Intracellular calcium concentration and hormone secretion are controlled differently by TRH in rat neonatal lactotrophs and somatotrophs

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Abstract

We studied the effects of TRH on the cytosolic free calcium concentration ([Ca²⁺]) of female rat pituitary prolactin-secreting (lactotroph) and GH-secreting (somatotroph) cells in the early postnatal period, i.e., at postnatal days 5 and 10. [Ca²⁺] of single identified lactotrophs and somatotrophs was recorded by dual-emission microspectrofluorimetry using the intracellular fluorescent calcium probe indo 1.

An application of TRH (100 nM, 10 s) induced a marked [Ca²⁺] increase in 65% of neonatal lactotrophs and 34% of neonatal somatotrophs while the remaining cells were unaffected. Most of the responsive cells, both lactotrophs and somatotrophs, exhibited a similar biphasic Ca²⁺ response, made up of an initial rapid large increase in [Ca²⁺], followed by sustained [Ca²⁺], fluctuations. In both cell types, removal of Ca²⁺ from the extracellular medium or addition of the Ca²⁺ channel blocker, cadmium chloride (500 µM), inhibited the second phase whereas the first phase persisted. Furthermore, in both cell types, protein kinase C (PKC) depletion by incubation in phorbol myristate acetate (1 µM) for 24 h abolished the second phase but did not inhibit the first phase. Conversely, when cells were pretreated with the Ca²⁺-ATPase inhibitor, thapsigargin (100 nM), all TRH-induced [Ca²⁺] changes in both cell types disappeared. TRH therefore induces a biphasic increase in [Ca²⁺], involving intra- and extracellular Ca²⁺ in neonatal lactotrophs and somatotrophs as it does in adult lactotrophs. The first phase is presumably due to mobilization of Ca²⁺ from intracellular stores whereas the second phase presumably results from a PKC-sensitive influx of Ca²⁺. TRH action on membrane potential was then investigated using the patch-clamp technique in the whole-cell mode. TRH-induced changes in membrane potential consisted of an initial hyperpolarization followed by depolarization and action potential firing.

We also investigated TRH action on prolactin and GH secretion by neonatal pituitary cells using RIA. Surprisingly, static assays of prolactin and GH revealed only stimulation of prolactin release by TRH but no effect on GH secretion, although, as expected, GH-releasing factor was a potent agonist of GH secretion.

Our results suggest that TRH regulates neonatal lactotrophs and somatotrophs differently, in that the [Ca²⁺] changes do not correlate with stimulation of exocytosis in the latter cell type.

Introduction

Calcium is thought to play a pivotal role in stimulus–secretion coupling in endocrine cells. Indeed, it has been demonstrated that an increase in the intracellular calcium concentration ([Ca²⁺]) by cytosol dialysis with buffers containing elevated Ca²⁺ dramatically stimulated secretory-granule fusion and thus hormone secretion (Ronning & Martin 1986, Sikdar et al. 1990, Zorec et al. 1991). Conversely, low [Ca²⁺], markedly slowed exocytosis. Recording intracellular Ca²⁺ with fluorescent Ca²⁺ indicators revealed that [Ca²⁺] in pituitary cells was modulated by a wide number of hypothalamic factors known to regulate hormone release (Winiger et al. 1987, Holl et al. 1988a,b, Malgaroli et al. 1988).

Among the releasing factors, thyrotrophin-releasing hormone (TRH) has a number of targets. It stimulates thyroid-stimulating hormone, prolactin (PRL) and, in some cases, growth hormone (GH) secretion (Welsh et al. 1986, Emerson 1988, Ben-Jonathan et al. 1989, Harvey 1990). It may thus regulate three cell types.

The intracellular transduction mechanisms after TRH binding to its receptor have been mainly studied in tumoral GH3 and related cell lines (Gershengorn 1986). In these cells, a biphasic Ca²⁺ response was observed after TRH application. Numerous studies have established that
activation of TRH receptors induced the cleavage of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol. The former causes a transient release of Ca2+ from internal stores. Subsequently, a phase of elevated [Ca2+]i occurs, resulting from increased Ca2+ influx. These [Ca2+]i changes are thought to cause the temporal pattern of PRL secretion in response to TRH (Albert & Tashjian 1984, Aizawa & Hinkle 1985, Kolesnick & Gershengorn 1986). Similar intracellular transduction pathways have been demonstrated in normal adult lactotrophs (Winiger et al. 1987, Akerman et al. 1991, Carew & Mason 1995, Ashworth & Hinkle 1996). However, little is known about the effects of TRH action during pituitary development in either lactotrophs or somatotrophs (Khorram et al. 1983, 1984, Welsh et al. 1986).

