Thyrotropin-releasing Hormone-induced Spike and Plateau in Cytosolic Free Ca\(^{2+}\) Concentrations in Pituitary Cells

RELATION TO PROLACTIN RELEASE*

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Using the acetoxyethyl ester of “Quin 2,” a fluorescent Ca\(^{2+}\)-indicator, we have loaded prolactin (PRL)-producing rat pituitary cells with non-toxic concentrations of Quin 2 and quantitated changes in cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_i\)) during stimulation of PRL release by thyrotropin-releasing hormone (TRH) and 40 mM K\(^+\). TRH induced a biphasic response, with an immediate (<1 s) spike in [Ca\(^{2+}\)]\(_i\), from basal levels (350 ± 80 nm) to a peak of 1–3 μM, which decayed rapidly (t\(_{1/2}\) = 8 s) to a near basal nadir, then rising to a plateau in [Ca\(^{2+}\)]\(_i\), of 500–800 nm. The TRH-induced spike phase was attenuated but not abolished by prior addition of EGTA, while the plateau phase was eliminated by EGTA. Addition of 40 mM K\(^+\) caused an immediate spike in [Ca\(^{2+}\)]\(_i\), to 1–3 μM which equilibrated slowly (t\(_{1/2}\) = 1 min) directly to a plateau of 600–800 nm. The K\(^+\)-induced spike and plateau phases were both abolished by prior addition of EGTA. The biphasic nature of TRH action on [Ca\(^{2+}\)]\(_i\) parallels the biphasic actions of TRH on Ca\(^{2+}\)-fluxes and the biphasic release of PRL by GH cells in suspension. These findings provide evidence that Ca\(^{2+}\)-dependent agonist-mediated increases in [Ca\(^{2+}\)]\(_i\), and hormone release are linked, and may generally have two modes: an acute “spike” mode, dependent primarily on redistribution of intracellular Ca\(^{2+}\) stores; and a sustained “plateau” mode, dependent on influx of extracellular Ca\(^{2+}\).

That changes in cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_i\)) are coupled to the mechanism of secretion has been suggested in a wide variety of tissues (1–3). Yet rarely have changes in [Ca\(^{2+}\)]\(_i\) during stimulation of secretion been actually measured.

We have used stimulation of PRL release by TRH as a model of hormone-mediated, Ca\(^{2+}\)-dependent secretion in rat pituitary GH cells (4, 5). Since TRH induces rapid changes in Ca\(^{2+}\)-fluxes (6, 7) and Ca\(^{2+}\)-dependent action potentials (8–12) in these cells, we have measured the effect of TRH on [Ca\(^{2+}\)]\(_i\) in GH cells loaded with Quin 2, a fluorescent Ca\(^{2+}\)-indicator (13–15).

Our initial description of an acute spike in [Ca\(^{2+}\)]\(_i\), upon addition of TRH to responsive cells has been confirmed in normal bovine pituitary cells (16), and in rat GH\(_2\) cells (17). However, these reports have failed to describe the full time course of TRH actions on [Ca\(^{2+}\)]\(_i\), have not related the TRH-induced changes in [Ca\(^{2+}\)]\(_i\), to hormone secretion, and have not shown TRH receptor mediation of these changes in [Ca\(^{2+}\)]\(_i\). Our results demonstrate that TRH has a biphasic effect on [Ca\(^{2+}\)]\(_i\), with an immediate rise in [Ca\(^{2+}\)]\(_i\), to micromolar levels (spike phase) followed by a rapid decline to near basal levels and recovery to a plateau phase of elevated [Ca\(^{2+}\)]\(_i\). This biphasic pattern of change in [Ca\(^{2+}\)]\(_i\), was correlated with a biphasic pattern of the rate of PRL release induced by TRH in the same cells in which [Ca\(^{2+}\)]\(_i\) was measured.

EXPERIMENTAL PROCEDURES

Materials

TRH and analogues were a gift from Dr. Eugene Woroch, Abbott Laboratories. Sera and culture media were from Gibco Laboratories, Grand Island, NY, and plastic culture dishes were from Falcon. Quin 2-AM was purchased from Lancaster Synthesis, Lancaster, England, lot 5145 and was stored in anhydrous dimethyl sulfoxide at −20 °C. [G\(^{3}\)H]Quin 2-AM (17 Ci/mmol in dimethyl sulfoxide) was purchased from Amersham Corp. D-[U\(^{14}\)C]Sorbitol (250 Ci/mmol) and D\(_2\)O (100 mCi/ml) were purchased from ICN, Irvine, CA. Digitonin (>80%) was from Sigma. All other chemicals were reagent grade or better.

