Published in final edited form as:

Cell Calcium. 2015 December ; 58(6): 598–605. doi:10.1016/j.ceca.2015.09.003.

Calcium Signaling Properties of a Thyrotroph Cell Line, Mouse T α T1 Cells

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Abstract

T α T1 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 T α T1 cells, an issue not previously addressed in these cells and incompletely described in native thyrotrophs. T α T1 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 T α T1 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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Conflict of interest

The authors declare no conflicts of interest.

Abstract

Keywords

ATP; Calcium influx; Calcium mobilization; P2Y receptors; Thyrotrophs; Thyrotropin-releasing hormone; Triiodothyronine

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 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], L β T2 [13, 14], and MMQ cells [15, 16].

Mellon and co-workers developed T α T1 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 T α T1 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. Material 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 T α T1 cells, provided by Dr. Pamela L. Mellon (University of California, San Diego, CA). T α T1 cells were plated during 24 or 48 h in DMEM containing 10% fetal bovine serum (FBS) treated (T_3 experiments) or not with charcoal, 4.5 mg/ml glucose, 110 μ g/ml pyruvate, 584 μ g/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% CO₂. 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 hours. 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 (50,000U; 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: GGCAAACCTGTTTCTTCCCAA;

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

TaT1 cells plated on matrigel-coated coverslips were bathed in KR-like medium containing 2.5 μ M 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 $\times$ 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 TaT1 cells using nystatin (300 μ g/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 MgCl₂, 2.5 CaCl₂, 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 MOhms 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 – 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.

3. Results

3.1. Electrophysiological properties of TαT1 cells

To address whether the TαT1 cells are excitable and how do they respond to TRH stimulation, we recorded the electrical activity in nystatin-perforated patch mode. After establishing a whole-cell patch, TαT1 cells showed a spontaneous electrical activity with a resting membrane potential of -55 ± 4 mV (Fig. 1A), characterized by small depolarizing events (<10 mV) with a mean frequency of 0.3 Hz, and big depolarizing non-overshooting APs with amplitudes of 40 ± 5 mV, 1.5 ± 0.3 s half width and 0.08 ± 0.03 Hz frequency. The acute stimulation with TRH causes a transient hyperpolarization due to activation of a calcium-controlled potassium channels (data not shown). The sustained stimulation with 100 nM TRH induced a depolarization of 10 mV and increased the firing frequency of the AP to 0.4 ± 0.1 Hz, with amplitudes of 35 ± 6 mV and decreasing the half width to 1.0 ± 0.2 s (Fig. 1B). The resting value of the membrane capacitance was 17 ± 2 pF before and 18.6 ± 2.3 pF after TRH stimulation. Altogether, these results indicate that TαT1 cells are spontaneously active and TRH stimulation depolarizes the resting membrane potential, leading to increase in the spiking frequency.

To determine the ionic conductance underlying the electrical activity, we used a voltage step protocol from -80 to $+100$ mV for 100 ms (Fig. 2A) in order to activate voltage dependent currents. With this protocol in the perforated-patch configuration, we observed a development of a fast inactivating Na^+ current starting at -20 mV, with a peak of -250 pA at 0 mV (Fig. 2A), the application of 1 μM tetrodotoxin (TTX) reduced the inward current by $96 \pm 2\%$ (Fig. 2B) indicating the presence of TTX-sensitive voltage-dependent sodium channels. On the other hand, at depolarized potential (> -20 mV), we observed the development of an outward slow-inactivating K^+ current with a peak of 300 pA (Fig. 2A and B) that was not inhibited by TTX. In summary, these results suggest that TTX-sensitive voltage-gated sodium channels and voltage-gated potassium channels contribute to TαT1 cell excitability.

To determine the rheobase current threshold for APs, we performed current steps protocols from 0 to 8 pA (Fig. 2C). This experiment shows that TαT1 cells start to fire APs at 2 pA of current injection, these spikes overshoot 0 mV, and at higher current injection, the amplitude of these events decrease. Altogether, this result shows that TαT1 needs small charge transfer to trigger an AP; this behavior is in accordance with the observed high input resistance (180 ± 12 MOhms).