During ontogeny, the five anterior pituitary cell types appear according to a precise schedule (Voss & Rosenfeld 1992, Dubois & ElAmraoui 1995). Somatotrophs and lactotrophs, detectable at embryonic day 18 (E18) and E21 respectively, are the last to develop. Several studies suggest that the two cell types are derived from a common progenitor cell population: targeted cell knock-out in transgenic mice has suggested that most (but probably not all) lactotrophs are derived from ‘primitive’ somatotrophs (Behringer et al. 1988, Borelli et al. 1989). Furthermore, during early development, some of the GH-secreting cells also secrete PRL, suggesting that these so-called mammosomatotrophs may be transition cells during differentiation of primitive GH-secreting cells to PRL-secreting cells (Hoeffer et al. 1985, Frawley & Boockfor 1991). In addition, in adulthood, lactotrophs and somatotrophs seem to undergo physiological interconversions. This may not involve cell division (Frawley & Boockfor 1991). For example, the proportions of PRL- or GH-secreting cells increased and decreased respectively to approximately the same extent from the beginning of pregnancy to the end of lactation (Porter et al. 1990). The two cell types therefore seem to be linked at all stages in development.

This study investigates the intracellular mechanisms underlying TRH control of normal pituitary lactotrophs and somatotrophs during postnatal development. As electrical activity correlates with [Ca2+]i, we first studied the effect of TRH on [Ca2+]i, and membrane potential on single pituitary cells. Because of the small percentage of lactotrophs in anterior pituitary cell populations during early postnatal development (Smets et al. 1989), we identified all the recorded cells by immunocytochemistry. Finally, we examined the effect of TRH on PRL and GH secretion.

Materials and Methods

Pituitary cell isolation

All animals used were female Wistar rats. They were either 5- or 10-day-old neonates or 3-month-old (mature) animals. The day of birth was designated as day 0 (D0) of life.

Pituitary glands were removed after rapid decapitation of the animals and placed into Ham F-10 medium (Gibco, Cergy Pontoise, France) containing 0.1% BSA (Sigma, La Verpillière, France) and antibiotics (penicillin-streptomycin, 50 IU/ml). The neurointermediate lobes were removed and discarded under the microscope. The anterior pituitaries cut in small pieces were digested by incubation in a solution of 0.1% trypsin (Sigma) at 37°C for 30 min and in a solution of DNase (Boehringer, Mannheim, Germany) for 1–2 min. Then, the pituitary lumps were washed and mechanically dispersed in a Ca2+- and Mg2+-free Hanks solution.

The dispersed cells were cultured in Ham F-10 nutrient medium supplemented with 10% heat-inactivated horse serum (Gibco) and antibiotics (penicillin–streptomycin, 50 IU/ml) and maintained at 37°C in an humidified atmosphere (95% air, 5% CO2).

All experiments were carried out between 24 and 48 h of culture.

For [Ca2+]i measurements, the cells were cultured on glass coverslips coated with polyornithine (5 g/l; Sigma) in a 35 mm Petri dish (300 000 cells/dish). The coverslips were scored manually in our laboratory, so as to form a grid for cell identification.

For secretory studies, cells were seeded on 24-well plates (100 000 cells/well).

Identification of recorded cells by immunocytochemistry

After [Ca2+]i measurements, the coordinates of each recorded cell were registered to identify the content of its secretory granules. Cells were first fixed in Zamboni fixative (4% paraformaldehyde, 15% saturated picric acid in 0.1 M phosphate buffer), and double immunostaining was performed according to the general procedure described by Vandersande (1983). We used rabbit antibody against rat PRL (rabbit anti-rPRL-IC5, diluted 1:3000) and monkey antibody against rat GH (monkey anti-rGH-IC1, diluted 1:4000) both provided by the National Institute of Diabetes and Digestive and Kidney Diseases of the USA (NIDDK), through the National Hormone and Pituitary Program. Cells were incubated with both antisera overnight at 4°C. The secondary antibodies, rhodamine goat anti-rabbit IgG (diluted 1:150) and fluorescein goat anti-mouse IgG (diluted 1:150), were applied for 2 h at room temperature. They were obtained from ImmunoTech (Marseille, France) and TEBU (Le Perray, France) respectively.

PRL- and GH-positive cells were visualized under a Nikon microscope equipped for transmitted light fluorescence (Nikon, Paris, France).