Methods

Cell Culture—These experiments were performed as follows. Cells were grown in monolayer as described (4, 5), harvested with 0.1% VioKase, and then propagated in suspension for 5–10 days to 1–2 × 10\(^6\) cells/ml in spinner medium with 15% horse serum and 2.5% fetal bovine serum and containing 0.47 mM Ca\(^{2+}\). (18). Cells fed 12–24 h prior to an experiment were harvested and washed by centrifugation (250 g × 3 min) in assay medium, Hepes-buffered balanced salt solution II (118 mM NaCl, 4.6 mM KCl, 1 mM CaCl\(_2\), 10 mM D-glucose, 20 mM Hepes, pH 7.2). Measurement of [Ca\(^{2+}\)]\(_i\) —Loading of Quin 2 was performed in 300–600 μl (1–2 × 10\(^6\) cells) of Hepes-buffered balanced salt solution II using 1–2 μl (25–30 nmol) of the acetoxyethyl ester of Quin 2. The cells were diluted to 10 μl with warm Hepes-buffered balanced salt solution II containing 0.1% VioKase and 25 mM EGTA

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1 The abbreviations used are: PRL, prolactin; TRH, thyrotropin; releasing hormone; Arg-TRH, pyro-Glu-Arg-Pro amide; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; AM, acetoxyethyl ester; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [X], cytosolic free concentration of X; t\(_{1/2}\), half-life; ED\(_{50}\), dose which gives 50% of maximum effect; K\(_{eq}\), equilibrium dissociation constant; [X]o, extracellular concentrations of X; [X]i, concentration of X at time t.

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solution II, pelleted, washed twice, and resuspended in the desired volume of Hepes-buffered balanced salt solution II. Fluorescence was measured at 37 °C using a Perkin-Elmer 650-10S spectrofluorometer at \( \lambda_{ex} = 339 \) and \( \lambda_{em} = 492 \) nm, slit widths both at 5 nm, with 3 ml of sample (2-5 \( \times 10^6 \) cells/ml). All tracings are recordings of fluorescence on a linear scale. Transformation of the fluorescent signal to the logit scale of [Ca\textsuperscript{2+}] was performed using the equation (see "Appendix" for derivation):

\[
[\text{Ca}^{2+}]= K_D \frac{F - F_{\text{min}}}{F_{\text{max}} - F}
\]

where \( K_D \) for [Ca\textsuperscript{2+}-Quin 2] is 115 nM (15) and \( F \) is the fluorescent signal in arbitrary units. \( F_{\text{max}} \) was the fluorescence after addition of 25-75 \( \mu \)M digitonin, which exposes the dye to 1.0 mM Ca\textsuperscript{2+} and gives the maximum fluorescence of the sample. \( F_{\text{min}} \) was the fluorescence after addition of 1 mM excess EGTA (as in experiments shown in Fig. 1, A and B) or 10 mM excess EGTA (as in Fig. 1, C and D) to suspensions adjusted to pH > 8.3 with Tris buffer. This brought [Ca\textsuperscript{2+}] to less than 1.0 nM, the minimum fluorescence of the sample.

Solving Equation 1 for \( F \), selected Ca\textsuperscript{2+} concentrations (e.g. 100 nM, 200 nM, etc.) were scaled onto the fluorescence recording by determining the value of \( F \) corresponding to these values of [Ca\textsuperscript{2+}], given \( F_{\text{max}}, F_{\text{min}}, \) and \( K_D \). Likewise, the precise [Ca\textsuperscript{2+}] of a fluorescent signal \( F \) can be determined from Equation 1.

The half-life of a decay in [Ca\textsuperscript{2+}], was determined by transforming 4-8 discrete fluorescent readings \( (F, \text{fluorescence at time } t) \) to equivalent [Ca\textsuperscript{2+}] at time \( t' \) (\([\text{Ca}^{2+}]_{t'}\)) and plotting \([\text{Ca}^{2+}]_{t'}\) vs. \([\text{Ca}^{2+}]_{t}\) (the final or minimum [Ca\textsuperscript{2+}] after the peak) as a function of time \( t \) after the peak [Ca\textsuperscript{2+}]. A good correlation \((r > 0.995)\) was consistently obtained, and \( t_{1/2} \) was calculated from the slope \((m)\) of the first order decay (see "Appendix").

Interruptions in the tracing correspond to periods of drug addition or gentle resuspension of the cells with a Pasteur pipette, except in the experiment shown in Fig. 1D where a small magnetic stirrer was used. All drugs were added from 200-fold concentrated stock solutions.

