

Thyrotropin-releasing Hormone Increases Cytosolic Free Ca^{2+} in Clonal Pituitary Cells (GH₃ Cells): Direct Evidence for the Mobilization of Cellular Calcium

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ABSTRACT Changes in the cytosolic free Ca^{2+} concentration following cell surface receptor activation have been proposed to mediate a wide variety of cellular responses. Using the specific Ca^{2+} chelator quin2 as a fluorescent intracellular probe, we measured the Ca^{2+} levels in the cytosol of clonal rat pituitary cells, GH₃ cells. We demonstrate that thyrotropin-releasing hormone (TRH) at nanomolar concentrations leads to a rapid and transient increase in cytosolic Ca^{2+} . This increase was found to occur in Ca^{2+} -free media in the presence of EGTA, thus at extracellular Ca^{2+} levels that are below the cytosolic concentrations, and was not prevented by verapamil, a Ca^{2+} channel blocker. Depolarization of GH₃ cells with K^+ , which can mimic the action of TRH on prolactin release, increased cytosolic Ca^{2+} levels only in the presence of free extracellular Ca^{2+} , and this increase could be blocked by verapamil. These data show that the mobilization of intracellular Ca^{2+} due to TRH action that has been proposed by previous studies actually leads to an increase in cytosolic free Ca^{2+} . The kinetic features of this response emphasize the key role of cytosolic free Ca^{2+} in stimulus–secretion coupling.

Thyrotropin-releasing hormone (TRH)¹ stimulates the secretion of prolactin and thyrotropin from the pituitary. Changes in intracellular Ca^{2+} distribution have been implicated in the mediation of TRH action (for review see reference 1). TRH increases the frequency of Ca^{2+} action potentials in pituitary cells (2) or in GH₃ cells (3, 4), a clonal rat pituitary cell line responsive to TRH (5). Depolarization of GH₃ cells with K^+ , which promotes the entry of Ca^{2+} , can mimic some of the actions of TRH (6). It has been proposed that TRH increases influx of extracellular Ca^{2+} (2–4, 6, 7), but it has also been demonstrated that TRH leads to an increased Ca^{2+} efflux from the cells (8–10) and can cause a net loss of Ca^{2+} from GH₃ cells (10). The use of digitonin made it possible to reveal distinct pools of intracellular Ca^{2+} and it was reported that TRH can lead to a rapid depletion of Ca^{2+} from two pools that have been tentatively identified as of the mitochondria

and of the endoplasmic reticulum (11). However, none of the studies so far could give direct information on a presumed key variable in stimulus–secretion coupling, namely, the concentration of free cytosolic Ca^{2+} , $[\text{Ca}^{2+}]_i$.

To measure the effects of TRH on $[\text{Ca}^{2+}]_i$ we used the fluorescent probe quin2, which was introduced by Tsien (12) and has been applied to lymphocytes (13, 14), platelets (15), neutrophils (16), and an insulin-producing cell line (17). quin2 can be introduced into cells as the lipophilic acetoxy methyl ester (quin2/AM) which is hydrolyzed by cellular esterases to yield hydrophilic free quin2. Its fluorescence increases in the presence of Ca^{2+} which is complexed selectively and with little interference from either Mg^{2+} or protons. The apparent dissociation constant for the fluorescent Ca^{2+} quin2 complex in the presence of 1 mM Mg^{2+} (the presumed cytoplasmic Mg^{2+} concentration) is 115 nM. Since this is close to cytoplasmic levels of Ca^{2+} in many cells (18, 19), the fluorescent probe monitors even relatively small variations in $[\text{Ca}^{2+}]_i$. The intracellular quin2 fluorescence can be calibrated, which allows a quantitative measure of $[\text{Ca}^{2+}]_i$. This present paper presents evidence, obtained with this technique, that TRH transiently increases $[\text{Ca}^{2+}]_i$ in both the presence and absence of extracellular Ca^{2+} .

¹ Abbreviations used in this paper: bis-oxonol, bis-(1,3-diethylthio-barbiturate)-trimethineoxonol; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; KRBH, HEPES-buffered Krebs-Ringer bicarbonate buffer; quin2/AM, quin2 acetoxy methyl ester; TRH, thyrotropin-releasing hormone.