[Ca2+]i measurement

The experiments were performed with the fluorescent dye indo 1 as described by Mollard et al. (1989). Cells
were incubated with 5 μm indo 1 acetoxymethyl ester and 0.02% pluronic in Hanks basic saline solution (HBSS) for 30 min at 37 °C. HBSS contained (in mM): NaCl, 136.7; KCl, 5; CaCl2, 2; MgCl2, 2; MgSO4, 0.9; NaHCO3, 4; NaH2PO4, 0.15; HEPES, 10; glucose, 10; pH was adjusted to 7.35 with NaOH and osmolarity to 310 mOsm with saccharose. After loading, the cells were rinsed with and kept in HBSS. Recording of \([\text{Ca}^{2+}]_i\) was performed at 37 °C on single cells using an inverted microscope (Nikon) equipped for microspectrofluorimetry. The fluorescence of indo 1 was recorded at two emission wavelengths (405 nm for the \(\text{Ca}^{2+}\)-bound form, 480 nm for the \(\text{Ca}^{2+}\)-free form) when excited at 355 nm.

immunocytochemistry. We found that fewer than 1% of lactotrophs or somatotrophs by post-recording immunocytochemistry, identified as lactotrophs or somatotrophs by post-recording immunocytochemistry.

Measurement of secretory responses

After 24 h of culture, the cells were first carefully rinsed with and incubated in 500 μl of Ham F-10 culture medium supplemented with 10% heat-inactivated horse serum, containing either vehicle or appropriate test substances. Incubation was carried out for 30 min at 37 °C in an humidified incubator. After the incubation period, the supernatants were removed and stored at −20 °C until PRL or GH were assayed.

RJAs for PRL and GH were performed using reagents donated by NIDDK, through the National Hormone and Pituitary Program. All samples from a given experiment were assayed in duplicate in the same assay.

Pharmaceutical reagents

Indo 1 acetoxymethyl ester, cadmium chloride, phorbol myristate acetate (PMA) and thapsigargin were purchased from Sigma. Pluronic was obtained from Molecular Probes (Eugene, OR, USA) and TRH from UCB (Bioproducts, Braine l’Alleud, Belgium). They were prepared as stock solutions and kept frozen at −80 °C until use. All stock solutions were diluted in bath medium just before use.

Data analysis

Values are expressed as mean ± s.e. for several independent experiments and compared using ANOVA, followed by Fisher’s least significant difference test. The difference was considered to be significant at \(P<0.05\).

Results

Characterization of isolated neonatal lactotrophs and somatotrophs

The main part of this study was performed in vitro on cells isolated from the pituitaries of neonatal rats. Immunocytochemistry was used to monitor the relative proportions of PRL and/or GH cells to total cells. Double immunostaining for rat (r)PRL and rat (r)GH was performed. GH was detected using an antibody coupled with fluorescein and PRL using an antibody coupled with rhodamine (Fig. 1). We found changes in the proportion of the two cell types between D5 and D10 and between D10 and maturity. At D5 and D10, there were fewer PRL-positive cells than GH-positive cells. Only 5 and 11.5% of the cells were PRL-positive, whereas GH-positive cells represented 36 and 42.5% respectively. In contrast, we found that 51% of cells from mature females were PRL-positive and 31% were GH-positive. Using this dual immunolabelling technique, we found that fewer than 1% of pituitary cells were immunonegative for PRL and GH, i.e. mammosomatotrophs.
As the anterior pituitary is a heterogeneous tissue, including five endocrine cell types, we identified each recorded cell. We have developed a post-recording identification method. The cells were cultured on coverslips scored in a grid pattern. The coordinates of each cell on the grid were registered after recording. Each recorded cell was found and checked for GH and/or PRL staining. Non-GH- and non-PRL-staining cells were eliminated from further analysis.

Basal $[\text{Ca}^{2+}]_i$ of neonatal lactotrophs and somatotrophs

We measured $[\text{Ca}^{2+}]_i$ using the fluorescent probe indo 1. In controls, two groups of cells were distinguished according to their resting $[\text{Ca}^{2+}]_i$ pattern: (i) cells with stable $[\text{Ca}^{2+}]_i$; (ii) cells exhibiting spontaneous oscillations in their basal $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ was recorded for 1 min in single lactotrophs or somatotrophs. The group with stable $[\text{Ca}^{2+}]_i$ included all cells exhibiting no change in $[\text{Ca}^{2+}]_i$ or $[\text{Ca}^{2+}]_i$ changes lower than 50 nM. The group with $[\text{Ca}^{2+}]_i$ fluctuations included all cells that presented $[\text{Ca}^{2+}]_i$ changes greater than 50 nM. Minimum ($[\text{Ca}^{2+}]_i$ min) and maximum ($[\text{Ca}^{2+}]_i$ max) were the lowest and highest $[\text{Ca}^{2+}]_i$ values measured in recording time respectively.

