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ARTICLES

Prolactin Secretion and Intracellular Ca^{2+} Change in Rat Lactotroph Subpopulations Stimulated by Thyrotropin-Releasing Hormone†

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Abstract Thyrotropin-releasing hormone (TRH) may stimulate lactotrophs to increase intracellular Ca^{2+} and to secrete prolactin (PRL). In this study, PRL contents in lactotrophs were determined by the sequential cell immunoblot assay (SCIBA) and their changes in intracellular Ca^{2+} was analyzed by confocal microscopy. Significant correlations were found in the corresponding parameters between TRH treatments with a recovery interval of 2 h. Measuring the PRL contents after the first TRH treatment and then determining the intracellular Ca^{2+} changes after the second TRH treatment revealed four lactotroph subpopulations. Type I cells (51%) showed significant responses of both PRL secretion and intracellular Ca^{2+} concentration. Type II cells (22%) increased in PRL secretion, but without changes in intracellular Ca^{2+}. Type III cells (17%) have increased in intracellular Ca^{2+}, but without changes in PRL secretion. Type IV cells (10%) did not show changes in PRL secretion and intracellular Ca^{2+}. J. Cell. Biochem. 87: 126–132, 2002.

Key words: intracellular Ca^{2+}; lactotrophs prolactin; thyrotropin-releasing hormone

Although inhibition of prolactin (PRL) secretion may be due to dopamine, stimulation of this hormone have been attributed to the PRL-releasing factors (PRFs), a heterogeneous group of hormones secreted by the hypothalamus. These factors included thyrotropin-releasing hormone (TRH) [Tashjian et al., 1971; Vale et al., 1973; Rivier et al., 1974; Boyd et al., 1976], vasoactive intestinal polypeptide (VIP) [Kato et al., 1978; Ruberg et al., 1978], and oxytocin. Among the PRFs, TRH may induce the release of thyroid-stimulating hormone (TSH) as well as the secretion of PRL and growth hormone (GH) [Blake, 1974; Chen and Meites, 1975; D'Angelo et al., 1975; De Lean et al., 1979; Lamberts and Macleod, 1990].

In lactotrophs, changes in intracellular Ca^{2+} have been demonstrated in the regulation of PRL secretion by dopamine and TRH [Malgaroli et al., 1987; Winiger et al., 1987; Winiger and Schlegel, 1988; Dufy-Barbe et al., 1993]. Moreover, these changes affect the activity of protein kinase, which may involve in dopamine inhibition [Chuang et al., 1993; Cui et al., 1994]. These findings suggest that intracellular Ca^{2+} concentration may play a role in the mechanism of PRL secretion. In addition, there are significant differences in the responses of lactotrophs to changes in intracellular Ca^{2+} [Shin et al., 1993]. Lactotrophs secrete different amounts of PRL in response to TRH treatment suggesting the heterogeneity of pituitary lactotrophs responsiveness to TRH [Arita et al., 1991, 1992; Duh et al., 1998]. It has been also identified that there exist different lactotroph types with significant variation to their responses to the intracellular Ca^{2+} level [Villalobos et al., 1996]. These findings raised an interesting question whether

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different PRL secretion levels in response to TRH treatment among individual cells is due to the change of intracellular calcium concentration. Accordingly, it is essential to investigate both the PRL secretion and intracellular calcium at the single cell level of lactotrophs.

In the studies of the mechanism of PRL secretion, it is generally based on models of whole tissue or cell populations. These models may only provide average values for whole population and information on individual cells is not accessible. In this study, PRL secretion in individual cells was determined by an improved sequential cell immunoblot assay (SCIBA). At the same time, intracellular Ca^{2+} concentrations in individual cells were also measured by confocal microscopy. This experimental design may enable us to clarify the responses of the subpopulations of lactotrophs treated after the stimulation of TRH. The result would provide an informative data to understand the molecular basis of regulation mechanism of PRL secretion in pituitary lactotrophs.

