Thyrotropin-releasing Hormone Stimulation of Prolactin Release from Clonal Rat Pituitary Cells

EVIDENCE FOR ACTION INDEPENDENT OF EXTRACELLULAR CALCIUM

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ABSTRACT Thyrotropin-releasing hormone (TRH) stimulates prolactin release and $^{45}Ca^{2+}$ efflux from GH_3 cells, a clonal strain of rat pituitary cells. Elevation of extracellular K^+ also induces prolactin release and increases $^{45}Ca^{2+}$ efflux from these cells. In this report, we distinguish between TRH and high K^+ as secretagogues and show that TRH-induced release of prolactin and $^{45}Ca^{2+}$ is independent of the extracellular Ca^{2+} concentration, but the effect of high K^+ on prolactin release and $^{45}Ca^{2+}$ efflux is dependent on the concentration of Ca^{2+} in the medium. The increment in $^{45}Ca^{2+}$ efflux induced by 50 mM K^+ during perifusion was reduced in a concentration-dependent manner by lowering extracellular Ca^{2+} from 1,500 to 0.02 μM (by adding EGTA), whereas 1 μM TRH enhanced $^{45}Ca^{2+}$ efflux similarly over the entire range of extracellular Ca^{2+} concentrations. Although 50 mM K^+ caused release of 150 ng prolactin from 40×10^6 GH_3 cells exposed to 1,500 μM Ca^{2+} (control), reduction of extracellular Ca^{2+} to 2.8 μM decreased prolactin release caused by high K^+ to <3% of controls and no prolactin release was detected after exposure to 50 mM K^+ in medium with 0.02 μM free Ca^{2+}. In contrast, TRH caused release of 64 ng of prolactin from 40×10^6 GH_3 cells exposed to medium with 1,500 μM Ca^{2+}, and release caused by TRH was still 50 and 35% of control in medium with 2.8 and 0.02 μM Ca^{2+}, respectively. Furthermore, TRH transiently increased by 10-fold the fractional efflux of $^{45}Ca^{2+}$ from GH_3 cells in static incubations with 1,500 or 3.5 μM Ca^{2+}, hereby confirming that the enhanced $^{45}Ca^{2+}$ efflux caused by TRH in both low and high Ca^{2+} medium was not an artifact of the perifusion system.

Data obtained with chlortetracycline (CTC), a probe of membrane-bound Ca^{2+}, were concordant with those obtained by measuring $^{45}Ca^{2+}$ efflux. Cellular fluorescence of CTC varied with the extracellular Ca^{2+} concentration and the duration of incubation. TRH decreased the fluorescence of cell-associated CTC in a manner strongly suggesting stimulus-induced mobilization of Ca^{2+}, and this effect was still demonstrable in GH_3 cells incubated in 50 mM K^+.

These data suggest that TRH acts to mobilize sequestered cell-associated Ca^{2+} reflected as a $^{45}Ca^{2+}$ efflux which is independent of the extracellular Ca^{2+} concentration. Mobilization of sequestered Ca^{2+} into the cytoplasm may elevate free intracellular Ca^{2+} and serve to couple stimulation by TRH to secretion of prolactin.

INTRODUCTION

Thyrotropin-releasing hormone (TRH)[1] stimulates release of thyrotropin and prolactin from pituitary cells (1), and these actions are dependent on Ca^{2+} (2, 3). A role for Ca^{2+} in TRH stimulation of release of prolactin was shown directly in the study of Tashjian et al. (3), which showed that chelation of Ca^{2+} specifically inhibited TRH-stimulated prolactin release and that this inhibition was completely reversed by adding Ca^{2+}. This finding for TRH action is consistent with the hypothesis (4) that Ca^{2+} is the coupling factor between stimulus and secretion. Indirect evidence in support of the contention that TRH may act by affecting cellular Ca^{2+} metabolism comes from studies demonstrating that TRH enhances $^{45}Ca^{2+}$ efflux both from rat anterior pituitary cells in vitro (5, 6) and from GH_3 cells, a clonal strain of rat pituitary cells that produce prolactin (7, 8). The increase in the rate of $^{45}Ca^{2+}$ efflux caused by TRH may reflect an increase in the concentration of free cytosolic Ca^{2+}; an increase which could

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[1] *Abbreviations used in this paper*: BSS, balanced salt solution; CTC, chlortetracycline; TRH, thyrotropin-releasing hormone.

be due to mobilization of Ca^{2+} from an intracellular pool (or pools), to enhanced entry of Ca^{2+} from the extracellular environment, or to both. A series of recent electrophysiological studies using GH_3 cells (9–12) and primary mixed cultures of normal rat anterior pituitary cells (13) have demonstrated that TRH also causes an increase in the frequency of Ca^{2+}-dependent action potentials, and it has been suggested that Ca^{2+} traversing the plasma membrane during action potentials may be involved in hormone secretion. In this regard, the action of TRH has been likened to that of depolarizing concentrations of K^+ which have been shown to release pituitary hormones by causing influx of extracellular Ca^{2+} (14).