The results summarized in Table 1 indicate that there was no significant difference in basal $[\text{Ca}^{2+}]_i$ between D5, D10 and 3-month lactotrophs nor between D5, D10 and 3-month somatotrophs. The fraction of oscillating

### Table 1 Basal $[\text{Ca}^{2+}]_i$ of neonatal identified pituitary cells

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<tr>
<th></th>
<th>Lactotrophs</th>
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<th>Somatotrophs</th>
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<tr>
<td></td>
<td>Cells with stable $[\text{Ca}^{2+}]_i$</td>
<td>Cells with large $[\text{Ca}^{2+}]_i$ oscillations</td>
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<td>Cells with stable $[\text{Ca}^{2+}]_i$</td>
<td>Cells with large $[\text{Ca}^{2+}]_i$ oscillations</td>
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<td></td>
<td>$[\text{Ca}^{2+}]_i$ (nM)</td>
<td>$[\text{Ca}^{2+}]_i$ min (nM)</td>
<td>$[\text{Ca}^{2+}]_i$ max (nM)</td>
<td>$[\text{Ca}^{2+}]_i$ (nM)</td>
<td>$[\text{Ca}^{2+}]_i$ min (nM)</td>
<td>$[\text{Ca}^{2+}]_i$ max (nM)</td>
</tr>
<tr>
<td>D5</td>
<td>183 ± 4</td>
<td>226 ± 29</td>
<td>289 ± 32</td>
<td>181 ± 5</td>
<td>218 ± 10</td>
<td>305 ± 23</td>
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<tr>
<td>(n=17)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=46)</td>
<td>(n=24)</td>
<td>(n=24)</td>
</tr>
<tr>
<td>D10</td>
<td>178 ± 13</td>
<td>224 ± 12</td>
<td>304 ± 25</td>
<td>160 ± 8</td>
<td>201 ± 8</td>
<td>341 ± 18</td>
</tr>
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<td>(n=31)</td>
<td>(n=23)</td>
<td>(n=23)</td>
<td>(n=23)</td>
<td>(n=38)</td>
<td>(n=38)</td>
<td>(n=38)</td>
</tr>
<tr>
<td>3-month</td>
<td>198 ± 9</td>
<td>249 ± 10</td>
<td>386 ± 20</td>
<td>210 ± 9</td>
<td>256 ± 18</td>
<td>419 ± 53</td>
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<tr>
<td>(n=28)</td>
<td>(n=46)</td>
<td>(n=46)</td>
<td>(n=46)</td>
<td>(n=15)</td>
<td>(n=17)</td>
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</table>

As the anterior pituitary is a heterogeneous tissue, including five endocrine cell types, we identified each recorded cell. We have developed a post-recording identification method. The cells were cultured on coverslips scored in a grid pattern. The coordinates of each cell on the grid were registered after recording. Each recorded cell was found and checked for GH and/or PRL staining. Non-GH- and non-PRL-staining cells were eliminated from further analysis.

![Figure 1](image-url) Lactotrophs and somatotrophs in a culture obtained from a 10-day-old rat. Double immunostaining for rPRL and rGH was performed, as described in Materials and Methods. PRL was detected using an antibody coupled with rhodamine and GH using an antibody coupled with fluorescein. Lactotrophs (PRL-positive cells) are indicated by arrowheads, and somatotrophs (GH-positive cells) by asterisks.
somatotrophs did not vary significantly between D10 and 3 months. On the other hand, the fraction of oscillating lactotrophs increased with age (23% at D5, 43% at D10 and 62% in adult (significant at $P<0.05$)).

$[Ca^{2+}]_i$ was also recorded in ten neonatal cells, later identified as mammosomatotrophs, from D10 female rats. Like lactotrophs and somatotrophs, some mammosomatotrophs exhibited spontaneous fluctuations in basal $[Ca^{2+}]_i$ (four of ten), ranging from 155 ± 8 and 253 ± 21 nm ($n=4$). The remaining six mammosomatotrophs had stable $[Ca^{2+}]_i$, without spontaneous fluctuations. Mean $[Ca^{2+}]_i$ was 145 ± 5 nm ($n=6$).