MATERIALS AND METHODS

Cell Culture

Culturing of the anterior pituitary cells were according to a previously described method [Arita et al., 1992]. Male Wistar rats (220–280 g) were purchased from the Animal Center (College of Medicine, National Taiwan University, Taipei). After sacrifice by capitation, anterior pituitaries were removed and minced in 2 ml DMEM solution (GIBCO, Grand Island, NY). After centrifugation at 600 rpm and 4°C, the pellet was re-suspended in a digestive solution containing 0.5% crystallized trypsin Type III (Sigma, St. Louis, MO) and incubated at 37°C for 15 min before adding 1 ml 0.004% deoxyribonuclease. This mixture was allowed to react for 1 min and then 1 ml 0.1% soybean trypsin inhibitor was added. Ten minutes later, the mixture was centrifuged at 600 rpm for 1 min. The pellet was incubated in 1 ml Hank's balanced salt solution containing 2 mM EDTA for 5 min and then in the same solution (1 ml) with 1 mM EDTA for 15 min. After counting, the cells were suspended at a density of 5×10^3 cells/plate (35 mm) in DMEM supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 1% PSN antibiotic mixture (GIBCO) before culturing at 37°C in a 5% CO_2–95% air atmosphere for 96 h. The culturing medium was replaced every 24 h and a phenol red-free medium was used 24 h before further processing.

SCIBA

The SCIBA was performed according to a previously described method [Arita et al., 1992]. After washing in triplicate with an oxygen-supplemented isotonic solution, the cells cultured for 96 h was suspended in 1 ml of this solution. A methanol-soaked PVDF membrane ($1.5 \times 1.3\ cm^2$) was used to absorb the solution in the plate and a 20-μl isotonic solution-pretreated PVDF membrane was then placed upon the cells for 15 min. The membrane was floated and picked up by adding 2-ml isotonic solution. Cells were transferred to another PVDF membrane by the same procedures for the determination of the basal levels of PRL contents. For TRH stimulation, 100-nM TRH-treated PVDF membranes were transferred from the cells with replacement of phenol red-free medium by repeating the same procedure for determining the amount of secreted PRL of lactotrophs treated with TRH.

All transferred membranes were incubated at room temperature for 2 h in a blocking buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20, and 3% (v/v) FBS. Antibodies to PRL were diluted in an antibody binding buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1%(v/v) Tween-20, and 1% (v/v) FBS and added to the membranes before incubating at room temperature for 4 h. The immunoblots were washed in triplicate with 50 ml blotting buffer for 10 min and then immersed in the secondary antibody solution containing alkaline phosphatase goat anti-rabbit IgG (Promega) for 1 h before diluting to 1,000-fold in the binding buffer. The blots were then washed in triplicate with a blotting buffer for 10 min. Color development was performed in a 20-ml mixture consisting of 7 mg nitro blue tetrazolium, 5 mg 5-bromo-4-chloro-3-indolyl-phosphate, 100 mM NaCl, and 5 mM $MgCl_2$ in 100 mM Tris-HCl, pH 9.5. Cell blots immunopositive for PRL were was quantified by a densitometer (Alphalmager 2000, Alpha Innotech Corporation). The amounts of PRL secreted from individual cells were calculated by reference to PRL standards.

Confocal Microscopy

Intracellular Ca^{2+} was determined using the fluorescent probe Fura Red-AM (Sigma). The

dye (5 mg) was dissolved in 25 μl DMSO and mixed with 25 μl 25% pluronic acid. This mixture was added to DMEM medium. After replacing the culture medium with this DMEM medium, the cells were incubated at 37°C for 30 min before washing in triplicate with fresh DMEM without the dyes and placing in a chamber with an isotonic 0.5 ml solution (0.5 ml, pH 7.4) containing 140 mM NaCl, 2 mM $CaCl_2$, 4.6 mM KCl, 1.0 mM $MgCl_2$, 10 glucose, and 10 mM HEPES. Fluorescence images of the cellular Ca^{2+} localization were observed with a laser-scanned confocal microscope (LSM 410 LSM 410 from Zeiss) equipped with a PI-Neofluar (2× and 20×) objective (NA = 1.4) by a modification of a previously described method [Burnier et al., 1994].

The maximal fluorescence (F_{max}) was estimated by adding non-fluorescent Ca^{2+} ionophore 4-bromo A-23187 (10^{-5} M) to optimize extracellular Ca^{2+} and to saturate the intracellular dye with Ca^{2+}. The minimal fluorescence (F_{min}) was estimated by adding 5 mM EDTA. Ca^{2+} concentrations were calculated according to the formula:

$$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$$

where F is the fluorescent intensity and K_d is the dissociation constant [Burnier et al., 1994].