MATERIALS AND METHODS

Cell Culture: GH₃ cells obtained from the American Type Culture Collection (Rockville, MD) were used throughout. Stock monolayer cultures were maintained as described (5). Before the experiment the cells were detached from the culture flasks with 0.02% EDTA and incubated in HEPES-buffered (30 mM, pH 7.4) Ham-F10 medium supplemented with 15% horse and 2.5% fetal calf serum in spinner flasks at 37°C (1) for 3 h or overnight.

Determination of [Ca²⁺]_i in GH₃ Cells with the Fluorescent Intracellular Probe quin2: The validity of the quin2 method for assessing [Ca²⁺]_i has been verified in detail with lymphocytes (13, 14). The experimental conditions for the application of this method to GH₃ cells were verified to assure a sufficient level of intracellular probe, the unchanged spectral characteristics of intracellular quin2 fluorescence (see Fig. 1) indicating complete hydrolysis of quin2/AM and negligible quenching of the signal, the cytosolic location of quin2, and the maintenance of cellular functions.

Before being loaded, the cells were centrifuged twice (100 g for 10 min) and resuspended in culture medium RPMI 1640 containing 25 mM HEPES (pH 7.4) and 0.5% BSA (fraction V). The final cell concentration was 20–30 × 10⁶ cells/ml. quin2/AM (Lancaster Synthesis, Morecambe, Lancashire, U.K.) was added to the cell suspension from a 10-mM stock solution in DMSO to a final concentration of 100 μM. After 10 min at 37°C, 4 vol of RPMI 1640 were added and the incubation was continued for 50 min. Control cells were incubated in parallel with 1% DMSO and then diluted as the test cells. After the incubation all cells were washed and resuspended in RPMI 1640 without serum or albumin and kept at room temperature until use.

Fluorescence of quin2 external standard and loaded cells was measured in 1-cm glass cuvettes thermostatted at 37°C in a Perkin-Elmer fluorescence spectrophotometer (LS-3; Perkin-Elmer Corp., Eden Prairie, MN) at wavelengths 339 nm for excitation and 492 nm for emission. Membrane potential was recorded with the fluorescent probe bis-(1,3-diethylthiobarbiturate)-trimethineoxonol (*bis*-oxonol), a lipophilic anion (20). *Bis*-oxonol fluorescence was measured at excitation and emission wavelengths of 540 and 580 nm, respectively. The slit widths were set to cover a spectral range of 10 nm (excitation) and 20 nm (emission), for both quin2 and *bis*-oxonol measurements. The cells (5 × 10⁵–2.5 × 10⁶ cells/ml) were suspended in 2 ml of a modified Krebs-Ringer bicarbonate buffer containing 25 mM HEPES, 5 mM NaHCO₃, 1.1 mM CaCl₂, 0.1 mM EGTA, and 2.8 mM glucose (KRBH), and were stirred continuously with a magnetic stirrer. Test agents were added from concentrated stock solutions.

The fluorescence signal was calibrated at the end of each individual trace as follows: To obtain the minimal fluorescence signal of quin2, 4 mM EGTA (pH 7.4) and 30 mM Tris were added to yield an approximate free [Ca²⁺]_i of 1 nM. These additions show the level of extracellular quin2 (13, 14), as the decrease of the extracellular [Ca²⁺]_i leads to a rapid (<10 s) initial decrease of the fluorescence of the extracellular dye. At this stage the hydrolysis of quin2/AM was usually verified by recording the emission spectrum (Fig. 1). After the cells were lysed with Triton X-100 (final concentration 0.1%), all the quin2 was exposed to [Ca²⁺]_i of ~1 nM and the minimal fluorescence signal, *F*_{min}, was obtained. The maximal quin2 fluorescence, *F*_{max}, was then determined by re-adding Ca²⁺ (4 mM) to the cuvette restoring a free [Ca²⁺]_i of ~1 mM. For quin2 measurements performed in Ca²⁺-free KRBH, the level of extracellular quin2 was determined by restoring the free [Ca²⁺]_i to 1 mM before following the procedure outlined above which was then used to obtain *F*_{min} and *F*_{max}. The difference between *F*_{min} and *F*_{max}, corrected for the dilution occurring by the addition of the various reagents, also comprises the extracellular quin2 fluorescence which has to be subtracted to obtain the maximal fluorescence due to intracellularly trapped quin2, *F*_{max}*; *F*_{max}* averages 71 ± 5% of *F*_{max} (± SEM, *n* = 10). To establish the scale of [Ca²⁺]_i relative to the fluorescence trace, *F*_{min} was corrected for the changes in fluorescence due to the detergent that were measured in nonloaded cells in parallel, and, in the case of Ca²⁺-containing KRBH, also for the contribution of extracellular quin2, yielding *F*_{min}*. [Ca²⁺]_i can then be calculated from the fluorescence *F* at each point of the trace as [Ca²⁺]_i = *K*_d(*F* - *F*_{min}*)/(*F*_{max}* - *F*), and *K*_d = 115 nM (for details see reference 13).

