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The Effect of Ghrelin on Ca^{2+} Concentration in Thyroid FRTL-5 Cells

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Ghrelin is a newly discovered peptide, which is released from the stomach and neurons in the hypothalamic arcuate nucleus (ARC), and potently stimulates growth hormone release and food intake. In the present study, we investigated the effect of ghrelin on $[Ca^{2+}]_i$ in thyroid FRTL-5 cells. Ghrelin (5 nM) increased $[Ca^{2+}]_i$ and TSH (1 unit/l) had an additive effect on $[Ca^{2+}]_i$ when extracellular normal solution was 1.1mM Ca^{2+} containing Coon's modified Ham's F12 medium. When Ca^{2+}-free medium containing 2 mM EGTA replaced the above normal solution, ghrelin also induced a similar rise in $[Ca^{2+}]_i$. In the middle of $[Ca^{2+}]_i$ increment by ghrelin, nifedipine (1 μM), nickel (100 μM) and La^{3+} (100 μM) had no effect on $[Ca^{2+}]_i$. After endoplasmic reticulum was depleted by cyclopiazonic acid (CPA;10 μM), ghrelin caused no visible change on $[Ca^{2+}]_i$ in Ca^{2+}-free/2 mM EGTA solution. These results suggest that ghrelin can increase $[Ca^{2+}]_i$ through endoplasmic reticulum in thyroid FRTL-5 cells.

Key Words: Ghrelin, Thyroid FRTL-5 cell, Endoplasmic reticulum

INTRODUCTION

Ghrelin, an endogenous ligand for the growth hormone secretagogue (GHS) receptor (GHSR), is abundantly synthesized in the stomach and to a much lesser extent in the hypothalamic arcuate nucleus (ARC) (Kojima et al, 1999). Peripheral or intracerebroventricular (ICV) injection of ghrelin releases growth hormone (GH), stimulates food intake, and increases body weight in mice, rats, and humans (Tschop et al, 2000; Asakawa et al, 2001; Inui et al, 2001; Nakazato et al, 2001). These findings have suggested that ghrelin plays a physiological role in the meal initiation. GHS receptors are detected mainly in the myocardium, but they are also present in thyroid cells (Papotti et al, 2000). Therefore, ghrelin might have an important role in thyroid cells.

In thyroid cells, as in several other cell types, changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) regulate a multitude of cellular functions (Ginsberg et al, 1997). Several studies have shown that changes in $[Ca^{2+}]_i$ evoked by ATP, carbachol, noradrenaline, and also by thyrotropin (TSH), may regulate the efflux of iodide (Weiss et al, 1984; Corda et al, 1985), cellular proliferation (Takada et al, 1990), the expression of receptors for TSH (Saji et al, 1991) and the generation of H_2O_2 (hydrogen peroxide) (Lippes & Spaulding, 1986).

Ghrelin-induced GH release was entirely dependent on extracellular Ca^{2+} influx through L-type voltage-sensitive channels (Malagon et al, 2003). The stimulatory effect of ghrelin on somatotropes was greatly attenuated in low-calcium saline and blocked by nifedipine, an L-type calcium channel blocker, suggesting involvement of calcium channels. In a zero Na^+ solution, the stimulatory effect of ghrelin on somatotropes was decreased, suggesting that besides calcium channels, sodium channels are also involved in ghrelin-induced calcium transients (Glavaski-Joksimovic et al, 2003). In the rat arcuate nucleus, ghrelin increased $[Ca^{2+}]_i$ concentration-dependently via PKA and N-type Ca^{2+} channel-dependent mechanisms (Kohno et al, 2003).

Ghrelin-induced GH release was also dependent on intracellular mechanisms. In somatotropes from pig pituitary cells, blockade of phospholipase C or protein kinase C inhibited ghrelin-induced GH secretion, suggesting that involvement of this route in ghrelin action (Malagon et al, 2003).

To understand pathophysiological mechanisms of thyroid diseases, researchers have usually used thyroid cell lines. Among thyroid cell lines, rat thyroid FRTL-5 cells proliferate and synthesize DNA by TSH and insulin/IGF-1 (Koide et al, 1998). According to recent reports, FRTL-5 cells proliferate in response to insulin/IGF-1 alone and the TSH/cAMP cascade amplifies this effect (Kimura et al, 2001). By contrast, however, Medina et al (2000) reported that FRTL-5 cells can proliferate in response to TSH alone.