3.2. Calcium mobilization and influx

When bathed in Ca^{2+} -containing medium, TαT1 cells exhibit small amplitude spontaneous $[\text{Ca}^{2+}]_i$ transients (Fig. 3A). These transients were abolished in cells bathed in calcium-deficient medium (Fig. 4D-F), and in the presence of nifedipine, a Ca_v channel blocker (data not shown). These data are consistent with electrophysiological experiments shown in Fig. 1A, indicating that spontaneous electrical activity leads to voltage-gated calcium influx.

When bathed in calcium-containing medium, application of TRH caused a rapid increase in $[\text{Ca}^{2+}]_i$, followed by a more gradual decline to a plateau. The spike amplitude was dependent on TRH concentration. Figure 3A shows representative traces and panel B shows mean $\pm$ SEM values for spike amplitude response. The rapid $[\text{Ca}^{2+}]_i$ response was preserved in cells bathed in calcium-deficient medium (Fig. 4A, D, E, and F), suggesting that the calcium mobilization pathway is operative in TαT1 cells. In contrast, the plateau phase was abolished in Ca^{2+} -deficient medium, indicating that TRH also facilitates calcium influx during the sustained agonist application. The influx can be further enhanced by Bay K 8644 (Fig. 5), indicating that L-type Ca_v channels are expressed and operative in these cells. In contrast, dopamine was not capable of suppressing it (Fig. 5), arguing against the expression of functional dopamine DA-2 receptors in this cell type.

3.3. The nature of TRH-activated calcium influx

To examine in more details TRH-induced calcium influx, cells were initially bathed in Ca^{2+} -deficient medium and stimulated with TRH, followed by introduction of calcium in bath solution in the presence of TRH. Figure 4 shows that, upon TRH-induced Ca^{2+} mobilization in extracellular Ca^{2+} deficient medium, Ca^{2+} influx can be re-initiated by returning Ca^{2+} to the extracellular medium. This rise was observed in both Fura-2 and Fluo-3 measurements. Figure 4A represents the mean value from over 20 cells using Fura-2 measurements, whereas Fig. 4D, E, and F show single cell traces using Fluo-3. Notice fluctuations in $[\text{Ca}^{2+}]_i$ in single cell recording during the plateau response.

Calcium influx could also be stimulated by non-receptor-mediated Ca^{2+} mobilization. Figure 4B illustrates depletion of the intracellular pool with thapsigargin, an inhibitor of SERCA type ATPase, in Ca^{2+} -deficient medium, followed by the secondary rise in $[\text{Ca}^{2+}]_i$ induced by return of bath calcium. Keeping the cells in calcium deficient medium for a prolonged time was also sufficient to provoke Ca^{2+} influx (Fig. 4C). Furthermore, TRH-induced $[\text{Ca}^{2+}]_i$ peak in cells that have spent several minutes in Ca^{2+} -deficient medium was much lower than in cells stimulated soon after removing Ca^{2+} from the medium (Fig. 6A). These observations indicate that TαT1 cells have extremely leaky intracellular Ca^{2+} pool and that its depletion in both receptor and non-receptor manner activates calcium influx.

In cells bathed in Ca^{2+} -deficient medium for a short period of time, return of bath calcium causes a gradual increase in $[\text{Ca}^{2+}]_i$ (Fig 6B, black trace). The same effect was observed in the presence of nifedipine, an L-type Ca_v channel blocker, but with smaller magnitude and at slower rate (Fig. 6B, gray trace). TRH-induced depletion of intracellular Ca^{2+} pool is also associated with a rise in $[\text{Ca}^{2+}]_i$ after return of bath calcium (Fig. 6C, black trace), the effect that was also attenuated by application of nifedipine (Fig. 6C, gray trace). These results

indicate that spontaneous and TRH-stimulated release of Ca^{2+} from intracellular pools triggers calcium influx through L-type Ca_v channels and other Ca^{2+} influx pathways.

3.4. The lack of effect of TRH on *Tshb* expression

To examine effects of TRH on *Tshb* expression, T α T1 cells in static cultures were exposed to 100 nM TRH or to medium alone for variable times. The RT-PCR analysis revealed a substantial basal *Tshb* expression and the lack of significant effect of TRH treatment on the gene expression during the first 6 h of stimulation (Table 1).

