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Calcium signaling properties of a thyrotroph cell line, mouse TαT1 cells

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ABSTRACT

TαT1 cells are mouse thyrotroph cell line frequently used for studies on thyroid-stimulating hormone beta subunit gene expression and other cellular functions. Here we have characterized calcium-signaling pathways in TαT1 cells, an issue not previously addressed in these cells and incompletely described in native thyrotrophs. TαT1 cells are excitable and fire action potentials spontaneously and in response to application of thyrotropin-releasing hormone (TRH), the native hypothalamic agonist for thyrotrophs. Spontaneous electrical activity is coupled to small amplitude fluctuations in intracellular calcium, whereas TRH stimulates both calcium mobilization from intracellular pools and calcium influx. Non-receptor-mediated depletion of intracellular pool also leads to a prominent facilitation of calcium influx. Both receptor and non-receptor stimulated calcium influx is substantially attenuated but not completely abolished by inhibition of voltage-gated calcium channels, suggesting that depletion of intracellular calcium pool in these cells provides a signal for both voltage-independent and -dependent calcium influx, the latter by facilitating the pacemaking activity. These cells also express purinergic P2Y1 receptors and their activation by extracellular ATP mimics TRH action on calcium mobilization and influx. The thyroid hormone triiodothyronine prolongs duration of TRH-induced calcium spikes during 30-min exposure. These data indicate that TαT1 cells are capable of responding to natively feed-forward TRH signaling and intrapituitary ATP signaling with acute calcium mobilization and sustained calcium influx. Amplification of TRH-induced calcium signaling by triiodothyronine further suggests the existence of a pathway for positive feedback effects of thyroid hormones probably in a non-genomic manner.

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1. Introduction

The anterior pituitary gland consists of at least five secretory cell types, corticotrophs, gonadotrophs, lactotrophs, somatotrophs and thyrotrophs, as well as of non-endocrine folliculostellate cells and vascular cells [1]. In vivo, these cells form a functional unit, called lobular structure, which is surrounded by extracellular matrix [2]. Within the lobule, five secretory cells are not randomly distributed but are organized with specific topographical affinities. For example, thyrotrophs are in close proximity with somatotrophs and especially with lactotrophs [3]. Folliculostellate cells are located in the center of lobule and form an excitable network that signals through gap junctions [4]. Extracellular matrix components, including laminin and collagens, contribute to the control of secretory cell types [5]. It also appears that at least some components of extracellular matrix are produced by secretory cell types, as indicated by ability of gonadotropin-releasing hormone to stimulate expression and secretion of dentin matrix protein-1 in gonadotrophs [6].

Numerous cell lines of all five secretory cell types of the anterior pituitary have been established. The most commonly used cell models are: mouse AtT-20 cells for corticotrophs, rat GC cells for somatotrophs, rat GH3/GH4C1 cells for lacto-somatotrophs, mouse LβT2 cells for gonadotrophs, and rat MMQ cells for lactotrophs [7]. These cells have provided valuable information on genealogy of the various cell lineages, the role of specific transcriptional factors determining each cell phenotype, cloning and characterization of receptors for hypothalamic neurohormones, feedback control of hormone synthesis and secretion, and signaling – transcription, and signaling – secretion coupling [7]. There is also a

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significant progress in understanding the calcium signaling properties and function of AtT-20 cells [8,9], GC cells [10], GH3/GH4-C1 cells [11,12], LβT2 [13,14], and MMQ cells [15,16].

Mellon and co-workers developed TαT1 cells, which represent differentiated thyrotrophs of the pars distalis [17,18] and became a useful model for studies on thyroid-stimulating hormone beta subunit gene (*Tshb*) expression and other cellular functions [19–23]. At the present time, however, there is no information about calcium signaling pathways in these cells and the role of calcium in their functions. Also, very little is known about calcium signaling in native thyrotrophs. Ashworth and Hinkle showed that thyrotropin-releasing hormone (TRH) caused increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in individual rat thyrotrophs and that the pattern of response was heterogeneous, as well as that potassium-induced depolarization of cells leads to increase in $[Ca^{2+}]_i$, indicating the presence of voltage-gated calcium (Ca_v) channels [24]. We also observed variable patterns of TRH-induced calcium signaling in identified thyrotrophs [3] as well as their spontaneous firing of action potentials (APs) [25].

Here we studied activation of calcium mobilization and influx pathways in single isolated TαT1 cells in response to application of TRH, a native hypothalamic agonist for thyrotrophs [26], and extracellular ATP, an intrapituitary agonist for two classes of receptors: G protein-coupled P2Y receptors and ATP-gated P2X channels [27]. We also examined contribution of voltage-gated and voltage-independent calcium influx pathway to calcium signaling, and the role of triiodothyronine (T_3) in cellular calcium homeostasis. Moreover, we studied the coupling of TRH receptors and thyroid-stimulating hormone beta subunit gene (*Tshb*) expression in these cells in vitro.