Nevertheless, the effect of ghrelin on $[Ca^{2+}]_i$ in the thyroid FRTL-5 cells has not yet been investigated. Therefore, we

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ABBREVIATIONS: GHS, growth hormone secretagogue; TSH, thyrotropin; HBSS, Hepes-buffered saline solution; CPA, cyclopiazonic acid; ER, endoplasmic reticulum.

undertook to investigate the effect of ghrelin on $[Ca^{2+}]_i$ in the thyroid FRTL-5 cells.

METHODS

Cell culture

Rat thyroid FRTL-5 cells were originally obtained from the Interthyr Research Foundation (Bethesda, MD, USA). The cells were grown in Coon's modified Ham's F12 medium, supplemented with 5% (v/v) fetal calf serum and six hormones [insulin, 10 μg/ml; transferrin, 5 μg/ml; cortisol, 10 nM; tripeptide Gly-l-His-l-Lys, 10 ng/ml; TSH, 10 m-unit/ml; somatostatin, 10 μg/ml (Ambesi-Impiombato et al, 1980)] in a water-saturated atmosphere of air/CO_2 (19 : 1) at 37°C. Cells from one donor culture dish were harvested with 0.1% trypsin solution, plated onto plastic 35-mm culture dishes, and grown for 4~5 days with 2~3 changes of the culture medium before experiment. Fresh medium was always added 24 h prior to an experiment.

Solution and drugs

Cells were transferred to an experimental chamber, and the chamber was mounted on the stage of an inverted microscope (Diaphot 300, Nikon, Japan) and superfused with Coon's modified Ham's F12 medium or Hepes-buffered saline solution (HBSS: 118 mM NaCl, 4.6 mM KCl, 10 mM glucose, 1mM $CaCl_2$ and 20 mM Hepes, pH 7.2). Ca^{2+} was excluded from normal solution, and 2mM EGTA was added to make the Ca^{2+}-free solution. To deplete endoplasmic reticulum, cyclopiazonic acid (CPA) was used. All the chemicals and drugs used in this study were obtained from Sigma (Sigma Chemical Co., USA)

Fura-2 loading and measurement of $[Ca^{2+}]_i$

Single cells were loaded with acetoxymethyl ester form of fura-2 (2 μM, diluted from 1 mM stock in dimethyl sulfoxide) in normal solution for 25 min at room temperature. Then, the cell suspension was briefly centrifuged (800 r.p.m., 5 min) and washed twice with Ca^{2+}-free normal solution. Fura-2 loaded cells were stored at 4°C until use. The recording of $[Ca^{2+}]_i$ was performed with a microfluorometric system consisting of an inverted fluorescence microscope (Diaphot 300, Nikon, Japan) with a dry-type fluorescence objective lens (X40, NA 0.85), a photomultiplier tube (type R 1527, Hamamatsu, Japan) and PTI-Deltascan illuminator (Photon Technology International Inc. USA). One drop of cell suspension was put on a superfusion chamber (100 μl). Cells were allowed to settle down and then superfused at a flow rate of 2 ml/min. Light was provided by a 75 W xenon lamp (Ushino, Japan) and to control excitation frequency, a chopper wheel alternated the light path to monochromators (340 and 380 nm) with a frequency of 5 or 10 Hz. A short-pass dichroic mirror passed emission light of <570 nm onto the photomultiplier tube, and intensity at 510 nm was measured. A mechanical image mask was placed in the emission path, thus limiting measurement to a single cell. Both data acquisition and control of light application were done by using a computer software (Felix v. 1.1, PTI). Because of uncertainties in calibrating the fura-2 signals in intact cells, no attempt was made to calibrate $[Ca^{2+}]_i$ and instead, all results are reported as changes in the 340 nm/380 nm signal ratio.

Statistics

All data are expressed as mean±S.E. *Student's t*-test for unpaired data was used to compare the control and the experimental groups. P value of less than 0.05 was considered to indicate statistically significant differences.