3.5. Expression of calcium-mobilizing P2Y1 receptors

The presence of calcium-mobilizing purinergic P2Y receptors was evidenced by the calcium signaling after administration of 10 μM ATP in T α T1 cells bathed in calcium-deficient (Fig 7A, left) or – containing (Fig 7A, right) medium. In parallel to TRH action, ATP generated a monophasic response in Ca^{2+} -deficient medium and subsequent application of TRH induced a small amplitude calcium signals. In cells bathed in Ca^{2+} -containing medium, ATP induced biphasic response and application of TRH in the presence of ATP induced high amplitude biphasic response, indicating the refilling of intracellular Ca^{2+} content during the first plateau response.

In further work, we focused on identification of purinergic receptors expressed in these cells using pharmacological tools. In the presence of 1 μM MRS2500 [2-Iodo-N6-methyl-(N)-methanocarba- 2'-deoxyadenosine-3',5'-bisphosphate], a potent and selective antagonist of P2Y1 receptor type, stimulatory effect of 10 μM ATP on $[\text{Ca}^{2+}]_i$ was abolished, while leaving TRH induced signaling unaffected in both Ca^{2+} deficient (Fig 7B, left panel) and Ca^{2+} containing (Fig 7B, right panel). This experiment clearly indicates the expression of P2Y1 and no other calcium-mobilizing P2Y receptor subtypes, as well as the absence of functional P2X receptor channels capable of generating global calcium signals detectable by methods used in this study.

3.6. Effects of T_3 on TRH-induced calcium signaling

For this experiment, T α T1 cells were cultured in DMEM containing 10% charcoal-treated fetal bovine serum to exclude influence of T_3 present in it. The pretreatment of T α T1 cells with 10 nM T_3 for 30 min prior to TRH application modified TRH-induced $[\text{Ca}^{2+}]_i$ signals, making the peaks broader and the decline to plateau levels slower (Fig. 8). The basal and plateau levels were not affected by T_3 . Because T_3 effects occur within 30 min of application, these results indicate its non-genomic effects on calcium signaling in T α T1 cells.

4. Discussion

Here we established that calcium-signaling pathways of the T α T1 cell line are complex and resemble that of differentiated secretory pituitary cells. T α T1 cells are excitable and fire APs spontaneously, accompanied with voltage-gated calcium influx. Their calcium-mobilizing pathway is also operative, causing a rapid calcium release in a non-oscillatory manner and sustained facilitation of electrical activity and voltage-gated calcium influx. It

appears that depletion of intracellular calcium pool in these cells not only provides a signal for voltage-independent calcium influx, but also facilitates the pacemaking activity accompanied with calcium influx. Such synchronized activity of two pathways results in a biphasic calcium signaling: the predominantly extracellular calcium-independent early spike phase and the extracellular calcium-dependent sustained plateau phase.

In general, endocrine pituitary cells are excitable and generate APs of different patterns. Some cells generate tall and narrow APs, with amplitudes of more than 60 mV, half-widths of less than 50 ms, and spiking frequencies that are typically ~0.7 Hz. Other cells generate bursting type of APs, consisting of periodic depolarized potentials with superimposed small-amplitude spikes, the membrane potential rarely goes above -10 mV during a plateau burst, and the spikes are quite small. Gonadotrophs usually fire the first type of APs [30] and somatotrophs and lactotrophs fire the second type [30-32]. However, cells from same lineage may exhibit both types of electrical activity [10, 11, 30, 33-35]. The type of APs we observed in T α T1 cells is more comparable to plateau bursting, with non-overshooting APs of 40 ± 5 mV amplitudes, 1.5 s half width, and 0.1 Hz frequency.