**Fig. 1.** Changes in [Ca\textsuperscript{2+}] in GH\textsubscript{3}C\textsubscript{2} cells treated with TRH or high extracellular K\textsuperscript{+} as measured by cytosolic Quin 2 fluorescence. **Ordinates** are fluorescence recordings on a linear scale and depict [Ca\textsuperscript{2+}] on a log scale. The tracings progress in time from left to right, with the scale of the abscissa indicated by the time bars. A change in the time scale occurs at the onset of a new time bar. A, TRH-induced changes in [Ca\textsuperscript{2+}] in the presence of [Ca\textsuperscript{2+}] = 1 mM. Addition of a saturating concentration of TRH (50 nM) caused an immediate elevation in [Ca\textsuperscript{2+}], from a basal level of 250 nM to 1.7 \( \mu \)M. This initial peak (TRH\textsubscript{1}) decayed rapidly (\( t_{1/2} = 4.5 \) s) to a near basal value of 330 nM (TRH\textsubscript{max}). Several minutes thereafter, the [Ca\textsuperscript{2+}], rose again to a plateau of 765 nM (TRH\textsubscript{delay}) which remained essentially constant for at least 15 min. [Quin 2], = 0.11 mM; 4.8 \( \times 10^6 \) cells/ml. B, high K\textsuperscript{+}-induced changes in [Ca\textsuperscript{2+}], in the presence of [Ca\textsuperscript{2+}] = 1 mM. Addition of depolarizing concentrations of K\textsuperscript{+} (40 mM) to cells caused an immediate elevation in [Ca\textsuperscript{2+}], from 260 nM (basal) to \( \sim 2.0 \mu \text{M (K\textsuperscript{+}max)} \) which slowly (note difference in time scale from A) decayed (\( t_{1/2} = 69 \) s) to a plateau of 560 nM (K\textsuperscript{+} plateau). [Quin 2], = 0.09 mM; 4.8 \( \times 10^6 \) cells/ml. C, TRH-induced changes in [Ca\textsuperscript{2+}], in the presence of 1.33 mM EGTA/1 mM CaCl\textsubscript{2}, [Ca\textsuperscript{2+}] = 200-300 nM. Addition of EGTA (1.33 mM) to GH\textsubscript{3}C\textsubscript{2} cells in 1 mM CaCl\textsubscript{2} caused a rapid drop in the basal [Ca\textsuperscript{2+}], from 440 to 300 nM. Addition of TRH 60 min later caused an immediate rise in [Ca\textsuperscript{2+}], to 760 nM, which decayed back to 300 nM. Addition of Ca\textsuperscript{2+} (1.33 mM) 5.7 min later caused a rise of [Ca\textsuperscript{2+}], to a plateau value of 900 nM. After replenishing extracellular Ca\textsuperscript{2+}, the cells responded to high K\textsuperscript{+} with \( K_{\text{max}} = 5 \mu \text{M and } K_{\text{pmax}} = 900 \) nM. [Quin 2], = 0.22 mM, 6 \( \times 10^6 \) cells/ml. D, lack of high K\textsuperscript{+}-induced changes in [Ca\textsuperscript{2+}], in the presence of 1.33 mM EGTA/1 mM CaCl\textsubscript{2}. Addition of EGTA caused a drop in [Ca\textsuperscript{2+}], from 310 to 158 nM. Addition of K\textsuperscript{+} (40 mM) 6.0 min after EGTA produced no change in [Ca\textsuperscript{2+}], except a transient dilution effect. Replenishment of Ca\textsuperscript{2+} resulted in expression of \( K_{\text{max}} = 900 \) nM, which decayed to a plateau of 600 nM. A TRH response was also present after Ca\textsuperscript{2+}-replenishment, with TRH\textsubscript{max} = 1.2 \( \mu \text{M (t1/2 = 14 s), TRH}_{\text{pmax}} = 436 \) nM. [Quin 2], = 0.17 mM; 4.3 \( \times 10^6 \) cells/ml. In B and C, the scale on the right ordinate is shifted to compensate for dilution after KCl addition (see "Methods"). DIG, digitonin.
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except Tris (100-fold) and KCl (25-fold in Fig. 1, B and C, and 100-fold in D). In the experiment shown in Fig. 1, B and C, a correction factor of 1.04 was applied to \(F_{\text{max}}\) and \(F_{\text{min}}\) to calibrate the fluorescence at times prior to KCl addition to the suspension. In all other drug additions the correction was negligible and no scale adjustment was made.

Cells incubated with dimethyl sulfoxide or saponified Quin 2-AM showed insignificant changes in fluorescence to all manipulations. [Quin 2], was measured using \(F_{\text{max}} - F_{\text{min}}\) to calibrate [Quin 2] (see “Appendix”), and an intracellular water volume of 2.0 µl/10^6 GH\(\times\)C\(_i\) cells as determined by the [Ca\(^{2+}\)]orbitol exclusion method (19). Cells were >90% trypan blue exclude before and after Quin 2 loading.

Basal and TRH-stimulated PRL release rates were the same in untreated and Quin 2 loaded cells up to [Quin 2] = 0.25 mM. At higher [Quin 2], there was some inhibition of basal PRL release, but TRH-stimulated release remained intact. In some PRL release experiments, GH cells were loaded with [H]<sup>3</sup>Trp 2-AM as described above for unlabeled Quin 2 [Quin 2] quantitated by measurement of cell-associated <sup>3</sup>H to similar that to measured fluorescence and was identical at all times up to 30 min in control and TRH-treated cells. Thus, TRH-induced fluorescence changes were not due to leakage of dye from the cells. Radioactivity was lost with a half-life of 41.3 ± 1.7 min (mean ± S.D.) in three separate experiments.