The intracellular concentration of quin2 was calculated by comparing *F*_{max}* to the fluorescence of a known quantity of quin2 added to control cells incubated under the conditions described to establish *F*_{max}. quin2 loading of GH₃ cells averaged 0.46 ± 0.07 mM (± SEM, *n* = 11) calculated with an intracellular volume of 1.4 μl/10⁶ cells (21); there was no apparent correlation between quin2 loading ranging from 0.24 to 0.9 mM and prestimulatory [Ca²⁺]_i.

The cytosolic localization of quin2 in GH₃ cells was confirmed by the correlation between the release of quin2 and the release of lactate dehydrogenase, a cytosolic enzyme, upon digitonin treatment. quin2-loaded GH₃ cells were exposed to increased concentrations of digitonin (2.5 μM to 1 mM) in Ca²⁺-free KRBH, 1 mM EGTA, for 5 min at 0°C; in the supernatant after centrifu-

gation (1500 g, 5 min), quin2 was determined by fluorescence and lactate dehydrogenase was measured following the change in absorbance at 340 nm with NADH and pyruvate. 85–90% of quin2 and >95% of lactate dehydrogenase were released at maximal digitonin levels, and for intermediate digitonin concentrations there was a strict correlation (*R* = 0.98, *n* = 16) between the release of quin2 and lactate dehydrogenase.

ATP levels were determined after extraction of GH₃ cells with 6% HClO₄ and neutralization, by high-performance liquid chromatography on a Partisil 10 SAX ion exchange column (4.6 × 25 cm) eluted isocratically with 0.5 M ammonium phosphate, pH 4.4, at a flow rate of 2 ml/min. It was found that quin2 loading led to a slight but not significant increase of the ATP levels in GH₃ cells (1.48 ± 0.13 [± SEM, *n* = 4] versus 1.24 ± 0.19 nmol/10⁶ cells). From these experiments we conclude that the quin2 loading does not cause a major disturbance of cellular homeostasis.

RESULTS

TRH Effects on Membrane Potential in GH₃ Cells

The lipophilic anion *bis*-oxonol shows an increase in fluorescence in a lipophilic environment. When added to a cell suspension, the distribution between the free and the cell-bound dye depends on the membrane potential, depolarization favoring the association of *bis*-oxonol with the cell and hence increasing overall fluorescence (20). *Bis*-oxonol fluorescence reflects the average membrane potential, since spontaneous action potentials occurring randomly in the cell population cannot be resolved. We used this method to determine whether TRH would affect the membrane potential in GH₃ cells in suspension. As shown in Fig. 2, TRH (10⁻⁷ M) leads to a slight hyperpolarization immediately after its addition, and to a transient, very slight depolarization thereafter. This observation is consistent with the biphasic change in membrane potential of GH₃ cells following TRH addition reported by Ozawa (22): using intracellular electrodes, he showed that TRH causes initial hyperpolarization, followed by an increase in action potential frequency. As can be seen in Fig. 2, the

