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Thyrotropin-releasing hormone mobilizes Ca^{2+} from endoplasmic reticulum and mitochondria of GH_3 pituitary cells: Characterization of cellular Ca^{2+} pools by a method based on digitonin permeabilization

(stimulus–secretion coupling/peptide hormone)

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ABSTRACT **Treatment of $^{45}Ca^{2+}$-loaded GH_3 pituitary cells with various concentrations of digitonin revealed discrete pools (I and II) of cellular $^{45}Ca^{2+}$ defined by differing detergent sensitivities. Markers for cytosol and intracellular organelles indicated that the two $^{45}Ca^{2+}$ pools were correlated with the two major cellular Ca^{2+}-sequestering organelles, endoplasmic reticulum (I) and mitochondria (II). Studies with various inhibitors were consistent with these assignments. Mitochondrial uncouplers preferentially depleted $^{45}Ca^{2+}$ pool II while trifluoperazine selectively depleted $^{45}Ca^{2+}$ pool I. Control experiments indicated that translocation of *in situ* organellar $^{45}Ca^{2+}$ during and after permeabilization was negligible. We used the digitonin-permeabilization method to examine the effect of thyrotropin-releasing hormone (TRH) treatment on intracellular Ca^{2+} pools of GH_3 pituitary cells. TRH was found to rapidly deplete both endoplasmic reticulum and mitochondrial exchangeable Ca^{2+} by 25–30%. The $^{45}Ca^{2+}$ loss from both pools was maximal by 1 min after TRH addition and was followed by a recovery phase; mitochondrial $^{45}Ca^{2+}$ content returned to control levels by 30 min. Previous treatment of cells with the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone blocked TRH-induced $^{45}Ca^{2+}$ efflux from mitochondria, while previous treatment with valinomycin, an agent that depleted both $^{45}Ca^{2+}$ pools, blocked any additional effect of TRH on these pools. We conclude that TRH rapidly promotes a net loss of exchangeable Ca^{2+} from GH_3 cells as a result of hormone-induced mobilization of Ca^{2+} from endoplasmic reticulum and mitochondria.**

The hypothalamic peptide thyrotropin-releasing hormone (TRH) stimulates the secretion of prolactin and thyroid-stimulating hormone from the pituitary. It has been proposed that TRH-stimulated hormone release is the result of a TRH-induced increase in cytosolic Ca^{2+} concentration (1). Some workers have suggested that TRH promotes influx of extracellular Ca^{2+} (1, 2) while others have proposed that the hormone mobilizes intracellular Ca^{2+} (3–5). In GH_3 cells, a clonal TRH-responsive rat pituitary tumor cell line, TRH-induced $^{45}Ca^{2+}$ efflux was shown to be independent of extracellular Ca^{2+} (3, 5). In addition, it has been found that stimulation of prolactin release by TRH may not require extracellular Ca^{2+} (2–5). These data imply that, if Ca^{2+} mediates TRH action, the source of Ca^{2+} is intracellular.

To test this hypothesis directly and to determine whether TRH affects a specific cellular Ca^{2+} pool, we have used a method to measure intracellular Ca^{2+} that avoids the technical difficulties inherent in homogenization and fractionation of tissue culture cells. The detergent digitonin complexes with membrane cholesterol (6) and can selectively permeabilize cells because cellular membranes vary in their cholesterol content [plasma membrane > endoplasmic reticulum > mitochondria (7)]. Previous workers have used digitonin to permeabilize the plasma membrane to examine Ca^{2+} uptake by intracellular organelles *in vitro* (8–10). In this paper, we present a method that uses digitonin permeabilization to define two discrete pools of cellular Ca^{2+} that correspond to endoplasmic reticulum (ER) and mitochondria and report that TRH treatment of GH_3 cells leads to a rapid net loss of exchangeable Ca^{2+} from both pools.

MATERIALS AND METHODS

All radiochemicals were obtained from New England Nuclear. $^{45}CaCl_2$ was 13–40 mCi/mg (1 Ci = 3.7×10^{10} becquerels). D600, prenylamine, and nifedipine were generously provided by Knoll (Ludwigshafen, Federal Republic of Germany), Hoechst-Roussel Pharmaceuticals (Somerville, NJ), and Pfizer, respectively. Trifluoperazine was donated by Smith Kline & French. All other chemicals were from Sigma. Ruthenium red was purified prior to use (11).