Measurement of PRL—Samples from the fluorescence cuvette were pelleted in a Beckman Microfuge B at 10,000 × g (<10 s), and PRL in the supernatant solution was measured by radioimmunoassay using materials supplied by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. The rate of PRL secretion (Δm/Δt) was calculated from the increase in PRL in the medium (Δm) within each 10-min interval.

RESULTS AND DISCUSSION

Actions of TRH and K\(^{+}\) on [Ca\(^{2+}\)]—Fig. 1A shows a biphasic response of [Ca\(^{2+}\)], to addition of a saturating concentration of TRH to GH\(\times\)C\(_i\) cells loaded with low, non-toxic concentrations (15, 19) of Quin 2 (see “Methods”). TRH induced an immediate spike phase (peak [Ca\(^{2+}\)] = 2.2 ± 1.2 μM (mean ± S.D.), n = 20), TRH\(_{\text{max}}\), followed by a rapid decay to a near basal level of 540 ± 90 nM (n = 10), and then recovery in minutes to a plateau phase ([Ca\(^{2+}\)] = 690 ± 140 nM, n = 18), TRH\(_{\text{plateau}}\). The uptake to TRH\(_{\text{max}}\) was as fast as mixing (<10 s) in magnetically stirred cell suspensions where TRH was added without interrupting fluorescence recording (data not shown). TRH\(_{\text{max}}\) reaches micromolar Ca\(^{2+}\) concentrations, consistent with levels required for activation of secretion (21) and contraction (22). The decay of [Ca\(^{2+}\)], from TRH\(_{\text{max}}\) was a first order exponential of \(t_{1/2} = 7.9 ± 1.8 s\) (n = 15).

As shown in Fig. 1B, addition of 40 mM K\(^{+}\) (but not 40 mM Na\(^{+}\) or choline\(^{+}\)) produced a temporal pattern in [Ca\(^{2+}\)], different from that induced by TRH. There was an immediate (<2 s) spike of [Ca\(^{2+}\)], to 1.7 ± 1.1 μM (n = 9), K\(^{+}\)_\(_{\text{max}}\), which decayed slowly as a first order exponential (\(t_{1/2} = 62 ± 6 s\), n = 6) to a plateau of elevated [Ca\(^{2+}\)] (630 ± 100 nM, n = 7), K\(^{+}\)_\(_{\text{plateau}}\). A TRH response was elicited after prolonged K\(^{+}\) depolarization (Fig. 1, B and D); likewise, a K\(^{+}\) response was elicited after TRH treatment (Fig. 1C).

Addition of 1.33 mM EGTA/1.0 mM CaCl\(_2\) to GH\(\times\)C\(_i\) cells blocks TRH-induced PRL release but not TRH-enhanced synthesis of PRL (23, 24). EGTA treatment lowered basal [Ca\(^{2+}\)], (Fig. 1, C and D). Replenishment of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]) to a 1.0 mM excess following EGTA restored basal [Ca\(^{2+}\)].

This mild EGTA treatment diminished TRH\(_{\text{max}}\) by 30–40%, while TRH\(_{\text{max}}\) was abolished (Fig. 1C), as were both K\(^{+}\)_\(_{\text{max}}\) and K\(^{+}\)_\(_{\text{plateau}}\) (Fig. 1D). Thus, the plateau phase of TRH action on [Ca\(^{2+}\)], and both phases mediated by K\(^{+}\) depolarization are dependent on the presence of Ca\(^{2+}\). Furthermore, each of these phases was recovered upon replenishing Ca\(^{2+}\), thus allowing Ca\(^{2+}\) influx to ensue via channels opened during secretagogue treatment in the presence of EGTA. In contrast, TRH\(_{\text{max}}\) was only partly sensitive to the lowered [Ca\(^{2+}\)], suggesting that release of cellular Ca\(^{2+}\) stores in part mediates this change in [Ca\(^{2+}\)].

The basal [Ca\(^{2+}\)], of freshly loaded cells (350 ± 80 nM, n = 34) was higher than the basal [Ca\(^{2+}\)], of 100–200 nM found in several other systems (14–17, 20–22, 25–31). Cells which maintain a basal secretion of hormone (e.g. anterior pituitary cells (16) and parathyroid cells (30)) may have higher basal [Ca\(^{2+}\)], than cells which do not secrete basally (e.g. adrenal medullary cells (31), lymphocytes (14), and platelets (21)). The relatively high basal [Ca\(^{2+}\)], in GH cells may support the steady basal PRL release observed in these cells (see below).

The basal [Ca\(^{2+}\)], of 100 nM reported in GH\(\times\)C\(_i\) cells is lower than that by which we have found. Using their method of cell preparation, we measure a basal [Ca\(^{2+}\)], of 310 ± 35 nM (n = 3); thus, the discrepancy in basal [Ca\(^{2+}\)], is not due to a difference in cell preparation. Gershengorn and Thaw (17) do not mention raising the pH during the addition of EGTA to calibrate F\(_{\text{min}}\) (see “Methods”). Such faulty calibration would cause an overestimation of F\(_{\text{min}}\) and hence a substantial underestimation of the basal [Ca\(^{2+}\)], similar to that reported.