RESULTS

Response of the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) to ghrelin in cells that prepared in 6 H medium

We prepared the thyroid FRTL-5 cells in the condition containing six hormone mixtures. Application of ghrelin (5 nM) to the thyroid FRTL-5 cells suspended in 1 mM Ca^{2+}-containing normal extracellular solution (HBSS) had no effect on $[Ca^{2+}]_i$ (Fig. 1A). Since proliferation of these cells was absolutely dependent on TSH, we applied TSH (1 unit/l) and then ghrelin to the thyroid FRTL-5 cells. There was no response on $[Ca^{2+}]_i$, either (Fig. 1B). Because of no response in HBSS bath solution, we repeated these experiments in the presence of 1.1 mM Ca^{2+}, containing Coon's modified Ham's F12 medium (normal medium). Application of ghrelin to the thyroid FRTL-5 cells increased $[Ca^{2+}]_i$ (Fig. 1C). Ratio-difference before and after the application of ghrelin for 100 s was 0.05±0.007 (mean± S.E.M., n=4, Fig. 1E). TSH increased $[Ca^{2+}]_i$ (ratio- difference: 0.01, n=3) as well as the effect of ghrelin on $[Ca^{2+}]_i$ (Fig. 1D). Ratio-difference before and after the application of ghrelin in the presence of TSH for 100 s was 0.14±0.005 (n=3, Fig. 1E). The results shown in Fig. 1 suggest that ghrelin could increase $[Ca^{2+}]_i$ and that TSH had additive effect with ghrelin on thyroid FRTL-5 cells that were prepared in 6H medium under normal medium.

Response of the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) to ghrelin in cells that prepared in 5H

We prepared the thyroid FRTL-5 cells in the same medium condition as above, but without TSH (5H medium). As proliferation of the thyroid FRTL-5 cells was absolutely dependent on TSH, the cells remained quiescent in 5H medium. Application of ghrelin (5 nM) to the thyroid FRTL-5 cells suspended in 1mM Ca^{2+}-containing normal extracellular solution (HBSS) had no effect on $[Ca^{2+}]_i$ (Fig. 2A). When TSH (1 unit/l) and then ghrelin were applied to the thyroid FRTL-5 cells, there was no response on $[Ca^{2+}]_i$ (Fig. 2B). Because of no response in HBSS bath solution, we repeated these experiments in normal medium containing 1.1 mM Ca^{2+}. Application of ghrelin to the thyroid FRTL-5 cells increased $[Ca^{2+}]_i$ (Fig. 2C). Ratio- difference before and after the application of ghrelin for 100 s was 0.05±0.02 (n=4, Fig. 2E). TSH increased $[Ca^{2+}]_i$ (ratio difference: 0.05, n=3) and the effect of ghrelin on $[Ca^{2+}]_i$ (Fig. 2D). Ratio-difference before and after the application of ghrelin in the presence of TSH for 100 s was 0.06±0.03 (n=3, Fig. 2E). The results in Fig. 2 suggest that ghrelin could increase $[Ca^{2+}]_i$ and that TSH had additive effect with ghrelin on thyroid FRTL-5 cells that were prepared

in 5H medium under normal medium. Because there was no difference in response to ghrelin and TSH between the cells prepared in 6H media and 5H media, we used thyroid FRTL-5 cells grown in 5H media for the following experiments. The above result suggests also a further study on the interaction between TSH and ghrelin under these conditions.

The release of an intracellular stored Ca^{2+} by ghrelin

The dependence of ghrelin-induced $[Ca^{2+}]_i$ increase on extracellular Ca^{2+} was examined in Fig. 3. First, we applied the L-type Ca^{2+} channel blocker, nifedipine (1 μM, Fig. 3A) (Segawa et al, 1999), the T-type Ca^{2+} channel blocker, Ni^{2+} (100 μM, Fig. 3B) (Mlinar and Enyeart, 1993) and non-specific blocker, La^{3+} (100 μM, Fig. 3C) (Lewis and Spalding, 1998). There were no responses on $[Ca^{2+}]_i$. When Ca^{2+}-free solution, which included 2mM EGTA, replaced the normal medium, the application of ghrelin induced also a similar