In this report, we compare the effects of TRH and a depolarizing concentration of K^+ (50 mM) on $^{45}Ca^{2+}$ efflux and prolactin release from GH_3 cells and the effects of TRH and 50 mM K^+ on the cellular fluorescence of chlortetracycline (CTC), a probe of membrane-bound Ca^{2+} (15, 16). We demonstrate that stimulation of $^{45}Ca^{2+}$ efflux and prolactin release from GH_3 cells caused by TRH, in contrast to that caused by depolarization by K^+, is not dependent on the concentration of Ca^{2+} in the incubation medium, and we suggest that TRH mobilizes cell-associated Ca^{2+} in GH_3 cells.

METHODS

Cell culture. GH_3 cells (American Type Culture Collection, Rockville, Md.) were grown as monolayer cultures in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum at 37°C as described (17, 18). 3–5 d before an experiment, the cells were harvested with 0.02% EDTA and incubated in Eagle's Minimal Essential Medium (Grand Island Biological Co., Grand Island, N. Y.) for suspension culture supplemented with 15% horse and 2.5% fetal bovine sera (19). Cells were exposed to 0.02% EDTA for 10 min one additional time before incubation in this medium with $^{45}Ca^{2+}$ (2–8 μCi/ml) for 16–40 h. Immediately before an experiment the cells were harvested by centrifugation at 180 *g* for 5 min and resuspended in a balanced salt solution (BSS)—135 mM NaCl, 4.5 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose, 2 g/liter ovalbumin, and 10 mM Hepes, pH 7.4—containing 1,500 μM added $CaCl_2$. The free Ca^{2+} concentration in BSS with no added Ca^{2+} was measured by colorimetric titration with arsenazo III dye (20); absorbance was measured at 650 nm in a double-beam spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif., model 25). The standard curve was constructed with EGTA/Ca EGTA buffer and [Ca^{2+}] free was calculated using dissociation constant of 0.15 μM for EGTA (21); reference cell contained BSS with 4 mM EGTA. BSS was prepared fresh and its Ca^{2+} concentration measured before use. BSS contained 3.0±0.2 μM Ca^{2+} (±SEM).

Cell perifusion. The perifusion system is our modification (8) of the method described by Lowry and McMartin (22). In most experiments, 15×10^6 cells were stirred with 1 ml settled resin (Bio-gel P-2, 200-400 mech, Bio-Rad Laboratories, Richmond, Calif.) and placed in a 3-ml disposable syringe on top of a nylon mesh (20 μm). 2 ml of additional resin was placed on top of the cell column. In one experiment, large columns were prepared by stirring 40×10^6 cells with 3 ml settled resin which was placed in a 10-ml syringe with 2 ml additional resin on top. Preparation of the cell column required ~20 min. The columns were perifused with BSS (0.5–0.8 ml/min, 26°C) containing various concentrations of Ca^{2+}, and the effluent was collected for 1-min intervals in a fraction collector. At the end of the experiment, cell viability as determined by exclusion of trypan blue was >98 and 95% for cells exposed to 1,500 and 2.8 μM Ca^{2+}, respectively. 1 μM TRH (Beckman Instruments, Inc.), 50 mM K^+ (prepared by substituting KCl for NaCl in BSS), or 20 μm A23187 (Eli Lilly & Co., Indianapolis, Ind.) was added to the perifusate for 1 min at the indicated times. ^{45}Ca radioactivity was analyzed and prolactin was measured in perifusate. Radioimmunoassay of rat prolactin (18) was performed using reagents kindly supplied by Dr. A. F. Parlow and the Pituitary Hormone Distribution Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases.

Static incubations. GH_3 cells were preloaded with $^{45}Ca^{2+}$ (4 μCi/ml) for 16 h, as described above. The cells were washed twice with BSS with 1,500 μM Ca^{2+} and then preincubated with BSS with 1,500 or 3.2 μM Ca^{2+}. After 20 min, the cells were centrifuged at 180 *g* for 5 min, resuspended in the same solutions (1×10^6/0.1 ml), and incubated at 26°C with constant stirring. After 5 min, 10 μl of TRH in 0.9% NaCl was added. At the times indicated, the cells (0.1 ml of suspension) were separated by centrifugation at 8,000 *g* for 1 min through a layer of silicone in a microfuge (23). ^{45}Ca radioactivity in the supernate and in the cell pellet was measured. These data are expressed as the fractional efflux (rate constant) which is defined as the fraction of $^{45}Ca^{2+}$ leaving the cells per minute (5, 24).