RESULTS

PRL Contents

Basal levels of PRL in lactotrophs are shown in Figure 1A. High PRL contents were observed in some lactotrophs before TRH treatment. After treating with 100 nM TRH, PRL secretion became apparent in many lactotrophs, whereas some maintained their basal levels (Fig. 1B). The PRL contents returned to their basal levels after incubating in a phenol red-free medium at 37°C in a 5% CO_2 environment for 2 h (Fig. 1C) and similar changes in the PRL contents in the corresponding cells were observed after another

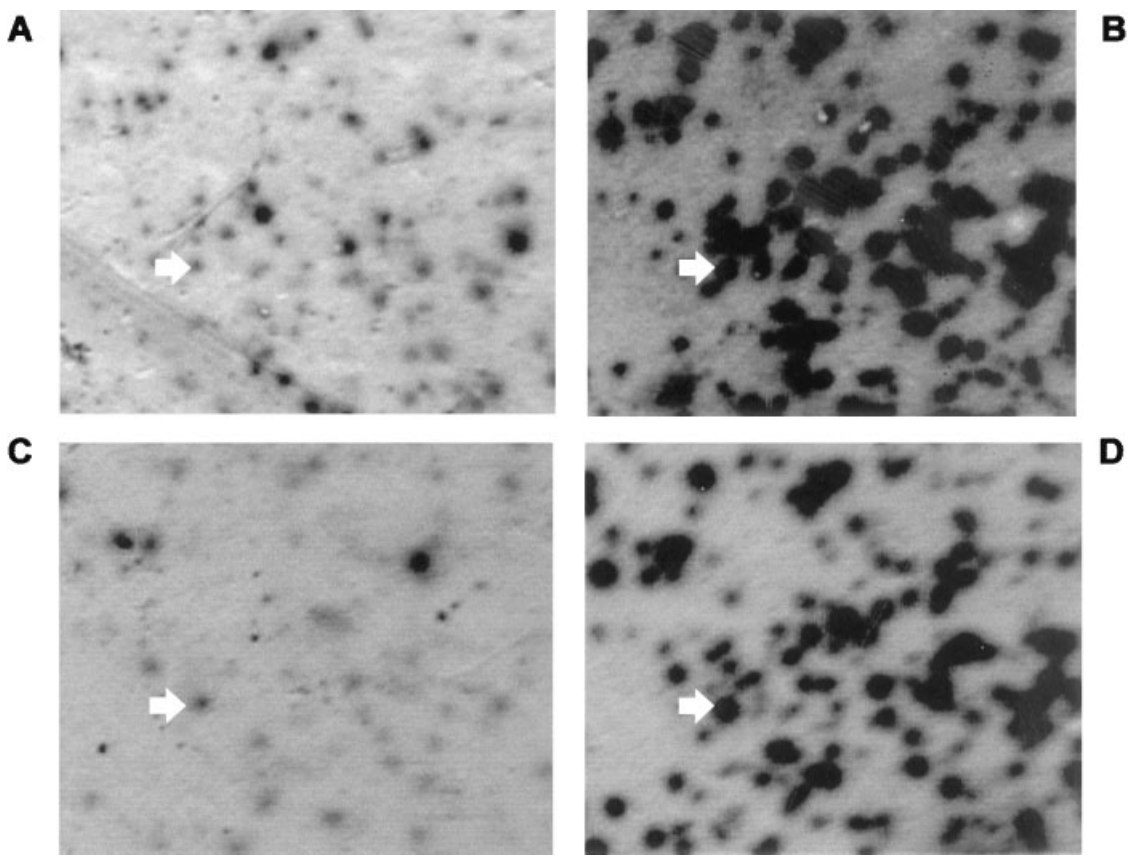

Fig. 1. Secretion of PRL determined by SCIBA in a lactotroph. **A**: Basal level. **B**: Level after the first administration of 100 nM TRH. **C**: Basal level after incubation for 2 h. **D**: Level after the second administration of 100 nM TRH. Arrows indicate the target lactotroph.

TRH treatment (Fig. 1C,D). There was a significant correlation between the PRL contents in individual cells between the two TRH administrations ($r = 0.80$, $P < 0.01$).

Intracellular Ca^{2+}

After treating the lactotrophs with 100 nM TRH, Ca^{2+} influxes were observed in most lactotrophs (Fig. 2A,B). However, the mobility of Ca^{2+} varied significantly among the cells (Fig. 2A,B). The Ca^{2+} influxes became unapparent after recovery for 2 h (Fig. 2C) and similar changes in the intracellular Ca^{2+} in the corresponding cells occurred after another TRH treatment (Fig. 2D). Correlation of intracellular Ca^{2+} between the two TRH administrations was found to be significant statistically ($r = 0.62$, $P < 0.01$).