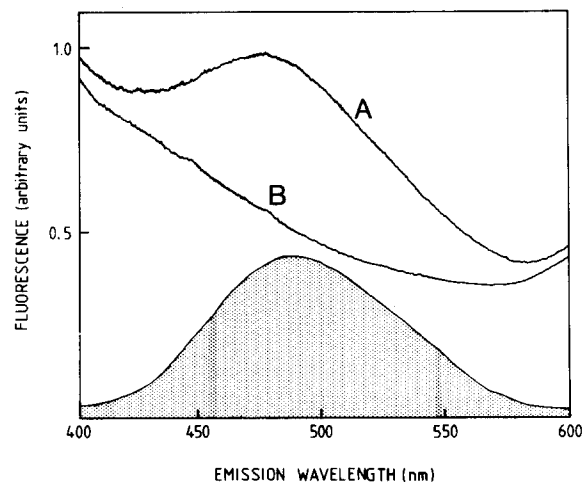


FIGURE 1 Intracellular quin2 fluorescence. The hydrolysis of the quin2 ester was usually verified by recording the emission spectrum of quin2 at excitation wavelength 339 nm in the loaded cells (A). To minimize the participation of extracellular dye the spectrum was recorded at low extracellular [Ca²⁺]_i, i.e., after addition of EGTA and Tris (see Materials and Methods). The emission spectrum of unloaded control cells was recorded under the same conditions (B). The differential spectrum (shaded area), which is due to the fluorescence of intracellular quin2, has a maximum at 490 nm; it corresponds to the emission spectrum of quin2 at pH 7.0 in the presence of 1 mM Mg²⁺ (13), indicating complete hydrolysis of quin2/AM and negligible quenching of the intracellular fluorescence signal. The emission spectrum of quin2/AM has its maximum at 430 nm (13).

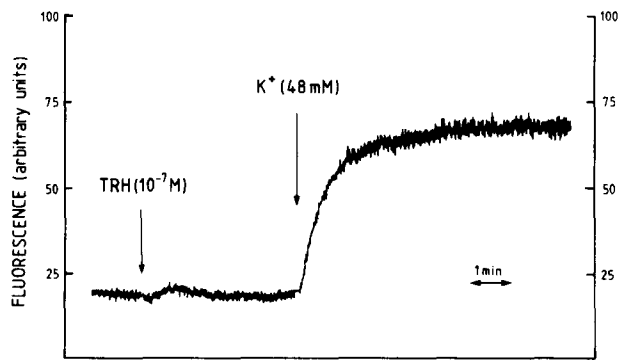


FIGURE 2 Effects of TRH (10^{-7} M) and K^+ (48 mM) on the membrane potential of GH₃ cells. Fluorescence signal of *bis*-oxonol (100 nM) in the presence of GH₃ cells (2×10^5 cells/ml). Upward deflection of the trace denotes depolarization of the cells. The final concentration of TRH was 10^{-7} M and that of K^+ 54 mM.

changes in membrane potential are small when compared with the effects of 48 mM K^+ , which leads to the expected depolarization. Gershengorn (21), with a method that requires the separation of cell-bound and free membrane potential probe, did not observe any change in membrane potential due to TRH. The results with *bis*-oxonol thus show that the changes in membrane potential of detached GH₃ cells are similar to those predicted from micro-impalement experiments on attached GH₃ cells. In view of this, the experimental set up seems well suited to correlate changes in membrane potential and $[Ca^{2+}]_i$.

Effects of TRH on $[Ca^{2+}]_i$ in Ca^{2+} -containing Medium

The fluorescence of quin2 was used to measure $[Ca^{2+}]_i$ in GH₃ cells suspended in KRBH containing 1 mM free Ca^{2+} ; typical experiments are represented in Fig. 3, and the mean values of $[Ca^{2+}]_i$ measured in a large series of similar experiments are given in Table I. Fig. 3A shows that TRH (10^{-7} M) leads to a rapid increase in $[Ca^{2+}]_i$; within seconds $[Ca^{2+}]_i$ increases from a mean prestimulatory level of 114 nM to a maximum that averages 293 nM (Table I). 3–5 min after TRH addition, a new steady-state $[Ca^{2+}]_i$ is reached that is significantly different from prestimulatory $[Ca^{2+}]_i$ (Table I). $[Ca^{2+}]_i$ can be further increased by depolarization of the cells with K^+ (Fig. 3A), and again the cells can buffer the change in $[Ca^{2+}]_i$ with time and reach a different steady state.

Fig. 3B shows that the increase in $[Ca^{2+}]_i$ can be obtained with low concentrations of TRH comparable to those that can elicit the cellular responses (1). TRH added to give 10^{-9} M final concentration increases $[Ca^{2+}]_i$. Subsequently, a steady-state $[Ca^{2+}]_i$ is reached that is not different from the prestimulatory level. $[Ca^{2+}]_i$ can be increased again by raising the TRH concentration to 10^{-8} M.