Cell Culture. GH_3 cells obtained from the American Type Culture Collection were used throughout. Stock monolayer cultures were maintained as described (12). On the day prior to the experiment, cells were placed in suspension culture (5×10^5/ml) in Eagle's minimal essential medium with spinner salts (GIBCO) supplemented with 7.5% serum (horse serum/fetal bovine serum, 6:1). The free Ca^{2+} of this medium was 65 μM as determined by a Ca^{2+} electrode. Cells used in a given experiment were from the same suspension culture.

$^{45}Ca^{2+}$ Labeling Experiments. All experiments were with suspension cells in culture as described above. Experiments were carried out either under "$^{45}Ca^{2+}$ steady-state" conditions, in which cells labeled to a stable specific activity with $^{45}Ca^{2+}$ were treated with test agents in the continued presence of $^{45}Ca^{2+}$, or under "$^{45}Ca^{2+}$ uptake" conditions, in which cells were treated with test agents prior to addition of $^{45}CaCl_2$.

$^{45}Ca^{2+}$ Steady-State Experiments. Cells were labeled to steady state while in suspension culture at 1 μCi of $^{45}Ca^{2+}$/ml. In initial experiments, 24-hr labeling was used but subsequently it was found that steady-state labeling was achieved within 60 min; cells labeled for various times between 1 and 24 hr had the same $^{45}Ca^{2+}$ content, and intracellular pool sizes were identical in cells equilibrated with $^{45}Ca^{2+}$ for 1 or 24 hr (data not shown).

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Abbreviations: TRH, thyrotropin-releasing hormone; HBSS, Hepes-buffered salt solution; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TFP, trifluoperazine; ER, endoplasmic reticulum.

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Therefore, in subsequent experiments, cells were labeled 60 min prior to use. Measurements of total cell calcium with arsenazo III indicate that >95% of cell Ca^{2+} is exchangeable with $^{45}Ca^{2+}$ in 1 hr. After labeling, cells were pelleted at room temperature, resuspended in labeling medium at 8–10 × 10^6/ml, and divided into aliquots in 15-ml conical test tubes. Test agents were then added from 100-fold concentrated stock solutions; control cells received the appropriate vehicle. In experiments testing K^+ depolarization, suspension cells were pelleted and suspended in Hepes-buffered salt solution (HBSS; 140 mM NaCl/5 mM KCl/1 mM $MgCl_2$/0.5 mM $CaCl_2$/10 mM glucose/0.1% bovine serum albumin/10 mM Hepes, pH 7.5) at 1.5 × 10^6/ml. After 60 min of equilibration in HBSS containing $^{45}Ca^{2+}$ at 1.5 μCi/ml, the cells were pelleted and suspended in 50 mM K^+ HBSS (formulated with NaCl reduced to maintain isotonicity) containing $^{45}Ca^{2+}$ at 1.5 μCi/ml.

$^{45}Ca^{2+}$ Uptake Experiments. Cells suspended in HBSS (1.5 × 10^6/ml) were incubated at 37°C for 60 min prior to addition of $^{45}Ca^{2+}$ (1.5 μCi/ml). Test agents were added 30 min prior to $^{45}Ca^{2+}$ and were present throughout the $^{45}Ca^{2+}$ uptake period (20 min).

Digitonin Permeabilization of Cells. Cells incubated as above ($^{45}Ca^{2+}$ steady-state or $^{45}Ca^{2+}$ uptake experiments) were centrifuged at 4°C (800 × *g*, 3 min) and washed once with ice-cold Na^+-, Ca^{2+}-free HBSS (formulated with choline chloride instead of NaCl). This buffer minimized $^{45}Ca^{2+}$ efflux during the wash. The washed pellets were resuspended at 5 × 10^6/ml in cold Na^+-, Ca^{2+}-free HBSS supplemented with 10 μM EGTA/0.2 μM ruthenium red, and 0.5-ml aliquots were distributed to a series of 1.5-ml Microfuge tubes on ice containing various concentrations of digitonin [5 μl of 100-fold-concentrated stocks (wt/vol) in dimethyl sulfoxide]. After addition of cells, the tubes were mixed on a Vortex briefly, incubated 5 min on ice, and centrifuged in an Eppendorf model 5413 Microfuge for 5 min. The resulting supernatants and cell pellets (pellets were solubilized in 0.2% $NaDodSO_4$) were mixed with RTU II scintillation fluid (Eastman Kodak) and radioactivity was determined. Total $^{45}Ca^{2+}$ content per 2.5 × 10^6 cells was at least 10,000 cpm for control cells.