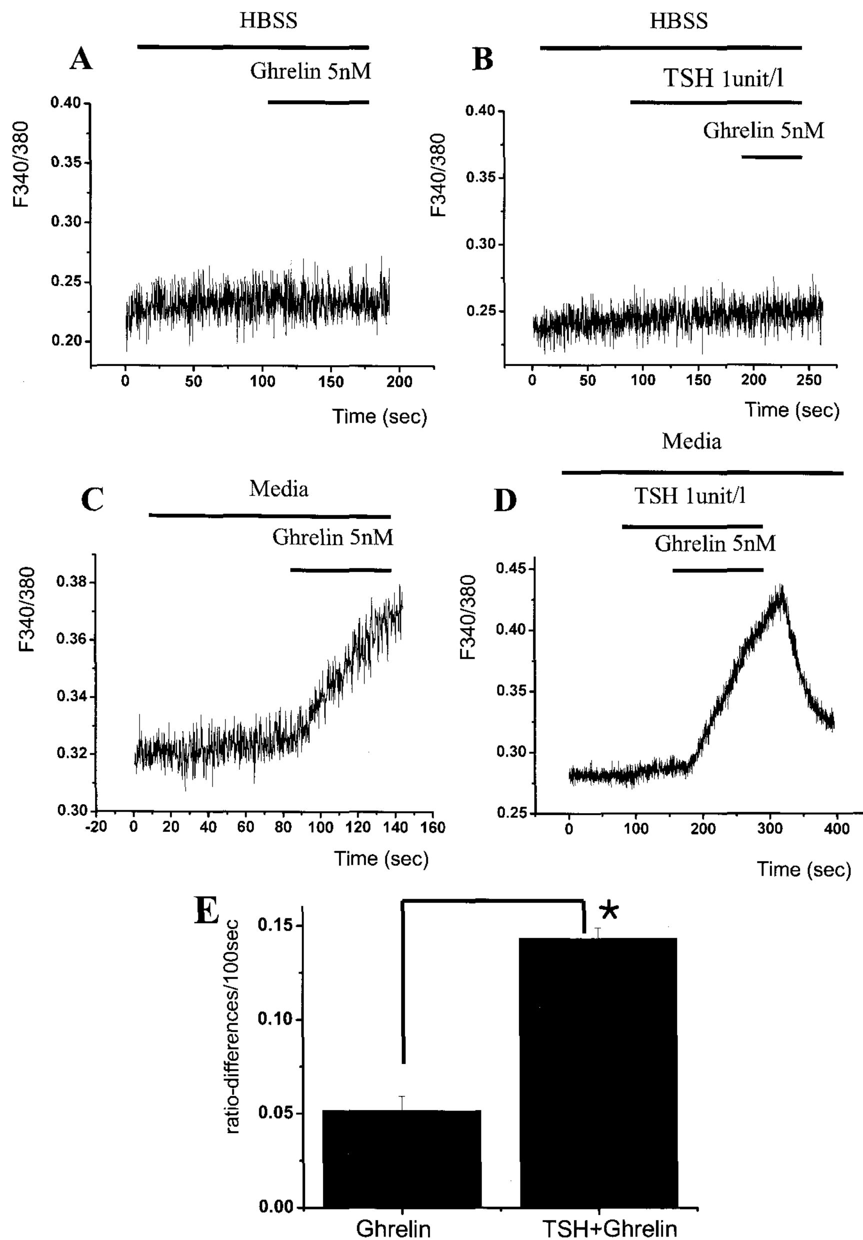

Fig. 1. Responses of intracellular Ca^{2+} level to ghrelin in thyroid FRTL-5 cell that were prepared in 6H medium. Ghrelin (5 nM) evoked no response in HBSS conditions (A and B). But, in normal media, 5 nM ghrelin caused sustained increase in $[Ca^{2+}]_i$ (C). TSH (1 unit/l) with ghrelin had an additive effect. (D). Ratio-differences before and after the application of ghrelin were calculated for 100 seconds (E). Asterisk(*) represents p value is less than 0.05.

rise in $[Ca^{2+}]_i$ (Fig. 4A), indicating that ghrelin-induced $[Ca^{2+}]_i$ increase resulted from mobilization of intracellular stored Ca^{2+}. Ratio-difference before and after the application of ghrelin for 100 s was 0.05 ± 0.01 (n=4) under Ca^{2+}-free/2 mM EGTA solution, which was not significantly different from the value of 0.05 ± 0.02 in the normal media. To confirm that the $[Ca^{2+}]_i$ increase by ghrelin resulted from the release of Ca^{2+} from internal Ca^{2+} store, we applied an ER Ca^{2+} pump inhibitor, CPA (10 μM). After ER was depleted by CPA, the perfusion of cells with ghrelin showed no increase on $[Ca^{2+}]_i$ in Ca^{2+}-free/2 mM EGTA solution (Fig. 4). The results in Fig. 3 and 4 suggest that ghrelin increases $[Ca^{2+}]_i$ by endoplasmic reticulum in thyroid FRTL-5 cells.