**TRH induces a $[Ca^{2+}]_i$ increase in single lactotrophs and somatotrophs**

When TRH (100 nm) was applied for 10 s in the vicinity of a neonatal D10 lactotroph, an increase in $[Ca^{2+}]_i$ was observed in 65% of cells (32 of 49) (Fig. 2 and Table 2). No change was observed in the other PRL cells (17 of 49).
even 120 s after TRH application. The former cells were therefore called responsive and the latter unresponsive. It should be noted that responsive cells were found in both of the groups defined above, i.e. oscillating and non-oscillating cells. A somewhat, but not significantly, higher percentage of responses to TRH was obtained in lactotrophs from 3-month-old rats (73%, P > 0.05, Table 2). Responses to TRH (100 nM, 10 s) were also found in a few D5 lactotrophs, but too few cells were recorded to calculate a significant percentage of responsive cells.

A transient application of TRH (100 nM, 10 s) to an isolated neonatal somatotroph induced a [Ca^{2+}] increase in 34 and 41% of the D10 and D5 cells respectively (Fig. 2 and Table 2). Again, both oscillating and non-oscillating cells were responsive. However, the response to TRH disappeared in mature somatotrophs whereas it was a major feature of mature lactotrophs. The percentage of TRH-responsive somatotrophs is therefore a significant distinguishing feature between neonatal and adult cells (Table 2).

After TRH (100 nM, 10 s) stimulation, an increase in [Ca^{2+}] was also observed in five of ten neonatal D10 mammosomatotrophs. No change was recorded in the remaining mammosomatotrophs.

From previous work performed on tumoral cell lines (Gershengorn & Thaw 1985) and normal pituitary cells (Winiger et al. 1987, Malgaroli et al. 1988), TRH is known to give rise to biphasic [Ca^{2+}] changes: the first phase is a rapid, transient increase in [Ca^{2+}], which reaches a peak at less than 10 s. The second is a moderate rise in [Ca^{2+}], which is maintained for several minutes. It is widely reported that the second phase is due to [Ca^{2+}] oscillations.

Biphasic responses to TRH were also observed in neonatal lactotrophs: in the recording shown in Fig. 2A, TRH increased [Ca^{2+}], from a basal value of 250 nM to a peak of over 1 µm within 10 s. It then decreased and formed a slowly declining plateau around 400 nM for 60 s. After that time, it slowly returned to its basal value. However, in some neonatal lactotrophs, only one phase was observed. Typical biphasic [Ca^{2+}] responses were observed in 23-5% of responsive PRL cells whereas 59 and 17-5% of responsive PRL cells presented only a first phase or a second phase respectively. Examples of these responses are given in Fig. 2B and C. Fig. 2B shows a PRL-secreting cell that only responded to TRH application by a rapid transient [Ca^{2+}] increase (i.e. a first phase). In another PRL-secreting cell, TRH only induced a moderate sustained [Ca^{2+}] increase (i.e. a second phase), as illustrated in Fig. 2C.

The average characteristics of the [Ca^{2+}] changes induced by TRH in D10 lactotrophs are summarized in Table 3.

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<th>First phase</th>
<th>Second phase</th>
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<tr>
<td></td>
<td>[Ca^{2+}] (nM)</td>
<td>Duration (s)</td>
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<tr>
<td>Lactotrophs</td>
<td>769 ± 124 (n=14)</td>
<td>40 ± 5 (n=14)</td>
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<tr>
<td></td>
<td>946 ± 90 (n=27)</td>
<td>35 ± 8 (n=27)</td>
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<tr>
<td>Somatotrophs</td>
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TRH regulation of neonatal lactotrophs and somatotrophs

A [Ca^{2+}] (nM)

B [Ca^{2+}] (nM)

Figure 3 TRH application (100 nM, 10 s) leads to a [Ca^{2+}] increase in single pituitary cells from 5-day-old rats, similar to that found in cells from 10-day-old rats. The biphasic Ca^{2+} responses in an identified lactotroph (A) and somatotroph (B) are shown.

TRH intracellular signalling: heterogeneity of Ca^{2+} sources

External Ca^{2+}-free solution and Ca^{2+} channel blockers were used to investigate the origins of the biphasic increase in [Ca^{2+}], induced by local TRH application.

When TRH (100 nM, 10 s) was applied in a Ca^{2+}-free 2 mM EGTA-containing medium, the first phase was maintained in only two of 12 responsive PRL-secreting cells (Fig. 4A) and in four of 14 responsive GH-secreting cells (Fig. 4B). This response rate differs from that of the cell group showing a first phase in control conditions (19 of 32 lactotrophs and 27 of 36 somatotrophs). On the other hand, the second phase was abolished in all recorded lactotrophs and somatotrophs (n=26). When Ca^{2+}-free solution was applied to TRH-induced biphasic Ca^{2+} responses, it transiently abolished the second phase (Fig. 4B). No second phase was ever recorded during local application of cadmium ions (500 µM, n=12), which are known to block T- and L-type voltage-operated Ca^{2+} channels in clonal pituitary cells (Cohen & McCarthy 1987). On the other hand, the number of cells showing a first phase with cadmium was similar to that found in controls (four of eight cells).