Permeabilization of cells and $^{45}Ca^{2+}$ release was half-maximal by 1 min and maximal by 4 min. Nominal zero time values of $^{45}Ca^{2+}$ release were obtained by centrifugation immediately after distribution of cells to Microfuge tubes containing 1% dimethyl sulfoxide (vehicle control). Control and agent-treated cells were always processed at the same time.

Results shown are representative of experiments repeated at least three times. Determinations were not carried out in duplicate in individual experiments because of the technical demands of the protocol and because experimental variation of the method was found to be very small. In a test repeated twice, four separate suspensions of cells washed and digitonin permeabilized in parallel generated $^{45}Ca^{2+}$ release profiles (see *Results*) that were virtually superimposable; supernatant $^{45}Ca^{2+}$ at a given digitonin concentration had a SD of less than 1.5%. The size of the symbols in Fig. 1 is equivalent to 1 SD.

[^{3}H]UTP Release. Suspension cells were incubated with [^{3}H]uridine (0.5 μCi/ml) for 15 min. Under these conditions, >90% of the cellular label is present as uridine nucleotides (13). Release of [^{3}H]UTP was determined under the digitonin-permeabilization conditions used to assess $^{45}Ca^{2+}$ release.

Enzyme Assays. Assays were carried out at 30°C on cell suspensions at 4 × 10^6 cells/ml. Glucose-6-phosphatase (EC 3.1.3.9) activity was determined as described (14), except that the incubation buffer was HBSS (pH 7.0) and reaction time was 30 min. Glutamate dehydrogenase (EC 1.4.1.3) activity was assayed by the method of Strecker (15); the incubation buffer was 0.3 M sucrose/0.1% bovine serum albumin/0.05 M KH_2PO_4, pH 7.6, and activity was measured after 15 min.

RESULTS

Effect of Digitonin on $^{45}Ca^{2+}$ Release from Labeled Cells. A representative profile of $^{45}Ca^{2+}$ released from labeled cells by various concentrations of digitonin is shown in Fig. 1*A*. The biphasic shape of the curve, with an inflection point at 0.03 (3 × 10^{-2})% digitonin, indicated that the bulk of cellular $^{45}Ca^{2+}$ resided in two discrete pools with different sensitivities to digitonin permeabilization. The first pool (pool I) was maximally released by about 0.01% digitonin, while the second (pool II) was released only at higher concentrations (0.03–0.1%). Permeabilization of the plasma membrane, as indicated by release of [^{3}H]UTP (Fig. 1*A*) and uptake of trypan blue (not shown), occurred over a low and narrow range of detergent (0.001–0.003%) that only partially overlapped the initial $^{45}Ca^{2+}$ release.

The $^{45}Ca^{2+}$ pool sizes were calculated as follows (Fig. 1*A*). The digitonin concentration that marks the boundary between pool I and pool II was routinely 0.02–0.03% (dependent on exact cell density). $^{45}Ca^{2+}$ released up to this inflection point was assigned to pool I. Pool II $^{45}Ca^{2+}$ was calculated by taking the difference between total digitonin-sensitive $^{45}Ca^{2+}$ and pool I $^{45}Ca^{2+}$. Zero-time $^{45}Ca^{2+}$ values were included in pool I because the source of this $^{45}Ca^{2+}$ was primarily efflux from cells of pool I $^{45}Ca^{2+}$ during the cold wash and resuspension steps prior to placing the cells in the Microfuge tubes. Pool II did not efflux at 4°C.

Separate cell populations displayed some variability in basal steady-state $^{45}Ca^{2+}$ content, primarily due to differences in pool II Ca^{2+} (see Fig. 2, for example). Average values for pools I and II, as percent total cell $^{45}Ca^{2+}$ (mean ± SD, n = 26), were 46.1 ± 5.8 and 38.1 ± 6.0, respectively. The remaining cell $^{45}Ca^{2+}$ (15.5 ± 2.8%) was not released by digitonin treatment. The digitonin-insensitive pool includes a background estimated at 5% (see Table 1, 0.1% digitonin), but the nature of the other 10% is unknown. It was not released by addition of 10 μM A23187 or 1 mM EGTA to 0.1% digitonin-treated cells (not shown) and may represent $^{45}Ca^{2+}$ bound to proteins.