2. Materials and methods

2.1. Cell culture

Coverslips and plates were coated with Matrigel (BD Biosciences, Bedford, MA) diluted 30-fold in PBS, to facilitate adhesion of TαT1 cells, provided by Dr. Pamela L. Mellon (University of California, San Diego, CA). TαT1 cells were plated during 24 or 48 h in DMEM containing 10% fetal bovine serum treated (T_3 experiments) or not with charcoal, 4.5 mg/ml glucose, 110 μg/ml pyruvate, 584 μg/ml glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Life Technologies, Grand Island, NY) and maintained at 37 C in an environment of 5% CO_2. After that, cells were used for qRT-PCR analysis and intracellular calcium or electrophysiological measurements according to protocols described below.

2.2. qRT-PCR analysis

At 60% of confluence, the cells were exposed to 100 nM TRH for 0–6 h. Afterwards, the cells were lysed and the total RNA was extracted using Trizol® reagent. Five hundred ng of total RNA were used for the Reverse Transcriptase (RT) assay by M-MLV Reverse Transcriptase (50,000 U; Promega®, EUA). The first-strand cDNA (product of the RT) was submitted to real-time quantitative PCR using GoTaq® qPCR Master Mix (Promega, WI, USA) and specific oligonucleotides for *Tshb* (forward: GGCAAACTGTTTCTTCCCAA; reverse: GTTGGTTTTGACAGCCTCGT; 198 bp) and *Gapdh*, used as an internal control (forward: GGGCTGCCCAGAACATCAT; reverse: CCGTTCAGCTCTGGGATGAC; 76 bp). The conditions for PCR were: 95 °C – 2 min, followed by 40 cycles of 95 °C – 15 s, 60 °C – 1 min, and 72 °C – 20 s. The reactions were performed in a Corbett Research system (Corbett Life Sciences, Australia). Ct values were recorded for each target gene, normalized to the results obtained with the internal control gene according to the ddCT method previously described [28]. The efficiency and slope values for *Tshb* and *Gapdh* amplification were close to the optimal values required for the ddCT analysis and the results were expressed as fold increase relative to *Gapdh* expression [29].

2.3. Intracellular calcium measurements

TαT1 cells plated on matrigel-coated coverslips were bathed in KR-like medium containing 2.5 μM Fura-2 AM or Fluo-3 AM for 1 h at room temperature. After that, the coverslips were washed in KR-like media and they were mounted on the stage of an inverted Observer-D1 microscope (Carl Zeiss, Oberkochen, Germany) attached to an ORCA-ER camera (Hamamatsu Photonics, Hamamatsu City, Japan) and a Lambda DG-4 wavelength switcher (Sutter, Novato, CA). Hardware control and image analysis were performed using Metafluor software (Molecular Devices, Downingtown, PA). Experiments were done under a 40× oil-immersion objective during exposure to alternating 340 and 380 nm excitation beams for Fura-2 or 488 nm for Fluo-3 loaded cells, and the intensity of light emission at 520 nm was followed simultaneously in about 20 single cells. Changes in $[Ca^{2+}]_i$ are presented by the ratio of fluorescence intensities F_{340}/F_{380} in case of Fura-2. For Fluo-3 measurements, measured fluorescence intensities are divided by the initial fluorescence at given region and further subtracted by the same ratio at the background (region with no cells). Since the illumination of cells with beams of 340 and 380 nm wavelengths caused $[Ca^{2+}]_i$ increase, measurements with Fura-2 had to be performed with the reduced sampling rate (about 1 point per second), while the measurements with Fluo-3 were done with 2 points per second. Traces shown are representative or mean values from at least 20 cells.

2.4. Electrophysiological measurements

Whole-cell currents and membrane voltage were recorded in single isolated TαT1 cells using nystatin (300 μg/mL) perforated patch clamp technique. Cells were seeded 4 h before the experiments in 12 mm round coverslips (10,000 per coverslip). Cells were transferred to a recording chamber and continuously perfused at 2 mL/min at room temperature with extracellular buffer containing (in mM): 140 NaCl, 5 KCl, 1. 3 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, 10 Glucose, pH 7.4 adjusted with NaOH, 295 mOsm. All experiments were recorded using a Multiclamp 700A or Axopatch 200B amplifiers (Molecular Devices, Sunnyvale, CA), voltage and currents were low pass filtered at 10 kHz and digitally sampled at 20 kHz using a Digidata 1440A A/D converter and data were acquired using pClamp 10.3 software (Molecular Devices). The whole cell pipettes were pulled to a tip resistance of 3–5 MΩ and filled with an intracellular buffer containing (in mM): 120 potassium gluconate, 10 KCl, 8 mM NaCl, 10 HEPES, 0.5 mM EGTA (270 mOsmol/kg^{-1}). All buffers and drugs were locally perfused with a peristaltic pump (BioRad, Hercules, CA) coupled to a perfusion system (ALA Scientific Instruments, Farmingdale, NY). Membrane potentials were corrected post hoc for liquid junction potential of 14.9 mV. In voltage-clamp experiments, whole cell capacitance and series resistance were compensated by 70%. To induce the activation of voltage-dependent current, we used a 20 mV steps from −80 to 100 mV (100 ms) from a holding potential of −60 mV. Leak currents were subtracted using a P/4 protocol. In current-clamp experiments the cells were held at zero and rheobase current was measured with a series of current 1 s, 2 pA current injections steps from 0 to 8 pA and defined as the smallest current injection needed to elicit an AP. Bridge balance and access resistance were monitored during the recording and experiments with >20% change were discarded.