The voltage-dependent Ca^{2+} channels can be blocked with verapamil. As can be seen in Fig. 3C, this inhibition of Ca^{2+} influx leads to a lowering of $[Ca^{2+}]_i$ indicating that the steady-state $[Ca^{2+}]_i$ is determined in part by Ca^{2+} influx via voltage-dependent Ca^{2+} channels. TRH added to cells in the presence of verapamil still causes a rapid and transient increase in $[Ca^{2+}]_i$. In contrast, depolarization with K^+ fails to raise $[Ca^{2+}]_i$, thus demonstrating that the channel blockage is complete. This indicates that TRH can act to increase $[Ca^{2+}]_i$ by a mechanism independent of voltage-dependent Ca^{2+} channels.

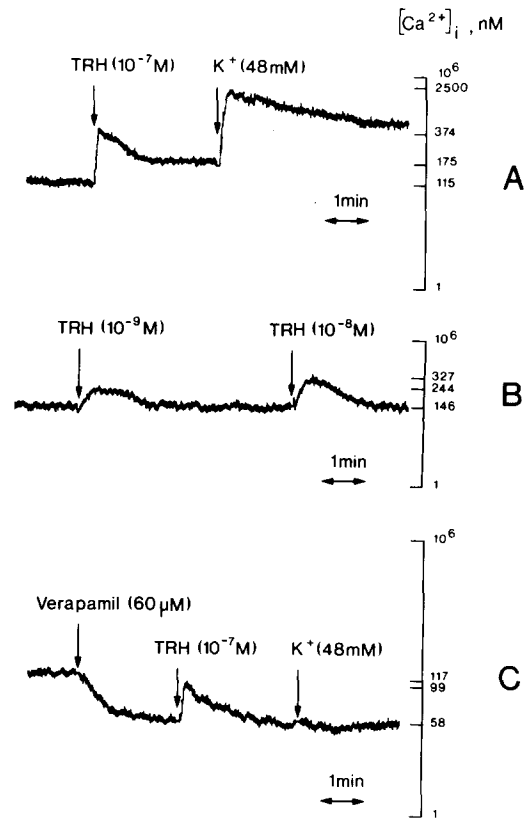


FIGURE 3 $[Ca^{2+}]_i$ in GH₃ cells in medium with 1 mM Ca^{2+} . quin2 fluorescence traces calibrated for $[Ca^{2+}]_i$, as described in Materials and Methods. Final concentrations are indicated in parentheses except for K^+ which was increased from 6 to 54 mM. (See text for a discussion of the individual traces, A–C.)

TABLE I
 $[Ca^{2+}]_i$ in GH₃ Cells

	KRBH, 1 mM Ca^{2+}	KRBH, Ca^{2+} free, 1 mM EGTA
	<i>nM ± SEM (n)</i>	
Prestimulatory	114 ± 7 (19)	95 ± 7 (13)
After TRH (10^{-7} M)		
Maximum	293 ± 44 (11)*	176 ± 20 (13)*
Steady state	160 ± 17 (9)*	ND*

$[Ca^{2+}]_i$ was determined from quin2 fluorescence in experiments like those shown in Figs. 3A and 4A. Results are expressed in nM as the mean ± SEM (*n* = number of experiments). quin2 loading of GH₃ cells averaged 0.46 ± 0.07 mM, SEM (*n* = 11) calculated with an intracellular volume of GH₃ cells of $1.4 \mu\text{l}/10^6$ cells (21). No correlation between quin2 loading and prestimulatory $[Ca^{2+}]_i$ was observed.

* Significantly elevated from prestimulatory $[Ca^{2+}]_i$, $P < 0.005$.

* ND, not determined; see footnote 2.

Effects of TRH on $[Ca^{2+}]_i$ in Ca^{2+} -free Medium

To determine whether TRH acts on $[Ca^{2+}]_i$ by an increase of the Ca^{2+} permeability of the plasma membrane leading to a net Ca^{2+} influx, we determined $[Ca^{2+}]_i$ in Ca^{2+} -free KRBH in the presence of 1 mM EGTA, i.e., at extracellular Ca^{2+} levels $<10^{-8}$ M. As shown in Fig. 4, TRH still increases $[Ca^{2+}]_i$. The prestimulatory $[Ca^{2+}]_i$ under these conditions averages 95 nM² and TRH raises $[Ca^{2+}]_i$ to 176 nM immedi-

² Exposure of GH₃ cells to Ca^{2+} -free KRBH with 1 mM EGTA leads to a progressive decrease in $[Ca^{2+}]_i$ of ~ 1.5 nM/min at 37°C. The cells reach an apparent steady-state $[Ca^{2+}]_i$ only after 30–60 min at which time the response of $[Ca^{2+}]_i$ to TRH is small or absent.