Marker enzyme analysis indicated that the $^{45}Ca^{2+}$ pools I and II could be correlated on the basis of digitonin sensitivity with the major Ca^{2+}-sequestering organelles of the cell, ER and mitochondria (16). Assays in cell suspensions for glucose-6-phosphatase [ER marker (17)] and glutamate dehydrogenase [mitochondrial matrix marker (17)] were designed so that increases in enzyme activity depended on substrate access to the cryptic enzyme. Thus, increases in enzyme activity depended on permeabilization of the organelle membrane and were a function of digitonin concentration. At 0.001–0.01% digitonin, glucose-6-phosphatase activity was detected; higher detergent concentrations (0.02–0.1%) were required to reveal glutamate dehydrogenase activity (Fig. 1*B*).†

Accurate measurement of *in situ* $^{45}Ca^{2+}$ distribution required that no translocation of $^{45}Ca^{2+}$ occur during or after permeabilization. As shown in Table 1 ("no additions" column), unlabeled cells permeabilized with 0.005–0.03% digitonin could accumulate ≈90% of $^{45}Ca^{2+}$ added during digitonin treatment. Higher digitonin concentrations (0.04–0.1%) progressively abolished this accumulation. Apparently, permeabilization of the plasma membrane and subsequent exposure of mitochondria (intact at low digitonin concentrations) to the trace

† Differences in cell density, temperature, and incubation time contributed to a slight leftward shift in digitonin sensitivities for the enzyme markers as compared with Ca^{2+} release.

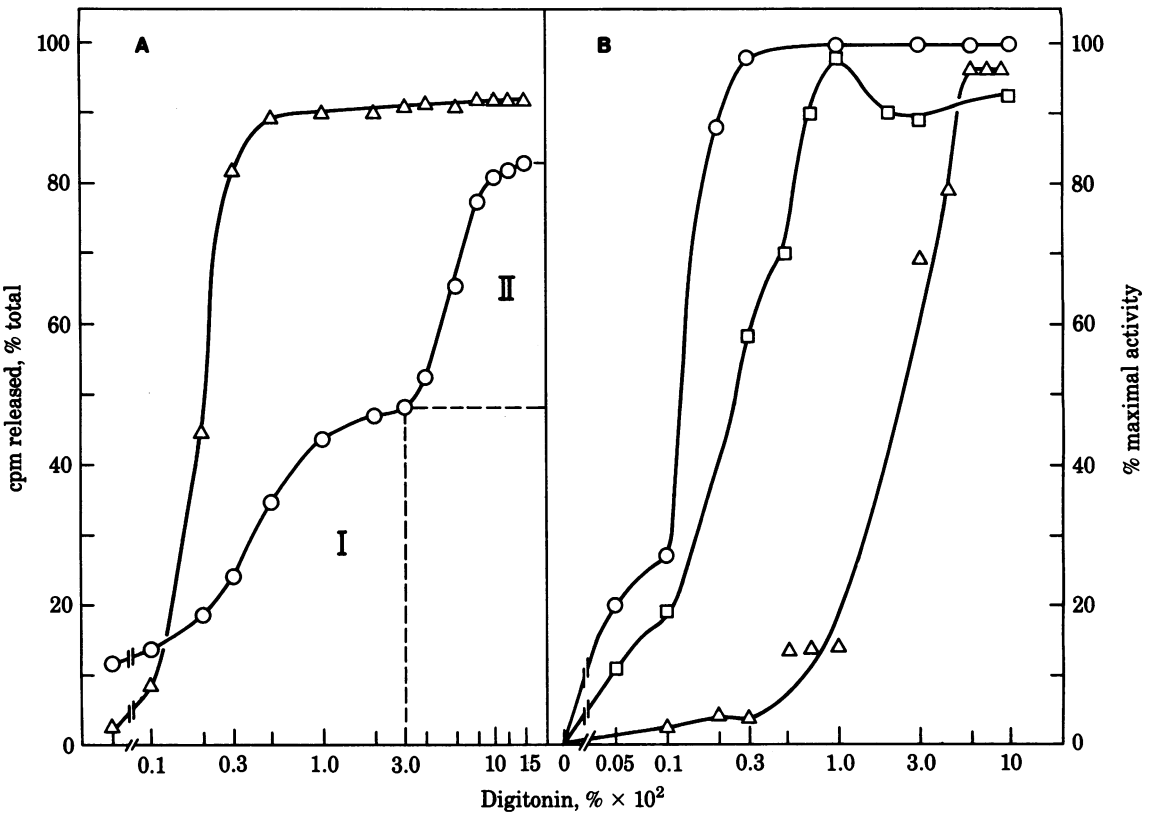

FIG. 1. Effect of digitonin concentration on small molecule release and enzyme activity. (*A*) Release of $^{45}Ca^{2+}$ (○) and [^{3}H]UTP (△). The dashed lines indicate the two $^{45}Ca^{2+}$ pools defined by digitonin permeabilization of cells. Cells were labeled as described for the $^{45}Ca^{2+}$ steady-state protocol. (*B*) Comparison of glucose-6-phosphatase (□) and glutamate dehydrogenase (△) activities with [^{3}H]UTP release (○).