Validation of Quin 2 for measurement of changes in [Ca\(^{2+}\)], in GH cells can be ascertained by internal controls (“Methods”), as well as by comparison with an intrinsic Ca\(^{2+}\)-dependent monitor. In these cells, the phosphorylation of a 97-kilodalton cytosolic protein (97 kDa) has been shown to be sensitive to Ca\(^{2+}\)-calmodulin in vitro (32). In intact cells, PRL depolarization caused an immediate (<15 s) and prolonged (>30 min) enhancement of 32P incorporation into the 97 kDa protein mirroring the changes in [Ca\(^{2+}\)],, we observed using Quin 2. TRH induced a spike in 97 kDa phosphorylation which decayed within 1 min coinciding with the spike phase in [Ca\(^{2+}\)]. The TRH-induced peak in 97 kDa phosphorylation was partially resistant to EGTA as was TRH\(_{\text{max}}\), while K\(^{+}\) induced phosphorylation was abolished by Ca\(^{2+}\) channel blockers. These changes in Ca\(^{2+}\)-sensitive 97 kDa phosphorylation parallel changes in [Ca\(^{2+}\)], and provide an independent validation of the changes we monitor with Quin 2.

Relation of Changes in [Ca\(^{2+}\)], to 45Ca\(^{2+}\) Fluxes—How are these responses in [Ca\(^{2+}\)], produced? High K\(^{+}\) treatment of GH cells causes an immediate and sustained depolarization (6, 33), opening of voltage-dependent Ca\(^{2+}\) channels (34, 35), Ca\(^{2+}\) influx (36, 37), increased [Ca\(^{2+}\)], (Fig. 1B), and PRL release (37–39). If the increased [Ca\(^{2+}\)], associated with K\(^{+}\) depolarization is entirely dependent on Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels, it should be abolished by EGTA (Fig. 1C) and Ca\(^{2+}\) channel blockers, as is the Ca\(^{2+}\) influx (36, 37) and PRL release (18, 37, 38). Preliminary results indicate that verapamil (50 µM) completely blocks all K\(^{+}\)-induced alterations in [Ca\(^{2+}\)], while 25 µM verapamil is only partly effective, consistent with concentrations of this Ca\(^{2+}\) channel antagonist required to block K\(^{+}\)-induced Ca\(^{2+}\) influx and PRL release (36, 37).

TRH has a biphasic effect on Ca\(^{2+}\) fluxes in GH cells. First, TRH produces a rapid and large efflux of Ca\(^{2+}\) from a “superfluidic” pool (6, 7), and net loss of cellular Ca\(^{2+}\) (39) (i.e. the Ca\(^{2+}\) efflux is not simply exchange with medium Ca\(^{2+}\); second, a subsequent uptake of Ca\(^{2+}\) into an enzyme-resistant (intracellular) pool (7). The TRH-induced efflux phase of Ca\(^{2+}\) fluxes starts in <2 s and is over within 1 min, a time course consistent with a causative relationship between this Ca\(^{2+}\) efflux and TRH\(_{\text{max}}\). As the Ca\(^{2+}\) efflux is resistant to EGTA (40) and Ca\(^{2+}\) channel blockers (7, 37, 39), so TRH\(_{\text{max}}\)
is resistant to EGTA (Fig. 1C) and 50 μM verapamil (data not shown). Thus, TRH_{max} could occur by transient release of Ca^{2+} from the superficial pool directly into the cytoplasm; net efflux could occur by concomitant release of this cell-associated Ca^{2+} directly into the medium or by the pumping of Ca^{2+} out of the cell. The rapid decay of TRH_{max} may be induced by sequestration of Ca^{2+} into depleted cell pools as well as by pumping of Ca^{2+} out of the cell. The mechanism by which TRH activates release of cell Ca^{2+} stores producing TRH_{max} and 45Ca^{2+} efflux is still unclear. The rapid onset, transient nature, ED_{50}, and EGTA resistance of TRH_{max} are consistent with recent data suggesting a redistribution of sequestered Ca^{2+} related to TRH-stimulated hydrolysis of polyphosphoinositides in GH cells (41-43).

The plateau phase in [Ca^{2+}], after TRH corresponds to a prolonged period of 45Ca^{2+} influx (7), which is associated with development of decreased K^{+} conductance (10, 12). The decreased K^{+} conductance depolarizes the cells and causes Ca^{2+}-dependent action potentials (8-12), allowing a net influx of Ca^{2+} to increase [Ca^{2+}]. Note that since the fluorescence monitored is an average over the population of cells, a given cell may actually show spikes of [Ca^{2+}], to micromolar levels, coinciding with Ca^{2+}-dependent action potentials. As with K^{+}-induced 45Ca^{2+} influx, TRH-induced [Ca^{2+}] influx is blocked by EGTA which also abolishes TRH_{max} (Fig. 1C), and is recovered upon addition of excess calcium.