CTC fluorescence measurements. Fluorescence measurements were made in a Spectrofluorometer Mark I (Farrand Optical Co., Valhalla, N. Y.) with excitation and emission wavelengths set usually at 400 and 515 nm, respectively, with slit widths of 5 nm. All measurements were made at 23°–26°C with cells ($2–3 \times 10^6$/ml) suspended in BSS without ovalbumin or Mg^{2+}, and with various concentrations of Ca^{2+} and CTC. For the uptake experiments, after a 20–30 min preincubation in BSS without ovalbumin or Mg^{2+} with the indicated [Ca^{2+}], the cell suspension was transferred to a cuvette, intrinsic fluorescence recorded, and 20 μl of a concentrated solution of CTC was added (zero time). Fluorescence intensity was measured at the indicated times after the cuvette was inverted twice to resuspend settled cells; intrinsic fluorescence which was <5% of the total was subtracted. For experiments in which effects of TRH and K^+ were measured, cells were loaded with CTC for the indicated times, centrifuged at 800 *g* for 2 min, and resuspended in 2 ml of fresh BSS without ovalbumin or Mg^{2+} containing the indicated [Ca^{2+}] and no CTC. The fluorescence intensity decreased in a biphasic fashion: an initial rapid loss of fluorescence which lasted <2 min followed by a slower sustained loss. The effects of TRH and 50 mM K^+ were studied during the second phase of fluorescence loss by means of dilution of the cell suspension with 1 ml of BSS without ovalbumin or Mg^{2+} (control), or with 1 ml of a solution in which NaCl was replaced by KCl. After an additional 3 min, 20 μl of a concentrated solution of TRH (the final concentration was 1 μM) or of 0.9% NaCl (control) was added, the cuvette was inverted twice, and the fluorescence loss was monitored.

RESULTS

$^{45}Ca^{2+}$ efflux studies. Previous studies (8) using this perifusion system have shown that TRH and 50 mM K^+

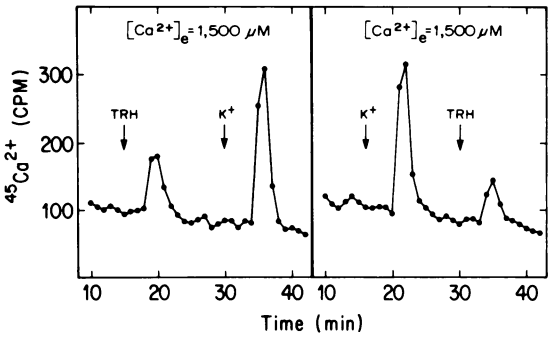

FIGURE 1 Effect of TRH and a depolarizing concentration of K^+ on $^{45}Ca^{2+}$ efflux. GH_3 cells, preincubated with $^{45}Ca^{2+}$ (2 μCi/ml) for 16 h, were perifused with BSS containing 1,500 μM Ca^{2+} (0.5 ml/min, 26°C) and 1 μM TRH or 50 mM K^+ was added to the perifusate for 1 min beginning at 15 or 30 min.

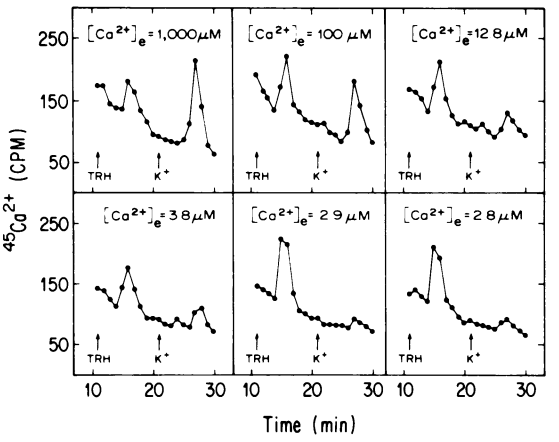

FIGURE 2 Effect of $[Ca^{2+}]_e$ on enhancement of $^{45}Ca^{2+}$ efflux by TRH and 50 mM K^+. GH_3 cells, preincubated with $^{45}Ca^{2+}$ (2 μCi/ml) for 16 h, were perifused with BSS containing the indicated concentrations of Ca^{2+} (0.5 ml/min, 26°C) beginning at time 0. TRH (1 μM) and 50 mM K^+ were present in the perifusate for 1 min beginning at 11 and 21 min, respectively. These results are representative of one of two similar experiments.

transiently enhanced the rate of $^{45}Ca^{2+}$ efflux from GH_3 cells in the presence of Ca^{2+} in the medium. Comparisons of the effects of these two secretagogues applied sequentially to GH_3 cells showed that the degree of enhancement was specific to each stimulus and was not affected by the sequence in which the stimuli were applied (Fig. 1). In the presence of 1,500 μM Ca^{2+}, the efflux induced by TRH was not as great as the efflux induced by 50 mM K^+ whether it was applied to the cells before or after the K^+. The apparent lag period between addition of TRH or K^+ to the perifusate and the increase in $^{45}Ca^{2+}$ efflux and the difference in duration of this period for TRH and K^+ is completely accounted for by the delay intrinsic to the perifusion system (8).