of Ca^{2+} in the buffer stimulated $^{45}Ca^{2+}$ uptake. The Ca^{2+}-uptake inhibitors tetracaine, Mg^{2+} (not shown), ruthenium red, La^{3+} (16), and EGTA inhibited the *in vitro* $^{45}Ca^{2+}$ accumulation (Table 1). Therefore, 10 μM EGTA/0.2 μM ruthenium red was included in all digitonin-permeabilization experiments with $^{45}Ca^{2+}$-labeled cells to prevent *in vitro* translocation of medium and pool I $^{45}Ca^{2+}$ into pool II (mitochondria). Another potential problem in assessing $^{45}Ca^{2+}$ pool sizes is efflux of mitochondrial $^{45}Ca^{2+}$. However, $^{45}Ca^{2+}$ loss from pool II during permeabilization (5–10 min, 4°C) was found to be less than 2% of the total pool, even in mitochondrial uncoupler-treated cells (not shown).

The studies described above indicated that pool I and pool II may be independent pools that represent ER and mitochondrial $^{45}Ca^{2+}$ content, respectively. Additional studies with various inhibitors supported these assignments. As shown in Table 2, treatment of steady-state $^{45}Ca^{2+}$-loaded GH_3 cells with the mitochondrial uncouplers, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and valinomycin, preferentially depleted pool II (>50% reduction in pool II, <25% reduction in

Table 1. *In vitro* $^{45}Ca^{2+}$ uptake by digitonin-permeabilized cells

	% $^{45}Ca^{2+}$ cpm in pellet			
% digitonin	No additions	2 μM ruthenium red	10 μM $LaCl_3$	10 μM EGTA
None	23.9	10.1	3.9	6.5
0.005	83.0	27.2	11.9	5.5
0.01	89.3	22.7	13.1	5.4
0.03	91.5	21.1	14.0	5.2
0.04	57.4	—	—	—
0.06	28.2	17.2	8.2	5.0
0.10	8.2	6.7	4.6	4.9

Suspension cells were washed, resuspended in ice-cold Na^{+}-, Ca^{2+}-free HBSS at 5 × 10^{6}/ml, and distributed to Microfuge tubes containing digitonin, other additions as indicated, and 10^{5} cpm of $^{45}Ca^{2+}$. After 5 min on ice, the tubes were centrifuged and the $^{45}Ca^{2+}$ radioactivity in pellets was determined. Note that, in this experiment, in contrast to all others presented, unlabeled cells were incubated with $^{45}Ca^{2+}$ during the permeabilization on ice.

Table 2. Effects of various agents on $^{45}Ca^{2+}$ pool sizes in steady-state labeled cells

Exp.	Agent	Conc., μM	Temp., °C	Time, min	Pool size, % control	
					Pool I	Pool II
1	FCCP	5	25	2	100	89
				10	92	50
				20	79	35
2	FCCP	10	37	15	82	36
	CCCP	10	37	15	76	42
3	Valinomycin	10	37	15	73	39
4	TFP	40	25	15	75	101
5	D600	100	25	10	75	84
	Prenylamine	100	25	15	79	87
6	Nifedipine	10	37	15	79	88
7	$CoCl_2$	1,000	25	10	75	68
8	KCl	50,000	25	15	121	214
9	TRH	1	25	2	72.8 ± 3.3	76.7 ± 4

Cells were treated with various agents at 25°C or 37°C under $^{45}Ca^{2+}$ steady-state conditions, and pool I and pool II $^{45}Ca^{2+}$ contents were determined. Data for Exps. 1–8 are from single experiments. Exp. 9 represents mean ± SD for 11 separate experiments. Conc., concentration; temp., temperature.