Calcium-mobilizing receptors are expressed in all endocrine pituitary cells. In gonadotrophs, Ca²⁺ mobilization is triggered by GnRH, endothelins, PACAP, substance P, and vasopressin/oxytocin. Lactotrophs express Ca²⁺-mobilizing receptors activated by acetylcholine, angiotensin II, ATP, ETs, oxytocin, 5-HT, substance P, TRH and galanin. In corticotrophs, the Ca²⁺-mobilizing pathway is activated by vasopressin, norepinephrine and potentially 5-HT. Somatotrophs express Ca²⁺-mobilizing ghrelin and ET_A receptors [1]. Here we show that, like native thyrotrophs and lactotrophs, T α T1 cells express calcium-mobilizing TRH receptor and TRH activates these receptors with an EC₅₀ value in subnanomolar concentrations. We also show that these cells express calcium mobilizing P2Y1 receptors, which are activated by extracellular ATP. Earlier studies with dispersed pituitary cells revealed the presence of this type of receptors in lactotrophs and other non-identified cell type [36]. Immunoreactive P2Y1 receptors were identified in rat thyrotrophs [37] and our experiments shows that mouse T α T1 cells also express functional P2Y1 receptors. In contrast to TRH-responsive lactotrophs, which express P2X4 receptor channels as well [38], the present data suggest that P2X receptor channels capable of generating global calcium signaling are not expressed in T α T1 cells.

In pituitary cells, the pattern of calcium mobilization is not determined by receptors but by cell type [1]. In lactotrophs, somatotrophs, thyrotrophs and cells from the GH cell lines, the initial rapid rise in [Ca²⁺]_i is typically followed by a slow decline to a plateau in [Ca²⁺]_i, with spike phase, predominantly reflecting the contribution of calcium mobilizing pathway, and the plateau phase, reflecting sustained activity of calcium influx pathway. This non-oscillatory pattern of Ca²⁺ response is termed biphasic. In mammalian gonadotrophs, the pulse is typically followed by large baseline [Ca²⁺]_i oscillations [39, 40]. The present data show that T α T1 cells also respond to TRH and ATP application with biphasic response and that the ratio between the peak amplitude of spike and plateau response is determined by agonist concentration.

The coupling between calcium mobilization and influx pathways during activation of calcium mobilizing receptors is well established in both non-excitable and excitable cells. In non-excitable cells, depletion of the intracellular pool via receptor activation and non-receptor manner triggers activation of store-operated calcium entry mediated by Stim1 and Orai1 [41]. In excitable cells, including pituitary cells, voltage-gated calcium entry is known to contribute to sustained calcium entry activated by calcium-mobilizing receptors. In pituitary cells, it has been suggested that M [42] or erg-like [43, 44] potassium channels account for sustained depolarization and associated facilitation of voltage-gated calcium entry.

However, here we show that non-receptor-mediated store depletion in T α T1 cells also facilitates voltage-gated calcium entry, suggesting that the store-operated calcium entry mechanism is responsible for cell depolarization. Consistent with this, we observed thapsigargin-induced inward depolarizing current in immortalized hypothalamic GT1 cells [45] and others reported that Stim and Orai proteins play important role in neuronal calcium signaling and excitability (reviewed in [46]). We suggest that in T α T1 cells store depletion facilitates Ca²⁺ influx through Orai channels, leading to depolarization of cells and activation of Ca_v channels. In that scenario, the sustained calcium influx activated by TRH and ATP reflects activation of both Orai and Ca_v channels, with Orai also playing a role of pacemaking due to a depolarizing nature of I_{CRAC}. Additional work is needed to clarify whether this feature is a unique characteristic of T α T1 cells, reflecting their embryonic origin or their immortalization, or is also operative in native thyrotrophs.

Further important information obtained in this study is a relatively rapid effect of T₃ on TRH-induced calcium signaling. Exposure of T α T1 cells to T₃ for up to 30 min has a small but consistent amplifying effect on biphasic response, caused by delaying the decay in [Ca²⁺]_i after the spike in calcium response was reached. The time course of T₃ effect is consistent with the non-genomic effect of this hormone on TRH-induced calcium signaling. In accordance with this hypothesis, previous studies using anterior pituitary dispersed cells have shown that perfusion of T₃ for only 30 min potentiated the TRH-induced TSH release [47]. T₃ also affects cardiomyocyte calcium handling [48]. In further agreement with our data, it has been shown that T₃ increases mitochondrial metabolism non-transcriptionally, leading to stimulation of 1,4,5-trisphosphate-mediated calcium waves [49].