Exposure of GH_3 cells to various $[Ca^{2+}]_e$ had different effects on stimulus-enhanced $^{45}Ca^{2+}$ efflux, depending on whether the stimulus was TRH or 50 mM K^+ (Fig. 2). There was no measurable effect of $[Ca^{2+}]_e$ on the enhanced $^{45}Ca^{2+}$ efflux caused by TRH. In contrast, decreasing $[Ca^{2+}]_e$ from 100 to 3.8 μM caused a dose-dependent decrease in the effect of 50 mM K^+ on $^{45}Ca^{2+}$ efflux. Depolarization of GH_3 cells by 50 mM K^+ (8) was not affected by varying $[Ca^{2+}]_e$ (unpublished observations). These data are consistent with the hypothesis that 50 mM K^+ increased plasma membrane permeability to Ca^{2+}, which resulted in influx of Ca^{2+} when $[Ca^{2+}]_e$ markedly exceeded that in the cytosol. The influx of Ca^{2+} may have elevated the free cytosolic Ca^{2+} and then was reflected as an increased efflux of $^{45}Ca^{2+}$ as the cellular response to lower free Ca^{2+} was accomplished, in part, by extruding Ca^{2+}. An alternate explanation, that K^+ depolarization increases Ca^{2+}–Ca^{2+} exchange without affecting free cytosolic $[Ca^{2+}]$ appears less likely since K^+ depolarization appears to increase Ca^{2+} uptake by many secretory cells (25). In any event, TRH did not appear to act by causing an influx of Ca^{2+} from the medium since its effect on $^{45}Ca^{2+}$ efflux was not dependent on extracellular Ca^{2+}.

The extracellular Ca^{2+} requirement for K^+-induced $^{45}Ca^{2+}$ efflux was paralled by a similar requirement for K^+ stimulation of prolactin release. Likewise, the lack of dependence of TRH-induced $^{45}Ca^{2+}$ efflux on Ca^{2+} in the medium was paralleled by a similar independence of TRH-induced prolactin release from extracellular Ca^{2+} (Fig. 3). Hence, TRH stimulated release of prolactin from cells perifused with medium with either 1,500 or 3.5 μM Ca^{2+}. In contrast, in this experiment, 50 mM K^+ enhanced prolactin release to a much greater extent than TRH in medium with 1,500 μm Ca^{2+}, whereas no stimulation of prolactin release by 50 mM K^+ was detected in medium with 3.5 μM Ca^{2+}. The inability of K^+ depolarization to enhance $^{45}Ca^{2+}$ efflux in low Ca^{2+} medium was not due to depletion of cell-associated $^{45}Ca^{2+}$ radioactivity because the calcium ionophore, A23187, caused similar increments in $^{45}Ca^{2+}$ efflux from cells exposed to either 1,500 or 3.5 μM Ca^{2+} (Fig. 3).

In an attempt to quantify the effect of extracellular Ca^{2+} in stimulation of prolactin release from GH_3 cells by TRH and 50 mM K^+, a perifusion column with 40×10^6 cells was employed. The effects of TRH and 50 mM K^+ on prolactin release were compared in perifusion medium containing 1,500 and 2.8 μM Ca^{2+} and in medium in which the concentration of unbound or free Ca^{2+} was lowered to 0.02 μM by adding 33 μM EGTA to BSS (Fig. 4). Between 10 and 15 min after beginning perifusion with medium containing 2.8 μM Ca^{2+} or 0.02 μM free Ca^{2+} there was a transient, but

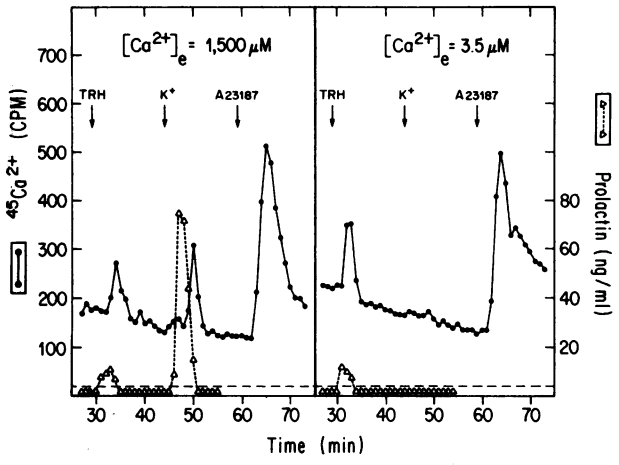

FIGURE 3 Comparison of the effects of TRH, 50 mM K^+, and A23187 on $^{45}Ca^{2+}$ efflux and TRH and 50 mM K^+ on prolactin release in medium with 1,500 and 3.5 μM Ca^{2+}. GH_3 cells used for perifusion in the presence of 1,500 μM Ca^{2+} (left-hand panel) were preincubated with 2 μCi/ml $^{45}Ca^{2+}$ for 16 h, and the cells used for perifusion without added Ca^{2+} (right-hand panel) were preincubated with 8 μCi/ml $^{45}Ca^{2+}$ for 16 h. Rate of perifusion was 0.6 ml/min at 26°C. TRH (1 μM), 50 mM K^+, or A23187 20 μM was present in the perifusate for 1 min beginning at 29, 44, and 59 min, respectively. The dashed lines at the lower part of both panels represent the limit of detectability of the prolactin immunoassay. These results are representative of one of three similar experiments.