Receptor and Cell Specificity of Responses in [Ca^{2+}].—The rapidity of the upstroke of [Ca^{2+}], to TRH_{max}, suggests that the spike phase may be tightly coupled to TRH-receptor occupancy. Fig. 2 shows that for TRH the ED_{50} for enhancement of peak [Ca^{2+}], is 2.5 nM, a value close to the K_{D} of [^{3}H]TRH for its receptor (44, 45) and to the ED_{50} values for stimulation of PRL production and release (19). Furthermore, the ligand specificity for TRH_{max} is identical with that seen for receptor binding, with methyl-TRH the most potent analogue, and TRH-free acid and Arg_{2}-TRH showing little or no activity (46, 47). Finally, in GH2C_{1} cells, which lack receptors for (45) and responses to (7) TRH, addition of 100 nM TRH or methyl-TRH produced no change in [Ca^{2+}], although GH2C_{1} cells responded normally to 40 mM K^{+}. In GH3 cells, which have TRH receptors (45), identical responses to those in Fig. 1 were obtained. These observations indicate that the observed TRH-induced changes in [Ca^{2+}], are mediated by the characterized membrane receptor for TRH.

[Ca^{2+}] and PRL secretion.—The results in Fig. 3 illustrate the relationship between [Ca^{2+}], and PRL secretion. Cells loaded with Quin 2 and not treated further show the steady rate of PRL release characteristic of untreated, control cells (about 0.20 ng/ml/min). PRL accumulation in the medium up to 30 min represents release of stored PRL (4, 48). In the untreated cells (Fig. 3, top), Quin 2 fluorescence increased slightly (corresponding to an apparent rise in [Ca^{2+}], of <100 nM) due to dye leakage (20) over this 30-min experiment. This slow leakage is insufficient to account for the higher plateau in [Ca^{2+}], seen after TRH and K^{+} in experiments of shorter duration (Fig. 1).

In Fig. 3, middle, TRH addition at 10 min to a separately loaded, duplicate suspension of cells caused a spike phase in [Ca^{2+}], (attenuated due to incomplete mixing in this experiment) followed by a plateau phase. Correspondingly, in the first 10 min after TRH (striped bar at 20 min) a burst of PRL release was induced (from 0.18 to 0.75 ng/ml/min) and then a lesser, but still enhanced rate of PRL release was observed between 20 and 30 min. These two phases of TRH action

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**Fig. 2.** Dose-response curves for TRH and TRH analogues on peak stimulation of [Ca^{2+}] during the spike phase (TRH_{max}). Two sets of GH2C_{1} cells were loaded with Quin 2-AM as described ("Methods") to a [Quin 2], = 0.19 ± 0.06 mM. Typically, basal fluorescence was recorded from the first set of cells for 5-10 min (basal [Ca^{2+}], = 380 ± 100 nM), then a dose of TRH or analogue was added and calibration performed 5-10 min later. This procedure was repeated on the second set at a new dose of TRH or analogue. Each symbol denotes the peak [Ca^{2+}], obtained from a single such group of cells setting the basal [Ca^{2+}], at 100%. The bars between points indicate mean values at a given dose, and crossed symbols indicate duplicates of a given value. The dashed line at 540% represents the average TRH_{max} with saturating TRH in 18 trials. ED_{50} values and plotted curves were calculated assuming a direct proportionality between TRH_{max} and receptor occupancy.

**Fig. 3.** Measurement of [Ca^{2+}], and PRL secretion in Quin 2-loaded GH2C_{1} cells in the absence and presence of TRH. Top and middle sections are consecutively recorded fluorescence tracings (as in Fig. 1) of a duplicate set of cells (3.7 × 10^{5} cells/ml) separately loaded with [Quin 2], = 0.65 mM. The bottom section is a histogram of PRL in the medium of 0.5-ml samples withdrawn from the fluorescence cuvettes during the recording at 10, 20, and 30 min. The twin bars centered at each sample time correspond to mean values of samples from untreated cells (top) and TRH-treated cells (middle), respectively, while the error bars represent standard deviations. Cells received TRH after the 10-min sample (indicated by the arrows). Mean values for PRL secretion rate in each 10-min interval, Δm/Δt (mg/ml/min) were: 1.6, 2.4, and 2.1 for the 10-, 20-, and 30-min samples of untreated cells, and 1.8, 7.5, and 2.7 for the TRH-treated samples. DIG, digitonin.
(burst and sustained) on PRL release were seen in each of three other experiments with Quin 2 loaded cells.