Although T α T1 cells express functional high affinity TRH receptors, their activation does not trigger *Tshb* expression up to 6 h of TRH administration. Such uncoupling, however, is not unique for these cells; the lack of the effect of TRH on *Tshb* expression was also observed in dispersed rat pituitary cells [3]. This observation reinforces the hypothesis that influence of other cell types and/or extracellular matrix proteins is/are needed for TRH-induced *Tshb* expression, which is commonly observed *in vivo* [50, 51].

In contrast to expression of two calcium-mobilizing receptor subtypes, it appears that T α T1 cells do not express functional dopamine DA2 receptors and/or sufficient number of receptors to alter spontaneous and receptor-stimulated voltage-gated calcium influx. These receptors are expressed in lactotrophs, which are also Pit-1-derived cell lineage [1], and provide critical control of spontaneous voltage-gated calcium influx and basal cAMP

production and prolactin secretion [52]. Because basal cAMP production in pituitary cells is substantial [53] and this messenger interacts strongly with calcium-secretion coupling in pituitary cells [54, 55], it is reasonable to speculate that basal cyclic nucleotide production in thyrotrophs influences spontaneous and receptor controlled TSH secretion.

In conclusion, the expression of functional TRH and P2Y1 receptors in T α T1 cells indicates that embryonic thyrotrophs are equipped to respond to feed-forward action of hypothalamic TRH and intrapituitary effects of released ATP with a rapid calcium mobilization and sustained calcium influx. Moreover, TRH-induced calcium mobilization in T α T1 cells is amplified by T₃, an action consistent with the positive feedback effect of this hormone on calcium-secretion coupling, presumably through a non-transcriptional mechanism, in contrast to the transcriptional and post-transcriptional negative effects of T₃ on *Tshb* expression. Further experiments are needed to clarify the status of cyclic nucleotide production in these cells and its influence on calcium signaling and calcium-secretion coupling.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institute of Child Health and Human Development (ZIA DH000195-20) and by a grant from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP: Proc. 2013/05629-4) to MTN. MTN is recipient of *Conselho Nacional de Pesquisa e Desenvolvimento* (CNPq) fellowships and PBS is recipient of a FAPESP fellowship (2012/06643-8).

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Highlights

- T α T1 thyrotrophs are excitable and generate spontaneous action potentials.
- These cells express functional calcium-mobilizing TRH and P2Y1 receptors.
- Their activation triggers rapid Ca²⁺ mobilization coupled to sustained Ca² influx.
- Both store-operated and voltage-gated calcium entry contribute to sustained calcium influx.
- Cellular calcium homeostasis is affected by triiodothyronine.

Fig. 1.

Characterization of spontaneous and TRH-stimulated electrical activity in T α T1 cells. (A) The electrical activity measured in current clamp mode in nystatin perforated patch showed a resting membrane potential of -55 ± 4 mV and spontaneous activity, characterized by small (< 10 mV; mean frequency 0.3 Hz) and big depolarizing events (> 10 mV; frequency 0.08 ± 0.03 Hz). (B) The TRH stimulation (100 nM) induced a depolarization of 10 mV and increased the firing frequency of the action potentials to 0.4 ± 0.1 Hz, with amplitudes of 35 ± 6 mV. (Inset) Frequency of spiking in unstimulated and TRH-stimulated cells.

Fig. 2.

Voltage-gated ion currents in TαT1 cells. (A) Representative traces in TαT1 cells elicited by 100 ms voltage steps from -80 mV to 100 mV, from a holding potential of -60 mV, showing an inward peak amplitude of -250 pA at 0 mV and an outward current peak amplitude of 300 pA at 100 mV. (B) The application of 1 μ M TTX inhibited the inward current. (C) Representative traces of current injection steps showing the rheobase at 2 pA.

Fig. 3. Concentration-dependent effects of TRH on $[Ca^{2+}]_i$ response in TαT1 cell line bathed in Ca^{2+} -containing medium. (A) Representative traces for each TRH concentration. The arrows indicate the start of TRH application. (B) Concentration-response curve depicting the dependence of the peak amplitude response subtracted by the basal values. The circles are averages calculated from three independent experiments with at least 20 cells in each one. The bars denote standard errors. The dye used was Fura-2 and changes in $[Ca^{2+}]_i$ are represented by the ratio of the fluorescence emitted at 340 and 380 nm.