very marked, efflux of $^{45}Ca^{2+}$ from GH_3 cells (data not shown). In this experiment the increment in prolactin release elicited by 50 mM K^+ in medium with 2.8 μM Ca^{2+} was <3% of that in medium with 1,500 μM Ca^{2+} which was 150 ng prolactin released; there was no detectable increment in medium with 0.02 μM free Ca^{2+}. In contrast, TRH enhanced prolactin release under all conditions; prolactin release stimulated by TRH was 50 and 35% of control of 64 ng (BSS with 1,500 μM Ca^{2+}) in medium with 2.8 μM Ca^{2+} and 0.02 μM free Ca^{2+}, respectively. These differences for TRH, however, may be due to loss of Ca^{2+} from a critical cell-associated pool (or pools) and not to changes in Ca^{2+} in the medium, because there was a marked efflux of $^{45}Ca^{2+}$ from cells before the addition of secretagogues. Hence, extracellular Ca^{2+} was necessary for K^+-stimulated, but not for TRH-stimulated prolactin release.

To confirm that the TRH-induced increase in $^{45}Ca^{2+}$ efflux from GH_3 cells was independent of $[Ca^{2+}]_e$ and that no artifact was introduced by the perifusion system, $^{45}Ca^{2+}$ efflux was measured from GH_3 cells in static incubations. Fig. 5 illustrates similar effects of TRH on the fractional efflux of $^{45}Ca^{2+}$ from cells incubated in medium with 1,500 or 3.5 μM Ca^{2+}. Basal fractional efflux from cells incubated with 3.5 μM Ca^{2+} ($0.027 \pm 0.003\ min^{-1}$) (±SEM) was more than twice that of cells incubated with 1,500 μM Ca^{2+} ($0.011 \pm 0.002\ min^{-1}$) ($P < 0.01$). In both instances, TRH caused a transient increase of ~10-fold in the fractional efflux of $^{45}Ca^{2+}$ which returned to basal levels within 4 min even though TRH was present continuously.

CTC fluorescence studies. The fluorescence emission spectrum obtained after incubating GH_3 cells with 100 μM CTC for 10 min was compared to emission

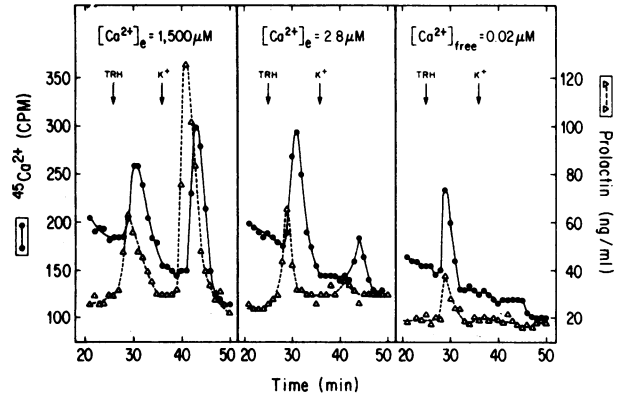

FIGURE 4 Comparison of the effects of TRH and 50 mM K^+ on $^{45}Ca^{2+}$ efflux and prolactin release in medium with 1,500 and 2.8 μM Ca^{2+} and 0.02 μM free Ca^{2+}. GH_3 cells (40×10^6/column) were preincubated with $^{45}Ca^{2+}$ (2 μCi/ml) for 16 h. Rate of perifusion was 0.8 ml/min at 26°C. TRH (1 μM) and 50 mM K^+ were present in the perifusate for 1 min beginning at 25 and 36 min, respectively. Medium containing 0.02 μM unbound or free Ca^{2+} ($[Ca^{2+}]_{free}$) was prepared by adding 33 μM EGTA to BSS.

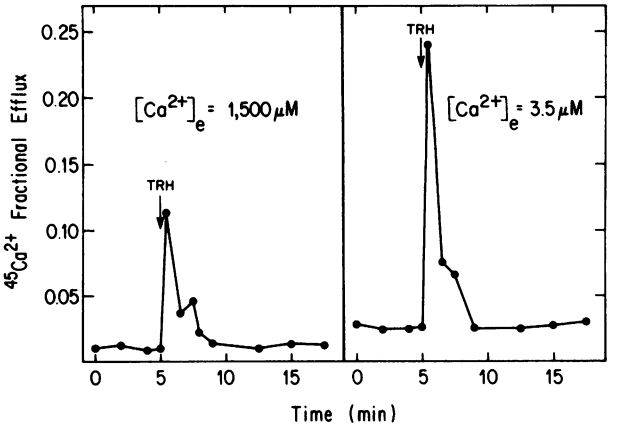

FIGURE 5 Effect of TRH on the $^{45}Ca^{2+}$ fractional efflux in the presence of 1,500 and 3.5 μM Ca^{2+} during static incubations. GH_3 cells, preloaded with $^{45}Ca^{2+}$ (4 μCi/ml) for 16 h, were preincubated with constant stirring with BSS containing 1,500 or 3.5 μM Ca^{2+}. After 20 min the cells were washed and resuspended in fresh solutions (time 0). Cells incubated in BSS with 3.5 μM Ca^{2+} contained 43% of $^{45}Ca^{2+}$ of cells incubated with BSS containing 1,500 μM Ca^{2+}. TRH was added at the time indicated. The points represent the mean of duplicate determinations. These results are representative of one of two similar experiments.