We have observed that the TRH-induced burst of PRL release in suspended GH cells occurs within 1 min of TRH addition, temporally consistent with mediation by the initial peak in \([Ca^{2+}]_i\). The sustained phase of enhanced PRL release which follows the burst phase corresponds with the plateau of elevated \([Ca^{2+}]_i\). In attached cells in monolayer, the TRH-induced burst release of PRL is not expressed, and TRH-enhanced PRL release occurs as a sustained enhancement of the secretion rate. In this case the mechanism controlling the plateau phase in \([Ca^{2+}]_i\), controls PRL release. In fact, in monolayer culture, low \([Ca^{2+}]_i\), EGTA, and \([Ca^{2+}]_i\)-channel antagonists block over 80% of the TRH-stimulated PRL release (11, 18, 37, 38), just as these treatments abolish TRH_{plat}. On the other hand, GH cells suspended in columns, show a prominent burst release of PRL which is resistant to EGTA (42) and 50 \(\mu\)M verapamil (49), providing evidence that TRH_{max} can mediate the burst phase of PRL release even in the absence of \([Ca^{2+}]_i\) influx. In our suspended GH cells, TRH-stimulated release of PRL was completely blocked by EGTA, possibly because our cells are more sensitive to EGTA and the peak of TRH_{max} falls below the threshold required to initiate release. Thus, the burst phase of PRL release is associated (at least in part) with redistribution of cell \([Ca^{2+}]_i\) stores, while the sustained phase is associated with \([Ca^{2+}]_i\) influx in part through voltage-dependent \([Ca^{2+}]_i\) channels (36, 37).

In the human platelet model (50, 51), both activation of protein kinase C (via phosphotidylinositol turnover) and \([Ca^{2+}]_i\) mobilization to a threshold \([Ca^{2+}]_i\), are required for normal secretion, suggesting by analogy the relevance of phosphotidylinositol turnover, both to mobilize cellular \([Ca^{2+}]_i\) and to activate protein kinase C, in the TRH-mediated burst release of PRL. Protein kinase activation may also be related to the decreased \(K^+\) conductance associated with TRH_{plat}, via phosphorylation over 3–10 min after TRH of release-related phosphoproteins (24), and thus play an indirect role in enhanced TRH-mediated sustained PRL release.

Conclusion—We have demonstrated a receptor-mediated, biphasic action of TRH on \([Ca^{2+}]_i\) using the Ca^2+ indicator Quin 2. The changes in \([Ca^{2+}]_i\), are different from those induced by high \(K^+\) depolarization. The initial spike phase of TRH action is partly resistant to EGTA, while the plateau phase, as well as all \(K^+\)-induced changes in \([Ca^{2+}]_i\), are abolished by EGTA. These results are consistent with TRH- and high \(K^+\)-induced changes in \([Ca^{2+}]_i\) fluxes and \([Ca^{2+}]_i\)-dependent phosphorylation, and they parallel TRH-induced patterns of PRL release. In GH cells in suspension, the TRH-induced spike phase of \([Ca^{2+}]_i\), and PRL release are due largely to redistribution of cellular \([Ca^{2+}]_i\), but may also be dependent on redistribution of \([Ca^{2+}]_i\). Prolonged enhancement of PRL release (e.g. 30-min release) by TRH appears to be sustained by influx of \([Ca^{2+}]_i\) producing a plateau phase in \([Ca^{2+}]_i\), similar to that seen after \(K^+\). Biphase hormon release has been described in other systems (e.g. \([Ca^{2+}]_i\)-dependent insulin release from pancreatic \(\beta\)-cells (52)) and the characteristics of the TRH-induced biphasic response in \([Ca^{2+}]_i\), may be relevant to these systems.

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Note Added in Proof—Snowdowne and Borle (55) have measured \([Ca^{2+}]_i\), using aequorin-loaded GH3 cells and have found values of basal peak TRH-stimulated and peak \(K^+\)-stimulated \([Ca^{2+}]_i\) identical to those that we measure with Quin 2 loaded GH cells.

APPENDIX

Derivation and Assumptions of Equation 1—Equation 1, essential for the calibration of \([Ca^{2+}]_i\), from \(F\), has been validated empirically, but a derivation from first principles has yet to be published.

The fluorescent signal \((F)\), measured in a cell suspension loaded with Quin 2, is described by

\[ F = \alpha[Q_2] + \beta[CaQ_2] + \epsilon \]  

where \(\alpha = K_{\phi_2}[Q_2]\) and \(\beta = K_{\phi_{CaQ_2}}\).

The constant \(K\) is determined by light intensity geometry, sensitivity, and absorption factors; \(\phi_2\) is the fluorescence efficiency of \(Q_2\) (Quin 2) with no \([Ca^{2+}]_i\) bound or \(CaQ_2\) (Quin 2) at excitation wavelength \(\lambda\) (53), and \(\epsilon\) is background fluorescence. The following assumptions are made: 1) \([Q_2]\) and \([CaQ_2]\) are less than 0.05/(\(a\), \(b\), where \(a\), is the molar extinction coefficient at \(\lambda_{max}\) = 339 nm, \(b\) = 1 cm). 2) \(\alpha\) and \(\beta\) differ only in fluorescence efficiency \((13, 15)\), at \(\lambda_{max}\) = 339 nm and \(\lambda_{max}\) = 492. 3) \(\epsilon\) is constant. Assumptions 1 and 2 are fulfilled since at all \([Q_{TOT}]\) used, a linear dependence of \(F\) on \([Q_{TOT}]\) is observed at all \([CaQ_2]/[Q_2]\). Assumption 3 is observed in a well stirred population of cells not loaded with Quin 2. Finally, at \(\lambda_{max}\) = 339 nm and \(\lambda_{max}\) = 492 nm, \(\beta > \alpha\).