Fig. 4.

The store depletion-activated Ca^{2+} influx in TαT1 cells. Store depletion in Ca^{2+} deficient medium with the help of 100 nM TRH (A, D-F), 1 μ M thapsigargin (TG), an inhibitor of SERCA type ATPase (B), or even by the simple Ca^{2+} leak from intracellular stores (C) is accompanied by significant Ca^{2+} influx once Ca^{2+} (2 mM) is returned to the medium. Panels A, B, and C show averaged values from at least 20 cells obtained from Fura-2 measurements, while panels D, E, and F show examples of single cell measurements obtained from Fluo-3 fluorescence. Notice fluctuations in $[Ca^{2+}]_i$ during plateau phase in single cell recording.

Fig. 5. Effect of Bay K 8644, an L-type Ca_v channel agonist, and the lack of effect of dopamine on $[\text{Ca}^{2+}]_i$ in cells bathed in calcium-containing medium and treated with TRH. Three representative traces from the same experiment are shown; single cells measurements obtained from Fura-2 fluorescence.

Fig. 6.

Involvement of Ca_v channels in Ca^{2+} influx induced by the intracellular store depletion. (A) Ca^{2+} leak from the intracellular pool is demonstrated by the reduction of TRH-induced $[Ca^{2+}]_i$ amplitude after ~6 min spent in Ca^{2+} -deficient medium. (B) Returning Ca^{2+} to the extracellular medium after a shorter time (~ 2 min) induced smaller Ca^{2+} influx (black trace) that was inhibited by 1 μ M nifedipine (gray trace). Notice the recovery of TRH-induced Ca^{2+} signal upon returning of Ca^{2+} to the medium. (C) Ca^{2+} influx induced by returning Ca^{2+} to the extracellular medium of cells with depleted pools by prolonged exposure to Ca^{2+} -deficient medium and TRH (black trace) was partially inhibited by 1 μ M nifedipine (gray trace). Experiments were done using Fura-2 as a Ca^{2+} indicator and traces shown are averages of at least 20 cells per experiment.

Fig. 7.

TαT1 cells express the Ca^{2+} -mobilizing P2Y₁ receptor. (A) ATP- and TRH-induced $[Ca^{2+}]_i$ responses in cells bathed in calcium-deficient (left) and calcium-containing (right) media. (B) ATP-induced rise in $[Ca^{2+}]_i$ was blocked by 1 μ M MRS2500 in both calcium deficient (left) and calcium containing medium (right), without affecting response to TRH application. Arrows indicate the time of application of agonists. Shown are representative traces of at least 20 cells. Experiments were done using Fluo-3 as a Ca^{2+} indicator.

Fig. 8.

Effects of triiodothyronine (T_3) on TRH induced $[Ca^{2+}]_i$ response in T α T1 cells. Prior to measurements, the cells were cultured for 48 h in medium containing fetal bovine serum treated with charcoal. Shown are average values of three control experiments (black traces) and three experiments where the cells were pre-treated for 30 min with 10 nM T_3 (gray traces). Average values are derived from at least 20 cells. The arrow indicates the time of application of 10 nM TRH. The dye used is Fluo-3 and $[Ca^{2+}]_i$ profiles are presented as ratios between fluorescence intensities and basal fluorescence at the corresponding region. Further background subtraction was also done.

Table 1

The lack of effect of TRH on *Tshb* expression in TαT1 cells in static cultures. Cells were exposed to 100 nM TRH for the time indicated. Values shown are mean $\pm$ SEM of *Tshb/Gapdh* expression.

Time (h)	Basal	N	TRH-stimulated	N
0	1.05 $\pm$ 0.27	6	0.95 $\pm$ 0.30	7
1	1.11 $\pm$ 0.06	3	1.70 $\pm$ 0.10	3
2	1.42 $\pm$ 0.25	6	1.72 $\pm$ 0.31	5
4	1.18 $\pm$ 0.19	4	1.61 $\pm$ 0.12	6
6	1.39 $\pm$ 0.13	5	1.86 $\pm$ 0.06	5