer as yet, is the physiological role of alkalinization in the action of EGF on cell proliferation. Further studies are required to establish whether alkalinization plays a functional role in the initiation of proliferation.

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