Relationship Between PRL Secretion and Intracellular Ca^{2+} Influx

Changes in the PRL contents of individual lactotrophs before and after administration of 100 nM TRH are shown in Figure 3A,B. Higher PRL contents were observed in some lactotrophs (Fig. 3B). After recovery for 2 h, intracellular Ca^{2+} concentrations were determined (Fig. 3C) after another treatment with 100 nM TRH. Some lactotrophs were observed to release PRL without intracellular Ca^{2+} changes (Fig. 3D). However, there was no significant correlation between PRL secretion and intracellular Ca^{2+} concentration ($r = 0.22$, $P > 0.05$).

Subpopulation of Lactotrophs

After examining 358 cells, lactotrophs were separated into four subpopulations according to their responses of PRL secretion and intracellular Ca^{2+} concentration after TRH administration (Table I). Type I lactotrophs consisted of 53% of the cell population and showed positive responses in both PRL secretion and intracellular Ca^{2+} elevation. Type II cells consisted of 17% of the population and did not have the response of PRL secretion, although they have changes in intracellular Ca^{2+} concentration.

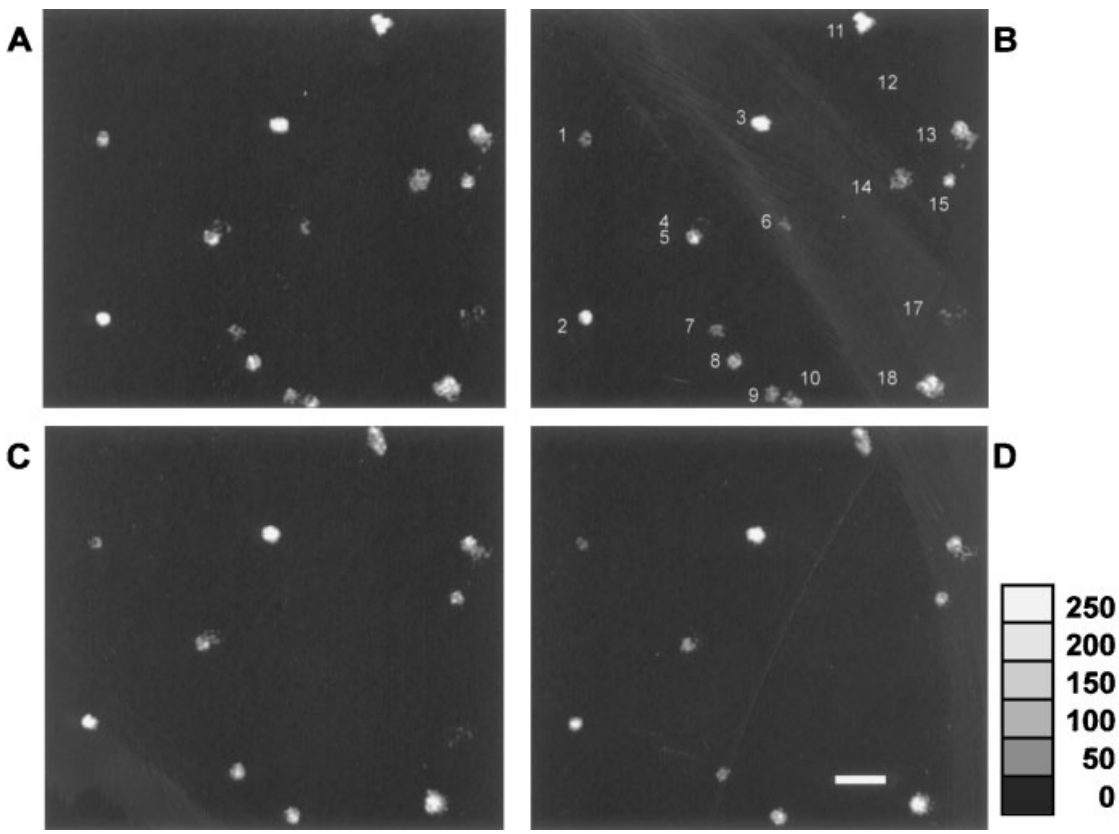

Fig. 2. Confocal microscope fluorescence images showing changes in the intracellular Ca^{2+} concentrations of individual lactotrophs treated with TRH. **A**: Basal levels. **B**: Levels at 20 s after administration of 100 nM TRH. **C**: Levels after incubation for 2 h. **D**: Levels at 20 s after another treatment with 100 nM TRH. Lactotrophs 1, 2, 3, 8, 9, 10, 13, 14, 15, 17, and 18 showed higher Ca^{2+} mobility, whereas lactotrophs 4, 5, 6, 7, 11, and 12 maintained their basal levels. (Bar = 20 μm).