DISCUSSION

Tissue slices from dog, calf, sheep, and human thyroid have been extensively used for functional studies. As they

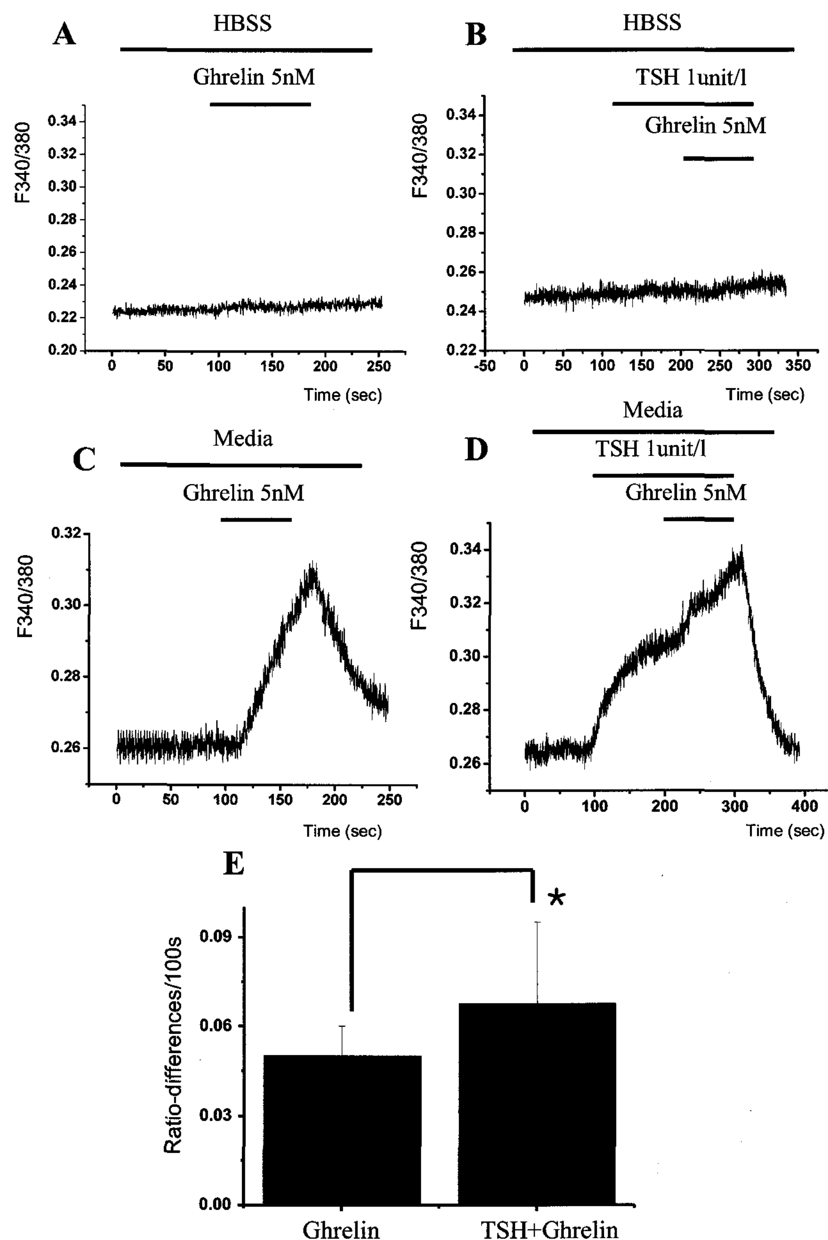

Fig. 2. Responses of intracellular Ca^{2+} level to ghrelin in thyroid FRTL-5 cell that were prepared in 5H medium. Ghrelin (5 nM) evoked no response in HBSS conditions (A and B). But, in normal media, 5 nM ghrelin caused sustained increase in $[Ca^{2+}]_i$ (C). TSH (1 unit/l) with ghrelin had an additive effect. (D). Ratio-differences before and after the application of ghrelin were calculated for 100 seconds (E). Asterisk(*) represents p value is less than 0.05.