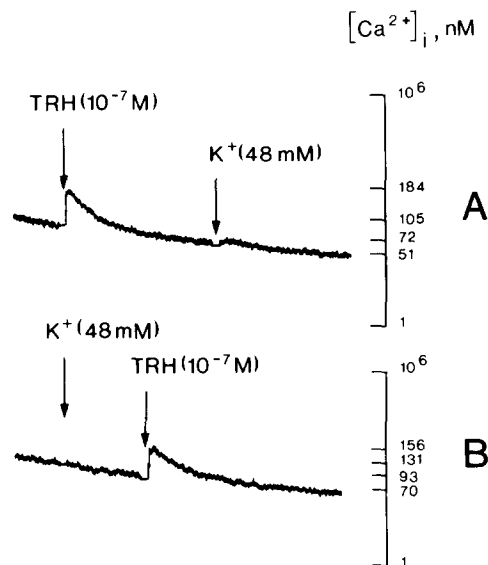


FIGURE 4 $[Ca^{2+}]_i$ in GH_3 cells in Ca^{2+} -free medium with 1 mM EGTA. quin2 fluorescence traces calibrated for $[Ca^{2+}]_i$ as described in Materials and Methods. quin2-loaded GH_3 cells were washed in Ca^{2+} -containing KRBH and resuspended in Ca^{2+} -free KRBH immediately preceding the fluorescence measurement, i.e., <3 min before the beginning of the traces shown. The final concentration of TRH was 10^{-7} M and that of K^+ 54 mM.

ately after its addition (Table I). Depolarization with K^+ before or after TRH stimulation no longer alters $[Ca^{2+}]_i$. Hence, changes in Ca^{2+} permeability and membrane potential that have important consequences on $[Ca^{2+}]_i$ in Ca^{2+} -containing medium (Fig. 3A) have no effect on $[Ca^{2+}]_i$ at low extracellular Ca^{2+} levels, a condition in which there is net efflux of calcium from GH_3 cells (10); in addition, depolarization does not interfere with the mechanism by which $[Ca^{2+}]_i$ is increased by TRH. The onset of the response of $[Ca^{2+}]_i$ is as rapid in Ca^{2+} -free media as it is in media containing Ca^{2+} , and the rise is similarly transient. The continuous decrease in $[Ca^{2+}]_i$ in Ca^{2+} -free medium² does not allow the observation of a change in steady-state $[Ca^{2+}]_i$ after TRH stimulation analogous to that seen in Ca^{2+} -containing medium (Table I).

DISCUSSION

The present work provides direct evidence of an increase of cytosolic free Ca^{2+} , $[Ca^{2+}]_i$, as an early event in stimulus-response coupling for TRH, using the recently developed Ca^{2+} probe quin2. Use of this novel methodology for the study of the second messenger role of Ca^{2+} has two distinct advantages: The fluorescent probe is readily introduced into the cells without the need to disrupt the plasma membrane, and the calibration of the fluorescent signal is straightforward and allows a quantitative measurement of $[Ca^{2+}]_i$ with a comparably small experimental error (Table I). Thus far it has been extremely difficult to measure $[Ca^{2+}]_i$, particularly in small mammalian cells (23). At present the only alternative to the quin2 method relies on a Ca^{2+} -sensitive photoprotein, aequorin, introduced into cells by a hypo-osmotic shock treatment (24); this method requires specialized equipment to measure luminescence and drastic manipulations of the cells for the introduction of the Ca^{2+} probe.