spectra of the Ca^{2+}- and Mg^{2+}-chelates of CTC in 90% methanol/10% BSS without ovalbumin, an amphipathic solvent in which the Ca^{2+} and Mg^{2+} complexes of CTC have spectra similar to those in membranes (15, 16). The emission maximum and shape of the emission spectrum of CTC-loaded GH_3 cells (data not shown) indicated that CTC in GH_3 cells was complexed with Ca^{2+} and Mg^{2+}. The effect of increasing $[Ca^{2+}]_e$ on the fluorescence associated with GH_3 cells after 10 min of incubation with 100 μM CTC is illustrated in Fig. 6. There was a concentration-dependent increase in fluorescence intensity between $[Ca^{2+}]_e$ of 100 μM and 10,000 μM. This suggests that there is a pool (or pools) of Ca^{2+} within these cells which can be altered by variations in the $[Ca^{2+}]_e$ and which can be probed by CTC. Since it has been shown that the enhanced fluorescence of CTC in biological specimens is due to its interaction with divalent cations in the lipid or hydrophobic domain of membranes, it was likely that this pool of Ca^{2+} was present within membranes of GH_3 cells.

To gain further insight into the pool of Ca^{2+} probed by CTC in GH_3 cells, we determined the time-course of uptake of CTC. Fig. 7 illustrates the change with time of fluorescence associated with GH_3 cells exposed to different concentrations of CTC in the presence of 100 and 3.5 μM Ca^{2+}. The intensity of fluorescence was dependent on the $[Ca^{2+}]_e$ (see above), the concentration of CTC, and the duration of exposure to CTC. For example, the fluorescence intensity at 10 min was only 40% of the intensity at 60 min in cells exposed to 100 μM CTC and $[Ca^{2+}]_e$ of 100 or 3.5 μM. This may have been due to increased labeling by CTC of the same Ca^{2+} pool (or pools) or to labeling of different pools with increasing duration of exposure.

The effects of TRH and a depolarizing concentration of K^+ on CTC fluorescence associated with GH_3 cells

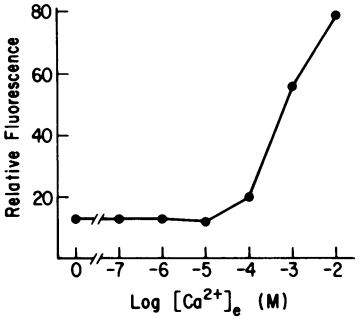

FIGURE 6 Dose-response effect of increasing $[Ca^{2+}]_e$ on the fluorescence of CTC associated with GH_3 cells. GH_3 cells (2×10^6/ml) were incubated in BSS without ovalbumin or Mg^{2+} containing the indicated concentrations of Ca^{2+} for 20 min and then 100 μM CTC was added. After 10 min the cuvette was inverted twice and the fluorescence intensity measured. Intrinsic fluorescence was subtracted from each value.

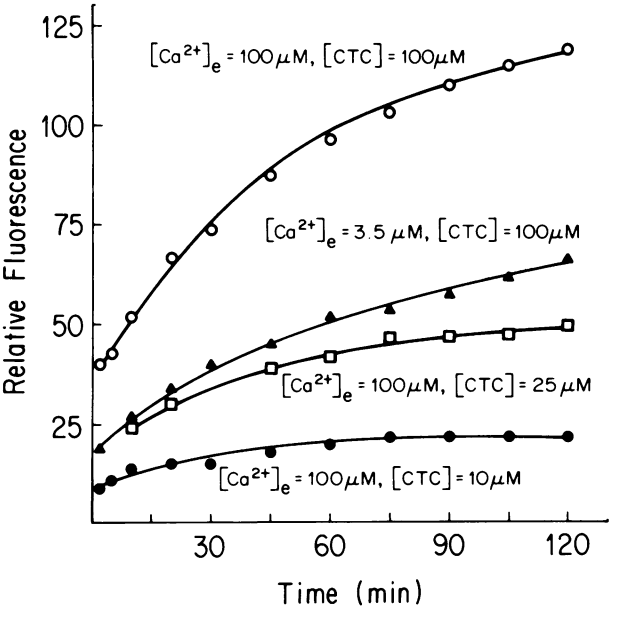

FIGURE 7 Time-course of the uptake of CTC into GH_3 cells. GH_3 cells (3×10^6/ml) were incubated in BSS without ovalbumin or Mg^{2+} with 100 or 3.5 μM Ca^{2+}. After 30 min (time 0), CTC in the indicated concentration was added and the fluorescence intensity measured at the times indicated. Intrinsic fluorescence was subtracted from each value.

were studied. For these experiments cells were exposed to CTC, centrifuged, and resuspended in fresh solutions without CTC. Fig. 8 illustrates the changes in fluorescence intensity of GH_3 cells exposed to 100 μM CTC for 10 min (uptake) and then, after removing CTC from the medium, to 50 mM K^+ (after 3 min), and to 1 μM TRH (after 6 min). K^+ depolarization caused a small increase in the rate of fluorescence loss which was shown in other experiments to persist for at least 10 min. TRH also increased the rate of fluorescence loss but this differed from that induced by 50 mM K^+ in two important ways. First, the effect of TRH was transient, lasting <1.5 min; that is, the rate of loss of fluorescence before TRH addition was reached again within 1.5 min after exposure. Second, the magnitude of the decrease in fluorescence achieved within the first 0.5 min after addition of TRH was much greater than after exposure to 50 mM K^+. The transient enhancement of fluorescence loss induced by TRH suggests that TRH displaces abruptly a portion of the membrane-bound Ca^{2+} monitored by CTC.