These results suggest that the increase in [Ca^{2+}], during the second phase involves an influx of Ca^{2+} from the extracellular medium, possibly through voltage-operated Ca^{2+} channels. They also suggest that the first phase is extremely sensitive to the removal of Ca^{2+} ions from the extracellular medium.

TRH accelerates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, generating two intracellular messengers, IP3 and diacylglycerol.

The first phase of the [Ca^{2+}] increase after stimulation by TRH is generally viewed as the result of mobilization of intracellular Ca^{2+} by IP3 acting on intracellular receptors on the endoplasmic reticulum membrane. We therefore examined the first phase in individual D10 cells in the presence of thapsigargin, an endoplasmic reticulum Ca^{2+}-ATPase inhibitor (Stojilkovic et al. 1988, Thastrup et al. 1990). Basal [Ca^{2+}] of both lactotrophs and somatotrophs was modified by a 45 min incubation in 100 nM thapsigargin. It was significantly higher than in control conditions (Table 4, P<0.05). Moreover, the oscillation frequency increased whereas the amplitude decreased (Table 4, P<0.05). Thapsigargin treatment abolished any TRH-induced [Ca^{2+}] changes in all the PRL- and GH-secreting cells tested (n=33). No first or second phase was ever found (Table 4).

The second phase of the TRH effect is thought to occur as the result of stimulation of protein kinase C (PKC) by the hydrophobic messenger diacylglycerol (Drust & Martin 1985, Gollash et al. 1993). We therefore investigated the possible involvement of PKC in the TRH-induced [Ca^{2+}] increase observed in neonatal lactotrophs and somatotrophs. D10 pituitary cells were treated with PMA (1 µM) for 24 h. This treatment induces PKC depletion in pituitary cells (Craig et al. 1989, Thastrup et al. 1990). [Ca^{2+}] measurements in lactotrophs and somatotrophs both revealed major modifications in basal [Ca^{2+}]. The number of spontaneously oscillating cells, as well as the amplitude of oscillations, were greatly reduced (Table 4, P<0.05), suggesting that PKC plays a role in the control of basal [Ca^{2+}], in these cells. However, 70% of PRL-secreting cells and 40% of GH-secreting cells still responded to TRH (100 nM, 10 s). All these recorded cells exhibited only a first phase (Fig. 4C and D). A second phase was never observed (Table 4).

Taken together, these results suggest that both intracellular and extracellular Ca^{2+} intervene in [Ca^{2+}] responses to TRH in neonatal lactotrophs and somatotrophs. Intracellular Ca^{2+} presumably comes from the endoplasmic reticulum as this is a thapsigargin-sensitive store. Extracellular Ca^{2+} forms a PKC-sensitive influx.

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Electrical response to TRH in neonatal lactotrophs and somatotrophs

Figure 5A presents a typical recording obtained in a D10 cell that stained for PRL after patch clamping. The whole-cell recording mode was used to monitor membrane voltage and input resistance. Before TRH application, membrane potential was $-35$ mV and input resistance $5 \, \text{G}\Omega$. TRH (100 nM for 10 s) initially induced a hyperpolarization to $-45$ mV accompanied by a reduction in input resistance to $0.3 \, \text{G}\Omega$. This first phase lasted for 20 s. The membrane voltage then depolarized. Voltage

Table 4 Effects of pretreatment with 100 nM thapsigargin and 1 \textmu M PMA on basal $\left[\text{Ca}^{2+}\right]_i$ and TRH-induced $\text{Ca}^{2+}$ response in D10 identified pituitary cells. Pretreatment with thapsigargin lasted 45 min. Pretreatment with PMA lasted 24 h

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<th>Lactotrophs</th>
<th>Somatotrophs</th>
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<tr>
<td></td>
<td>Control (n=49)</td>
<td>Thapsigargin (n=13)</td>
</tr>
<tr>
<td>$\left[\text{Ca}^{2+}\right]_i$ min</td>
<td>224 $\pm$ 12</td>
<td>280 $\pm$ 11*</td>
</tr>
<tr>
<td>$\left[\text{Ca}^{2+}\right]_i$ max</td>
<td>304 $\pm$ 25</td>
<td>340 $\pm$ 21*</td>
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<tr>
<td>Oscillations (frequency/min)</td>
<td>12</td>
<td>16*</td>
</tr>
<tr>
<td>Percentage of oscillating cells</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>Percentage of cells responding to TRH</td>
<td>65</td>
<td>0*</td>
</tr>
<tr>
<td>Percentage of cells showing a first phase</td>
<td>59</td>
<td>0*</td>
</tr>
<tr>
<td>Percentage of cells showing a second phase</td>
<td>17.5</td>
<td>0*</td>
</tr>
<tr>
<td>Percentage of cells showing biphasic responses</td>
<td>23.5</td>
<td>0*</td>
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*Significantly different from control at $P<0.05$. 