\[ F_{max} = \beta[Q_{TOT}] + \epsilon \]  

\[ F_{min} = \alpha[Q_{TOT}] + \epsilon \]  

From Equation 1b,

\[ \beta = \frac{F_{min} - \epsilon}{[Q_2] + [CaQ_2]} \]

and from Equation 1c,

\[ \alpha = \frac{F_{max} - \epsilon}{[Q_2] + [CaQ_2]} \]

Substituting \(\alpha\) and \(\beta\) in Equation 1a yields:

\[ F = \frac{[Q_2](F_{min} + [CaQ_2](F_{max})}{[Q_2] + [CaQ_2]} \]

\[ [CaQ_2] = \frac{(F - F_{max})}{(F_{max} - F)} \]

Like EGTA, Quin 2 binds \([Ca^{2+}]_i\) with a 1:1 stoichiometry (15), thus

\[ K_d[CaQ_2] = \frac{\frac{[Ca^{2+}]_i}{[Ca^{2+}]_i}}{K_d + \frac{[Ca^{2+}]_i}{[Ca^{2+}]_i}} \]

from Equations 1g and 1e

\[ \frac{[CaQ_2]}{[Q_2]} = \frac{K_d}{K_d + \frac{[Ca^{2+}]_i}{[Ca^{2+}]_i}} \]

The assumptions used for the empirical determination of \(K_d\) have been discussed (15).

Note that \(F_{max} - F_{min} = (\beta - \alpha)[Q_{TOT}]\) (from Equation 1b – 1c). This relation is used to calibrate \([Q_{TOT}]\) in each cell suspension.

Model for Determining Half-lives of Decay in \([Ca^{2+}]_i\)—The model is based on the a priori assumption that the rate of decay of \([Ca^{2+}]_i\), is dependent on \([Ca^{2+}]_i\), i.e. is mediated by processes with nonsaturable, first order dependence on \([Ca^{2+}]_i\), at the levels of \([Ca^{2+}]_i\), measured.
Using notation described under “Methods,”

$$-\frac{d[Ca^{2+}]}{dt} = k[Ca^{2+}],$$

(2a)

where $k$ is the rate constant.

$$\int_{[Ca^{2+}]_{\text{initial}}}^{[Ca^{2+}]_{\text{final}}} \frac{d[Ca^{2+}]}{[Ca^{2+}]} = \int_{t_m}^{t} -k \, dt$$

(2b)

$$\ln([Ca^{2+}]_{\text{final}}) - [Ca^{2+}]_{\text{initial}} = -kt$$

(2c)

In order to set $\Delta = 0$, the ordinate was shifted such that:

$$[Ca^{2+}] = [Ca^{2+}]_{\text{initial}} - [Ca^{2+}]_{\text{final}}$$

(2d)

and

$$t_{1/2} = \frac{\ln 2}{k} \text{ since } m = -k$$

(2f)

The model described by Equation 2 assumes that only $Ca^{2+}$-dependent processes are responsible for the decay in $[Ca^{2+}]$, although more than one process may occur. It is suggested that pumping of $Ca^{2+}$ out of the cell by a $Ca^{2+}$-ATPase is sufficient to account for the $t_{1/2}$ of 8 s for the decay of TRH$_{\text{max}}$. Recently, such a model has been used to describe the half-life of 4 s in decay of $[Ca^{2+}]$, following a brief pulse of $Ca^{2+}$-influx into the squid axon (54). The longer half-life in the case of TRH$_{\text{max}}$ may be due to a larger influx of $Ca^{2+}$ into the cytosol, a different density of $Ca^{2+}$-ATPases, pumping into more than one noncytosolic compartment, etc.; nevertheless, the model suggests that activation of $Ca^{2+}$-ATPase alone can account for the rapid half-life of TRH$_{\text{max}}$.

The slower “equilibration” of the $K^+$-induced peak in $[Ca^{2+}]$, has a half-life of 1 min, very similar to that observed for uptake of $^{45}Ca^{2+}$ into the superficial pool (7) and to the rapid phase of $^{45}Ca^{2+}$ uptake following high $K^+$ treatment of GH$_3$C1 cells (36). It is suggested that, in the face of the steady $Ca^{2+}$ influx induced by high $K^+$, the decay from K$_{\text{max}}$ is due to accumulation of $^{45}Ca^{2+}$ in the superficial pool. Tan and Tashjian (36) have described a second component of $Ca^{2+}$ uptake after $K^+$ depolarization of $t_{1/2} = 50$ min, corresponding to uptake into the “intracellular” pool ($t_{1/2} = 38$ min) (7). Although a slight downward drift in $[Ca^{2+}]$, sometimes persists after high $K^+$, the small magnitude, and the problem of leakage of Quin 2 out of the cells make it difficult to document a second, slow component of decay in $[Ca^{2+}]$.

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