Further evidence supporting the contention that TRH mobilizes Ca^{2+} from a cellular membrane-associated pool, and not via influx of Ca^{2+} from the medium, may be derived from the observation that the effect of TRH on CTC fluorescence was observed even in GH_3 cells that had been exposed 3 min earlier to a depolarizing concentration of K^+ (Fig. 8, lowest tracing in each panel).

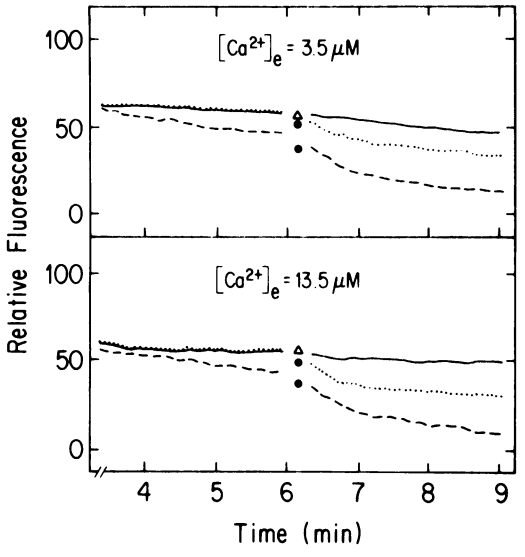

FIGURE 8 Effect of TRH and 50 mM K^+ on CTC fluorescence associated with GH_3 cells. GH_3 cells (2×10^6/ml) were incubated in BSS without ovalbumin with 13 or 3.5 μM Ca^{2+}. After 30 min, 100 μM CTC was added. After 10 min of uptake, the cells were centrifuged, divided into three portions and resuspended in 2 ml of fresh solution without CTC. After 3 min of incubation without CTC, 1 ml of BSS without ovalbumin or Mg^{2+} was added to two cuvettes (solid and dotted lines) and 1 ml of 150 mM KCl was added to the last cuvette (50 mM K^+ final concentration, dashed line). After 6 min, 30 μl of TRH (final concentration 1 μM, ●) or 0.9% NaCl (△, control) was added where indicated. The break in the tracing indicates where the light source was turned off and the factor added. Each tracing is representative of four similar experiments.

TRH appeared to mobilize Ca^{2+} only from a pool (or pools) that was monitored completely by incubating GH_3 cells with 100 μM CTC for 10 min. This conclusion was based on the finding that TRH caused a similar absolute decrease in CTC fluorescence irrespective of the duration of incubation with CTC between 10 and 120 min, even though the total fluorescence intensity increased with longer incubations (data not shown). Hence, it seems likely that TRH mobilized Ca^{2+} specifically from a cell-associated pool (or pools) that is readily probed by CTC.

DISCUSSION

These data demonstrate that TRH stimulation of prolactin release and $^{45}Ca^{2+}$ efflux from GH_3 cells was not dependent on the concentration of Ca^{2+} in the incubation medium and suggest that TRH mobilizes Ca^{2+} from a cell-associated pool. TRH increased $^{45}Ca^{2+}$ efflux from cells studied in a perifusion system and in static incubations even when there was only 0.02 μM free Ca^{2+} in the medium. In the perifusion system, TRH increased prolactin release at all $[Ca^{2+}]_e$. These findings for TRH were in marked contrast to those observed when GH_3 cells were exposed to a depolarizing concentration of K^+ (50 mM) since the K^+-induced increase in $^{45}Ca^{2+}$ efflux and prolactin release were both dependent on $[Ca^{2+}]_e$. Additional evidence supporting the contention that TRH and K^+ depolarization affected cellular Ca^{2+} metabolism differently was obtained from studies of GH_3 cells incubated with CTC, a fluorescent probe of membrane-bound Ca^{2+} (15, 16). During incubation of cells preloaded with CTC, TRH caused an abrupt, marked, but transient increase in fluorescence loss. The limited nature of the fluorescence loss, even in the continued presence of TRH, paralleled the transient increase in $^{45}Ca^{2+}$ efflux induced by TRH. In contrast, K^+ depolarization caused only a small and persistent increase in the rate of CTC fluorescence loss. These observations confirm our previous suggestion, based on findings that TRH did not depolarize GH_3 cells (8), and those of Milligan and Kraicer (26) and Eto et al. (27), that the mechanisms by which TRH and a depolarizing concentration of K^+ may lead to prolactin release are clearly different.