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fluctuations of a few mV appeared. The current steps caused off action potentials. Later on, spontaneous action potentials appeared. At 4 min and 30 s after the application of TRH, the membrane potential repolarized and input resistance was similar to values before TRH stimulation.

Figure 5B shows a recording of a similar experiment on a GH-secreting cell. In this cell, the first phase displayed a decrease in input resistance (2.6 GΩ before compared with 0.4 GΩ 3 s after the beginning of the TRH application) with little, if any, hyperpolarization. Marked depolarization occurred, with superimposed high frequency action potentials. The action potential amplitude was 30 mV. The TRH-induced depolarization lasted for 1 min and 30 s. The cell then repolarized to its prestimulatory value.

Patched D10 cells thus show changes in voltage and input resistance reminiscent of those reported for tumoral GH3 cells. The first phase may be absent and the second phase is readily observed. This is in agreement with our [Ca^{2+}]_i recordings.

**TRH modulates PRL but not GH secretion from neonatal pituitary cells in vitro**

The ability of TRH to stimulate PRL secretion from immature pituitary cells in vitro is shown in Fig. 6A. Incubation in 100 nM TRH for 30 min significantly increased PRL release from cells obtained from D5, D10 and mature female rats. The basal secretion level after TRH stimulation was increased at least 3-fold in immature cells and 2-fold in mature cells. However, no significant statistical difference was found in the percentage increase in PRL release due to TRH throughout development.

On the other hand, when pituitary cells were incubated with TRH (100 nM) for 30 min, no stimulation of GH secretion was observed, whatever the age group (Fig. 6B). Nevertheless, the cells were able to release GH upon stimulation as shown by the potent stimulatory effect of GH-releasing factor (GRF) in the same cells. The mean GH release induced by GRF from cells obtained from D5, D10 and 3-month-old female rats was 300, 700 and 500% of basal secretion respectively (data not shown).

**Discussion**

Our results clearly demonstrate that TRH induces an increase in the intracellular Ca^{2+} concentration of both lactotrophs and somatotrophs during early postnatal development. The intracellular pathways leading to this increase are similar to those found in mature lactotrophs. However, TRH does not stimulate GH but only PRL secretion in immature cells.

All our single-cell results were obtained in identified cells, i.e. cells in which the secretory content was later assessed by immunocytochemistry. Simultaneous post-recording immunostaining for rat GH and PRL showed
the intracellular hormone peptides (GH and/or PRL) stored in the cells. The percentage of somatotrophs and lactotrophs was consistent with that reported in the literature (Chen 1987, Smets et al. 1989). Nevertheless, it should be borne in mind that this type of identification procedure has at least two drawbacks. On the one hand, it does not provide data on a labelled cell’s capacity to release the hormones, either spontaneously or under stimulation. On the other hand, as immunostaining was performed after the TRH assay, some cells may have released most, if not all, of their secretory granules upon stimulation and may therefore appear PRL- or GH-negative.