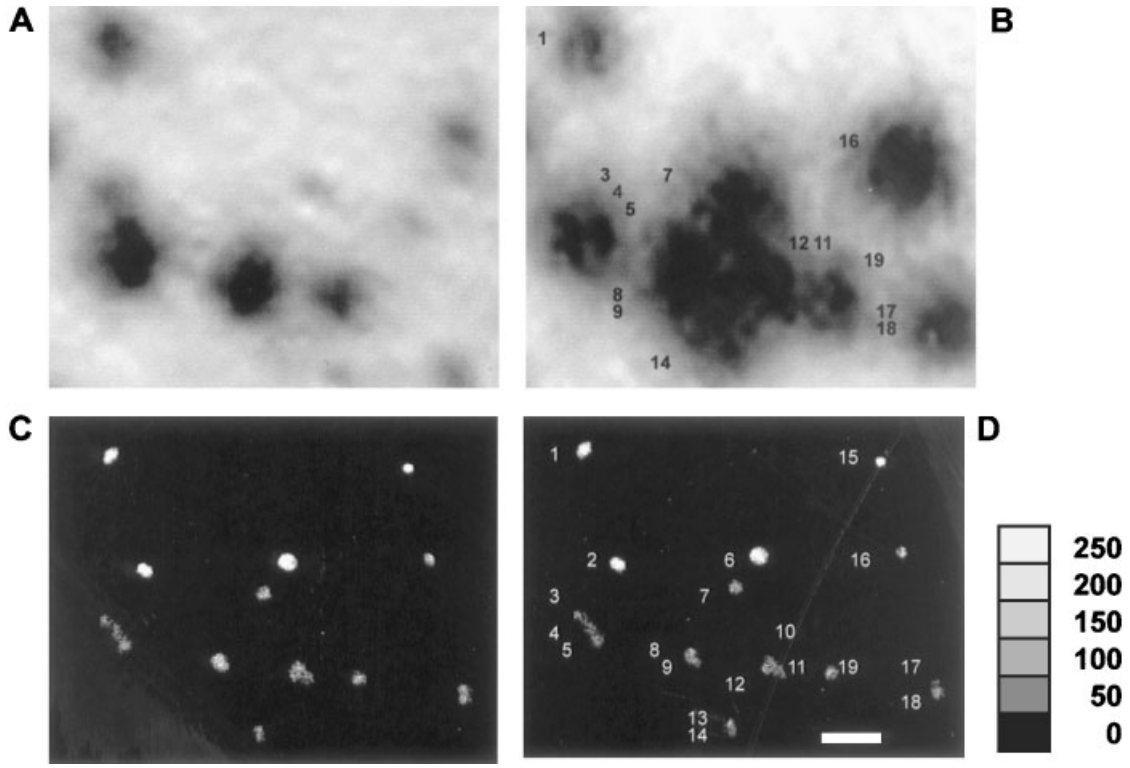

Fig. 3. Changes in PRL secretion and intracellular Ca^{2+} concentration in individual lactotrophs treated with 100 nM TRH. **A**: Basal levels of PRL secretion. **B**: Levels of PRL secretion after administration of 100 nM TRH. High levels of PRL secretion were observed in lactotrophs 7, 10, 11, 12, 13, 14, 16, 17, 18, and 19. **C**: Intracellular Ca^{2+} concentrations after recovery for 2 h. **D**: Intracellular Ca^{2+} concentrations after another treatment with 100 nM TRH. Lactotrophs 3, 4, 5, 13, 14, and 19 released PRL without intracellular Ca^{2+} changes. (Bar = 20 μm).

Type III cells consisted of 18% of the population and did not have the response of intracellular Ca^{2+} change, but have increased PRL secretion after TRH administration. Type IV cells consisted of 11% of the population and showed neither PRL secretion nor intracellular Ca^{2+} change.