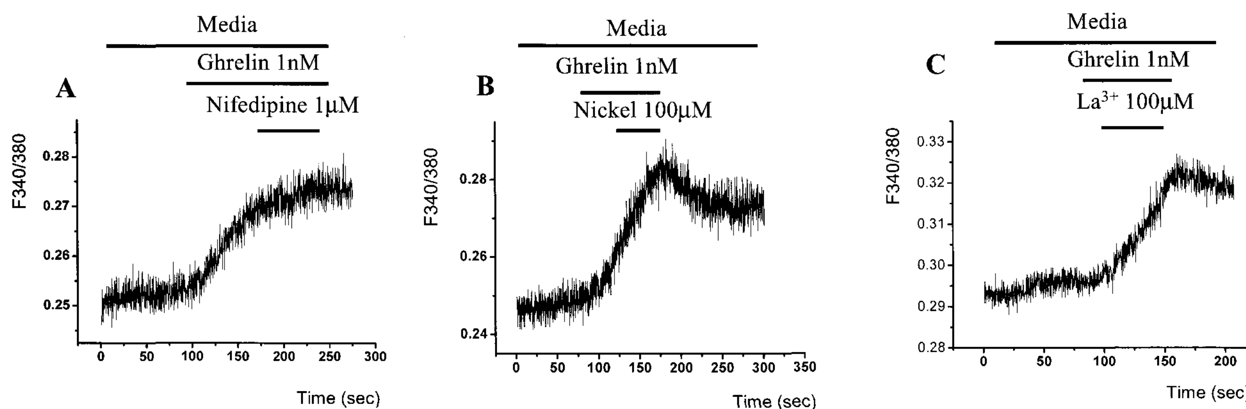

Fig. 3. The effect of blocker on Ca^{2+} increment by ghrelin. We applied the L-type Ca^{2+} channel blocker, nifedipine (1 μM, A), the T-type Ca^{2+} channel blocker, Ni^{2+} (100 μM, B) and nonspecific blocker, La^{3+} (100 μM, C). But there were no responses in $[Ca^{2+}]_i$.

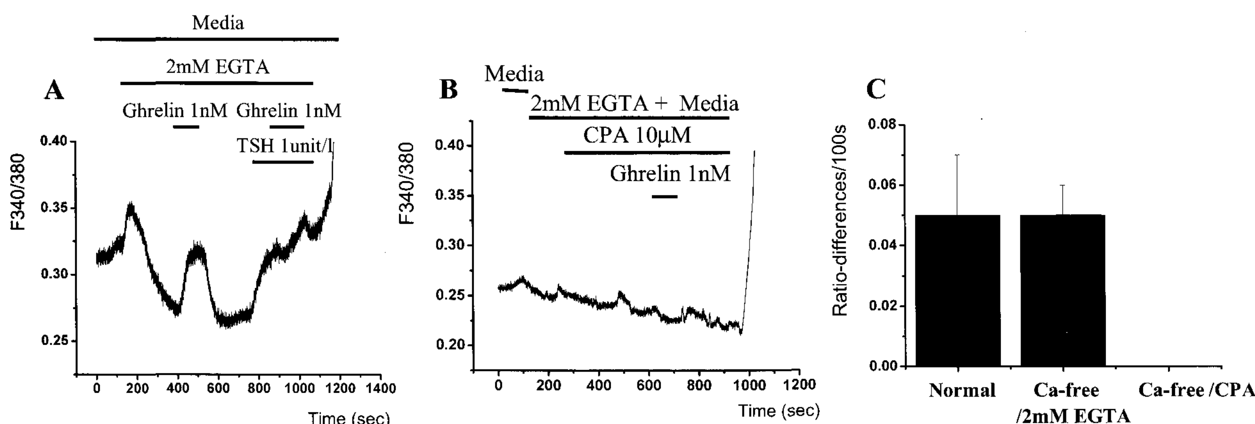

Fig. 4. The release of an intracellular stored Ca^{2+} by ghrelin. When Ca^{2+}-free solution, including 2mM EGTA, replaced normal medium, the application of ghrelin induced a rise in $[Ca^{2+}]_i$ (A). To confirm that the $[Ca^{2+}]_i$ increase by ghrelin resulted from the release of Ca^{2+} from internal Ca^{2+} store, we applied an ER Ca^{2+} pump inhibitor, cyclopiazonic acid (CPA,10 μM). After ER was depleted by CPA, the perfusion of cells with ghrelin showed no increase of $[Ca^{2+}]_i$ in Ca^{2+}-free/2 mM EGTA solution (B). The summary of results was plotted (C).