Very few data are available in the literature on intracellular Ca²⁺ concentration control of neonatal pituitary cells (Cuttler et al. 1992, 1993, Felix et al. 1993). In agreement with Felix et al. (1993), we found a smaller percentage of spontaneously oscillating PRL-secreting cells in 5- or 10-day-old rats than in adults, where this type of cell accounted for over 60% of the population (Lorsignol et al. 1994). This small number of oscillating cells is likely to be responsible for the smaller basal PRL release in neonatal cells (Chen 1987, Felix et al. 1993, A Lorsignol unpublished results). We show that basal [Ca²⁺]i and TRH-induced [Ca²⁺]i changes in lactotrophs and somatotrophs obtained from immature rats are similar to those found in lactotrophs from mature rats. First, basal [Ca²⁺]i is heterogeneous as was reported for adult lactotrophs (Winiger et al. 1987, Malgaroli et al. 1988, Lorsignol et al. 1994). Some neonatal cells exhibit oscillating basal [Ca²⁺]i, whereas others exhibit stable basal [Ca²⁺]i, without spontaneous fluctuation. Secondly, TRH receptor activation in the neonatal cells often induced a biphasic increase in [Ca²⁺]i, involving Ca²⁺ release from internal stores and/or Ca²⁺ entry. Moreover, our patch-clamp recordings revealed a biphasic response, with hyperpolarization followed by depolarization, with superimposed action potentials. These results are in agreement with those already reported for mature lactotrophs and GH3 cells. We can therefore assume that, as in adult lactotrophs and GH3 cells, TRH receptors are coupled to a phospholipase C. Cuttler et al. (1995) have reported the existence of a functional phospholipase C in neonatal somatotrophs, consistent with this hypothesis. TRH receptor activation may therefore generate IP₃ and diacylglycerol. Nevertheless, no direct demonstration of IP₃ involvement in immature cells has yet been reported. To our knowledge, this is the first time that a thapsigargin-sensitive intracellular Ca²⁺ pool, which may be mobilized by TRH receptor activation, has been demonstrated in neonatal lactotrophs and somatotrophs. However, we cannot preclude the involvement of thapsigargin-sensitive but IP₃-insensitive Ca²⁺ stores. Our experiments on PKC-depleted cells revealed a PKC-dependent TRH-induced Ca²⁺ influx. Nevertheless, in the presence of extracellular Ca²⁺, TRH-induced Ca²⁺ responses were fully abolished by treatment with thapsigargin. The depletion of a Ca²⁺ pool by thapsigargin thus suppresses the TRH-induced Ca²⁺ influx. Two hypotheses may be put forward: (i) this is due to inactivation of Ca²⁺ channels by the high [Ca²⁺]i induced by thapsigargin, as already demonstrated in GH3 cells (Kalman et al. 1988); (ii) TRH stimulates a thapsigargin-sensitive Ca²⁺ influx component, i.e. a capacitative Ca²⁺ influx, activated by store depletion, as proposed by Carew & Mason (1995) for mature lactotrophs. In this case, TRH would have no effect on thapsigargin-treated cells in normal external medium, as capacitative Ca²⁺ entry would already be maximally activated.

Our measurements of PRL release by neonatal pituitary cells demonstrate that TRH stimulates PRL secretion during postnatal development, as is the case in mature animals. This is in agreement with the results of our single-cell study. The amplitude of the PRL peak is similar...
to that observed with adult cells. As the number of PRL-secreting cells is smaller during postnatal development than in adults and a significant proportion of lactotrophs in neonatal rats do not spontaneously secrete measurable amounts of PRL (Chen 1987), this finding supports the hypothesis of Khorram et al. (1984), who suggested that TRH was a more potent PRL-releasing factor in early postnatal development than in adults.

By contrast, no modification of GH release was observed when either postnatal or mature pituitary cells were stimulated with TRH for 30 min. It should be noted that GH release was strongly stimulated by GRF at the same stages of development. It has been reported that TRH stimulated GH secretion during prenatal and early postnatal development, but only with long-term stimulations, presumably by inducing protein synthesis (Khorram et al. 1984, Welsh et al. 1986). Moreover, the TRH-induced GH stimulation diminished with age and disappeared at the end of postnatal development. Our results on GH secretion were unexpected as TRH significantly increased [Ca^{2+}]_i in neonatal GH-secreting cells. It is worth noting that TRH-induced [Ca^{2+}]_i regulation disappears in adult somatotrophs. Although Ca^{2+} ions play a fundamental role in stimulation-secretion coupling, it has also been demonstrated that exocytosis may also be Ca^{2+}-independent (Penner 1988). Moreover, some cells undergo a large transient agonist-induced [Ca^{2+}]_i increase, without any change in secretion (Cheek et al. 1989). It is thought that, in cells of this type, second messengers other than Ca^{2+} ions may be involved in regulating exocytosis. It is well known that GRF and somatostatin both control somatotrophs by regulation of intracellular cAMP levels. It is thus possible that GH release is more dependent on cAMP concentration than on [Ca^{2+}]_i. We suggest that at D5 or D10, even though TRH increases [Ca^{2+}]_i, GH secretion is not stimulated because some late steps in the exocytic process, downstream of Ca^{2+} ions, have already disappeared. The control of somatotrophs by TRH would therefore lose its physiological significance during postnatal development. It has been shown that it may appear again in adults as the result of pathological disorders (Harvey 1990), indicating that it depends on reversible cell regulation(s). As somatotrophs and lactotrophs are derived from a common progenitor cell (Behringer et al. 1988, Borelli et al. 1989), we further suggest that TRH regulation of neonatal somatotrophs is a residual feature of the progenitor cell status.

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