DISCUSSION

Technically, it is possible to determine PRL secretion in individual lactotrophs by SCIBA [Arita et al., 1992]. Moreover, visual observation of the influxes of intracellular Ca^{2+} in individual cells is feasible by confocal microscopy [Burnier et al., 1994]. However, it is operationally difficult to determine the two phenomena simultaneously. In this study, we determined PRL contents after the first and second TRH stimulation in lactotrophs, and significant correlation was shown between both data. Similar results were also shown in the measurement of intracellular Ca^{2+} in lactotrophs treated with TRH twice. Consequently, it is possible to define the correlation between the PRL contents and changes in intracellular Ca^{2+} in these cells. Therefore, we first measured the PRL levels after the first TRH administration and then determined the intracellular Ca^{2+} changes translated from the level of fluorescence with a recovery interval of 2 h. By this special design, it is revealed that lactotrophs may be divided into four subpopulations.

It has been reported that lactotrophs stimulated by TRH may release PRL [Tashjian et al., 1971; Mueller et al., 1973; Vale et al., 1973; Rivier et al., 1974; Boyd et al., 1976; Drouin et al., 1976; Nakano et al., 1976]. It was also found that a lactotroph subpopulation showed different responses of intracellular Ca^{2+} to four well-established hypothalamic-releasing hormones (HRHs: TRH, LHRH, CRH, and GHRH) indicating the multiresponse of HRH action in lactotrophs [Villalobos et al., 1997]. In this study, about 53% of the Type I cells showed positive responses of both PRL releasing and intracellular Ca^{2+} influx. In Type II (17%) and III (18%), the PRL releasing was not

TABLE I. Subpopulations of Lactotrophs Based on the Responses of Ca^{2+} Release and PRL Secretion After Administering 100 nM TRH

Type	No. (%) of cells examined	Treatment with TRH	Ca^{2+} release	No. (%) of cells with PRL secretion (pg/cell)			
				0–0.5	0.6–1.0	1.1–1.5	1.6–2.0
I	203 (53)	No		141 (6931)	62 (28)	0	0
		Yes	Yes	0	55 (27)	122 (60)	26 (13)
II	66 (17)	No		25 (38)	25 (38)	16 (24)	0
		Yes	Yes	25 (38)	25 (38)	16 (24)	0
III	71 (18)	No		58 (82)	13 (18)	0	0
		Yes	No	0	13 (18)	20 (28)	38 (54)
IV	44 (11)	No		18 (41)	26 (59)	0	0
		Yes	No	18 (41)	26 (59)	0	0

associated with cellular Ca^{2+} concentration. Moreover, a minor group (Type IV, 11%) had neither PRL releasing nor intracellular Ca^{2+} change after TRH stimulation. It has been reported that lactotrophs with lower basal PRL secretion may have higher secretion and those with higher basal secretion with lower response to TRH stimulation [Arita et al., 1991, 1992]. This may be corresponded to the Type I and III lactotrophs in this study. Although there was no significant change in the intracellular Ca^{2+} in Type III cells, PRL secretion still increased in these cells after TRH stimulation. This finding indicates that the PRL secretion is not mediated through intracellular Ca^{2+} change in this subpopulation. It is also consistent with the results that EGTA-treated cells partially alter PRL secretion, indicating TRH action has been contented a Ca^{2+}-independent secretary mechanism [Yajima et al., 1990]. In addition, TRH induce a biphasic Ca^{2+} response in which a sustained level of calcium maintaining longer time followed a Ca^{2+} spike staying short time [Akerman et al., 1991]. The maintenance of sustained level of Ca^{2+} was identified to the PRL secretion in lactotrophs. However, according to previous study [Ashworth and Hinkle, 1996], the period of sustained level of Ca^{2+} caused by the administration of 10 nM or less TRH was longer than that of 100 nM TRH treatment that was applied in our protocols, although the response of PRL secretion to TRH concentration is positive correlation in general lactotrophs. The mechanism is unclear. It is possible that high concentration of TRH will stimulate more Ca^{2+}-independent cells.

In this study, we did not observe a significant correlation between PRL secretion and intracellular Ca^{2+} change. However, we revealed four subpopulations of lactotrophs with different responses to these two factors after stimulation (Table I). Although change in intracellular Ca^{2+} concentration may have influence in some subpopulation of the cells, it should not be the only factor for PRL secretion. Further studies are required to identify the density of TRH receptors on lactotroph membranes or other signaling pathway without Ca^{2+} involvement for the mechanism PRL secretion.

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