have no time to reprogram themselves, they are very good replicas of the tissue in vivo. However, they survive for 24 h for the most, and the cells do not enter DNA synthesis. The in vitro models used for the study of thyroid cell proliferation and differentiation belong to two main classes; cell lines and primary cultures. Cell lines are derived from normal and cancer tissues. Obviously, human cancer cell lines can not be used for studying the normal process of thyrocyte growth and division. Differentiated sheep thyroid cell lines (OVNIS) have little been used for investigations of proliferation. The most studied models are immortal rat thyroid cell lines FRTL-5, which present a very appealing set of properties that resemble those ascribed to normal differentiated thyrocytes (Ambesi-Impiombato et al, 1987), such as TSH dependence for growth and differentiated functions, iodide uptake, and thyroglobulin and thyroperoxidase gene transcriptions.

FRTL-5 cells proliferate rapidly (doubling time: 36 h) in the presence of 5% FCS and the six hormone mixture (6H) containing TSH, high concentrations of insulin that activate IGF-I receptors (insulin/IGF-I), transferrin, somatostatin, gly-his-lys acetate, and hydrocortisone. According to the initial characterization, this proliferation was absolutely dependent on TSH, whereas the cells remained quiescent in the same medium as above without TSH (5H medium). However, many reports on growth stimulation in the absence of TSH by insulin/IGF-I or serum, and additive effects of insulin and serum have been published, which represent present-day characteristics of the cell line in most laboratories (Tramontano et al, 1986; Zakarija et al, 1989; Yamamoto et al, 1996).

In thyroid cells, including FRTL-5 cells, calcium-mediated mechanisms are of crucial importance in regulating thyroid hormone synthesis (Raspe et al, 1991). The activity of a calcium/NADPH-dependent thyroid peroxidase in the plasma membrane is considered to be the rate-limiting step in the iodination of tyrosine groups and the production of thyroid hormones. Therefore, agonists which increase $[Ca^{2+}]_i$ potently activate this system.

Growth hormone secretagogues (GHSs) are synthetic peptide and nonpeptide molecules with strong, dose-dependent, and reproducible growth hormone (GH)-releasing activity

even after oral administration. GHs specifically bind to normal and neoplastic follicular derived human thyroid tissue and inhibit the proliferation of follicular-derived neoplastic cell lines. The discovery of ghrelin, 28 amino acid peptide synthesized in the stomach and also in other tissues, has opened new fascinating research perspectives in growth hormone secretagogues (GHSs) field. GHS receptors are detected mainly in the myocardium and also in thyroid cells (Papotti et al, 2000), thus suggesting an important role of ghrelin in thyroid cells. Ghrelin can increase $[Ca^{2+}]_i$ and may also produce thyroid hormones.

In the present study, we investigated the effect of ghrelin on Ca^{2+} concentration in thyroid FRTL-5 cells. Ghrelin increased intracellular Ca^{2+} via releasing Ca^{2+} from the endoplasmic reticulum, but not via L-type Ca channels, T-type Ca channels and nonselective cation channels. In normal medium, ghrelin increased intracellular Ca^{2+} by releasing Ca^{2+} from the endoplasmic reticulum. In normal HBSS solution, there were no responses in $[Ca^{2+}]_i$. This result suggests that some elements in normal medium have an important role in increase of $[Ca^{2+}]_i$ in thyroid FRTL-5 cells by ghrelin. The effect of ghrelin on other Ca^{2+} internal store, especially mitochondria, needs to be studied in further.

In conclusion, this study have shown that ghrelin induces a $[Ca^{2+}]_i$ increase in thyroid FRTL-5 cells by Ca^{2+} release from endoplasmic reticulum. From the physiological point of view, this study, therefore, provides the valuable data to understand the underlying complex mechanisms of Ca^{2+} homeostasis in thyroid FRTL-5 cells.

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