Beside the advantages mentioned above, the quin2 method has one potential drawback; in order to obtain a sufficient

fluorescence signal, cells have to contain up to millimolar concentrations of quin2. This adds a considerable Ca^{2+} -buffering capacity to the cells. Despite this, the cells maintain $[Ca^{2+}]_i$ at distinct levels in steady state and respond to regulatory ligands with rapid changes of $[Ca^{2+}]_i$ and new steady-state $[Ca^{2+}]_i$ distinct from prestimulatory levels (13–17). This clearly indicates that the presence of quin2 does not overrule cellular mechanisms for the regulation of $[Ca^{2+}]_i$. However, it is likely that rapid changes in $[Ca^{2+}]_i$ are attenuated, particularly if they rely on a limited pool of calcium to be mobilized. Indeed we find in preliminary experiments that while basal prolactin secretion of GH_3 cells is hardly changed by quin2 loading, the stimulation of the release of prolactin by TRH is attenuated in quin2-loaded cells compared with nonloaded control cells. This could indicate that the rapid alterations of $[Ca^{2+}]_i$ measured with quin2 are smaller than the changes occurring in nonloaded cells. Such a consideration as well as slight modifications of steady-state $[Ca^{2+}]_i$ due to quin2 have to be kept in mind.

The distinct rise in $[Ca^{2+}]_i$ of GH_3 cells elicited by TRH consolidates the view that $[Ca^{2+}]_i$ can serve as an intracellular second messenger for the action of this releasing hormone (1). The data indicate that $[Ca^{2+}]_i$ could provide both an acute and a chronic signal, since $[Ca^{2+}]_i$ is markedly changed from control immediately after stimulation and remains slightly enhanced at steady state; this pattern contrasts with the larger and more sustained increase in $[Ca^{2+}]_i$ due to depolarization with K^+ (Fig. 3 and Table I). Experiments with quin2 allow the direct measurement of a key variable in the complex regulation of hormone synthesis and secretion and the control of cellular activities. The comparison of the kinetics of the $[Ca^{2+}]_i$ response with the secretory or other cellular responses to various stimuli will help to determine the relative role of changes in $[Ca^{2+}]_i$ for stimulus-response coupling. It has been reported that protein phosphorylation in GH_3 cells is affected differently by either TRH or depolarization with K^+ (25); the clear difference in the magnitude and the kinetics of the $[Ca^{2+}]_i$ response (Fig. 3A) to the two stimuli of prolactin secretion could explain the difference in protein phosphorylation without the need to evoke further second messengers for TRH action (25).

There are many possible mechanisms by which a rise in $[Ca^{2+}]_i$ following hormonal stimulation could occur. Since TRH increases $[Ca^{2+}]_i$ in Ca^{2+} -free medium, it must be able to mobilize intracellular calcium, as proposed previously (1, 9–11). The possibility that TRH raises $[Ca^{2+}]_i$ merely by blocking Ca^{2+} efflux is ruled out by the observation of an enhanced Ca^{2+} efflux from GH_3 cells after TRH stimulation (8–10). Direct stimulation of Ca^{2+} extrusion should lead to a lowering of $[Ca^{2+}]_i$; hence the increase in Ca^{2+} efflux is probably a consequence of the increase in $[Ca^{2+}]_i$. The intracellular site from which calcium is mobilized and the mechanisms linking TRH-receptor interaction to this early event in stimulus-secretion coupling will have to be determined.

The data presented above cannot exclude that TRH also changes the Ca^{2+} permeability of the plasma membrane. In fact, the increase in $[Ca^{2+}]_i$ due to TRH is larger with Ca^{2+} in the extracellular medium than in its absence (Table I). This difference suggests increased Ca^{2+} influx after TRH action, e.g., due to Ca^{2+} action potentials (2–4, 22). There is, however, experimental evidence that GH_3 cells lose calcium rapidly when exposed to a Ca^{2+} -free medium and this loss could diminish the hormone-sensitive calcium pool(s) (26). The relative importance of changes in Ca^{2+} influx versus mobili-

zation of intracellular calcium for the transient rise and/or the alterations of steady-state $[Ca^{2+}]_i$ remains to be established. But it is likely that the latter process mediates the rapid changes, since the TRH-induced increase in Ca^{2+} action potential frequency of GH₃ cells occurred only after a lag time of ~1 min (4, 22).

In conclusion, the data on $[Ca^{2+}]_i$ in GH₃ cells presented above suggest that steady-state levels of $[Ca^{2+}]_i$ are determined predominantly by the regulation of transmembrane Ca^{2+} fluxes, whereas the rapid and transient increase in $[Ca^{2+}]_i$ after hormonal stimulation relies on the mobilization of cellular stored calcium.

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