In this study, CTC, which has been used successfully to monitor changes in membrane-associated Ca^{2+} in isolated mitochondria (28, 29), sarcoplasmic reticulum vesicles (30), and intact cells (30–34), was employed to complement the $^{45}Ca^{2+}$ efflux studies to determine indirectly whether TRH mobilized Ca^{2+} from a cellular membrane-associated pool (or pools). This was important since the enhanced $^{45}Ca^{2+}$ efflux caused by TRH could have been due simply to release of $^{45}Ca^{2+}$ from secretory granules during the exocytotic process. Although other factors could have affected CTC fluorescence, the very close parallels between the effects of TRH on $^{45}Ca^{2+}$ efflux and CTC fluorescence suggest that the decrease in fluorescence caused by TRH is best explained by displacement of membrane-bound Ca^{2+}, as has been concluded for other secretagogues (28–35).

It has been suggested that stimulation of release of adenohypophyseal hormones may be dependent on extracellular Ca^{2+} (36, 37). This notion was based on observations that anterior pituitary cells in vitro would not respond to secretatogues when incubated in Ca^{2+}-free medium. In most previous studies, however, cells were washed extensively with, or bathed for prolonged periods in, Ca^{2+}-free buffers or buffers containing divalent cation chelators before experimental incubation. We, as well as others (38), have found that exposure of anterior pituitary cells to medium without Ca^{2+} or with Ca^{2+} chelators, such as EDTA, caused a marked loss of Ca^{2+}. For example, GH_3 cells exposed to BSS with no added Ca^{2+} or to 0.02% EDTA for 20 min contained only 40 (Fig. 5) or 15% of control $^{45}Ca^{2+}$, respectively. We propose, therefore, that previous observations of lack of response to TRH were not due to absence of extracellular Ca^{2+} but were caused instead

by loss of Ca^{2+} from a critical cell-associated pool (or pools). In our experiments, in which care was taken to minimize loss of cellular Ca^{2+}, TRH increased $^{45}Ca^{2+}$ efflux under conditions that completely abolished the increment due to Ca^{2+} influx usually induced by K^+ depolarization.

The relation of the observations reported here to those of electrophysiological studies with adenohypophyseal cells in culture in which the frequency of Ca^{2+} action potentials was increased by TRH (9–13) is uncertain. Two lines of evidence suggest that these phenomena are separate. First, when Ca^{2+} is not added to the incubation medium, action potentials in GH_3 cells which depend on a Ca^{2+} mechanism are abolished (10), whereas under these same conditions TRH still causes an increase in $^{45}Ca^{2+}$ efflux and prolactin release (Figs. 3 and 4). Second, in the presence of verapamil, which is a Ca^{2+} channel blocker, Ca^{2+} action potentials are abolished in GH_3 (12) and in normal adenohypophyseal cells (13), whereas TRH still enhances $^{45}Ca^{2+}$ efflux and prolactin release from GH_3 cells, although K^+-induced efflux and release are inhibited (unpublished observations). It appears, therefore, that the effect of TRH to increase the frequency of action potentials and, perhaps in so doing, to increase Ca^{2+} influx is not a necessary mechanism for TRH-induced prolactin release.

The data reported here are consistent with the stimulus-secretion coupling hypothesis (4) as regards TRH regulation of prolactin release from anterior pituitary cells. According to this hypothesis, TRH may act to increase free intracellular Ca^{2+}, which then induces secretion of prolactin. The increased $^{45}Ca^{2+}$ efflux from anterior pituitary cells caused by TRH (Figs. 3 and 4) (5–7) appears to reflect this elevation in free cytosolic Ca^{2+} (36). We conclude, differently from previous investigators (2, 9–13), that the possible increase in free Ca^{2+} concentration is due, not to net influx of Ca^{2+} from the medium, which would be associated with an increase in cell-associated Ca^{2+}, but to mobilization of sequestered cell-associated Ca^{2+} into the cytoplasm. Since TRH interacts with a plasma membrane receptor (18, 39–42) and may cause mobilization of Ca^{2+} from a cellular membrane-associated pool (or pools), this may be analogous to the loss of Ca^{2+} from the plasma membrane of neutrophils after exposure to secretagogues (43); but subcellular localization of the Ca^{2+} pool affected by TRH requires further study.

In summary, we have shown that TRH affects cellular Ca^{2+} metabolism in a manner independent of the concentration of Ca^{2+} in the incubation medium. We suggest that displacement of Ca^{2+} from a cellular membrane-associated pool (or pools) may lead to an increase in the intracellular free Ca^{2+} concentration and to prolactin release. Our studies do not, however, allow any conclusion concerning the role of extracellular Ca^{2+} in maintaining responsiveness chronically of pituitary cells to TRH, because experiments performed with cells in medium with no added Ca^{2+} were, by design, only of short duration. They also do not determine whether TRH may increase free intracellular Ca^{2+} simultaneously by increasing Ca^{2+} influx during a series of action potentials or whether these separate mechanisms may act synergistically to affect stimulation of prolactin release.

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