pool I). Antimycin A (10 μM) action resembled that of CCCP (not shown). Treatment with trifluoperazine (TFP) resulted in depletion of pool I $^{45}Ca^{2+}$ without affecting pool II $^{45}Ca^{2+}$ (Table 2). The selectivity of TFP and FCCP action was more marked when $^{45}Ca^{2+}$ uptake into pools I and II of cells previously treated with these agents was assessed (Fig. 2, *A* and *B*). TFP treatment reduced pool I size by 50% without affecting pool II $^{45}Ca^{2+}$ content (Fig. 2*A*) whereas FCCP abolished uptake into pool II while diminishing pool I only slightly (Fig. 2*B*). Treatment of steady-state $^{45}Ca^{2+}$-loaded cells with calcium channel blockers reduced both $^{45}Ca^{2+}$ pools (Table 2, Exps. 5–7). Apparently, inhibition of plasma membrane Ca^{2+} influx decreased the cytosolic Ca^{2+} concentration so that the balance between uptake and efflux from organelles shifted, resulting in a net loss of $^{45}Ca^{2+}$ from both pools. Treatment of cells with the depolarizing agent K^+ (50 mM) doubled the pool II $^{45}Ca^{2+}$ content within 15 min while the pool I $^{45}Ca^{2+}$ content was affected to a smaller extent (Table 2, Exp. 8).

Effect of TRH on ER and Mitochondrial $^{45}Ca^{2+}$ Content. The data presented above indicate that ER and mitochondrial exchangeable Ca^{2+} can be measured by digitonin permeabilization as pools I and II, respectively. In the experiments described below, we used this method to determine whether TRH treatment of steady-state $^{45}Ca^{2+}$-loaded GH_3 cells results in a net loss of $^{45}Ca^{2+}$ from either of these organelles. As shown in Fig. 2*C*, TRH treatment resulted in loss of $^{45}Ca^{2+}$ from both pools. ER $^{45}Ca^{2+}$ was reduced by 25% and mitochondrial $^{45}Ca^{2+}$ was reduced by 30%. In most experiments, TRH depleted ER $^{45}Ca^{2+}$ to a somewhat greater extent than mitochondrial $^{45}Ca^{2+}$ (Table 2, Exp. 9). A similar TRH effect was obtained with cells equilibrated in HBSS (not shown). Ca^{2+} pool depletion induced by TRH was found to be half-maximal at 3 nM and maximal by 10 μM (not shown).

The time course of the effect of TRH on $^{45}Ca^{2+}$ pools is shown in Fig. 3. $^{45}Ca^{2+}$ loss from both organelles was maximal by 1 min after TRH addition and was detectable by 10 sec. Such rapid mobilization of Ca^{2+} was unique to TRH action; the Ca^{2+} loss

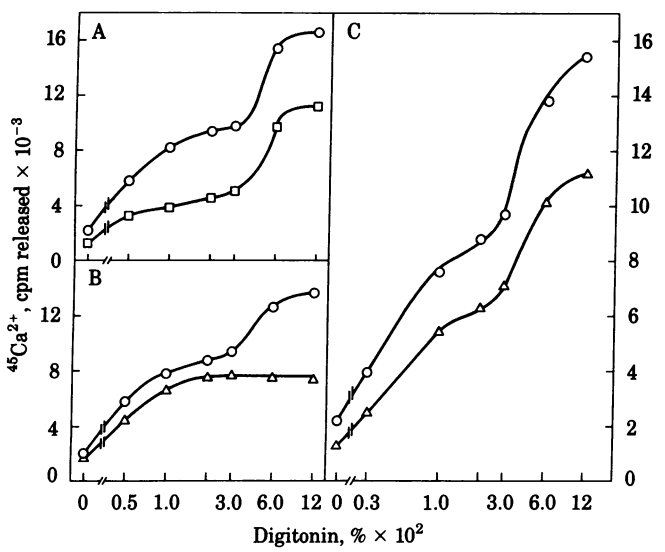

FIG. 2. Inhibition of $^{45}Ca^{2+}$ uptake into cellular pools by 10 μM TFP (*A*) and 5 μM FCCP (*B*). $^{45}Ca^{2+}$ uptake conditions were used to label cells. Total cell $^{45}Ca^{2+}$ (cpm per incubation mixture): (*A*) control (○), 19,200; TFP (□), 13,200; (*B*) control (○), 16,200; FCCP (△), 8,820. (*C*) Effect of TRH on cellular ^{45}Ca pools. TRH (1 μM) treatment was for 2 min at 37°C under $^{45}Ca^{2+}$ steady-state conditions. Total cell $^{45}Ca^{2+}$ (cpm): control (○), 17,600; TRH (△), 13,600. The ordinate represents $^{45}Ca^{2+}$ released from labeled cells by digitonin permeabilization.

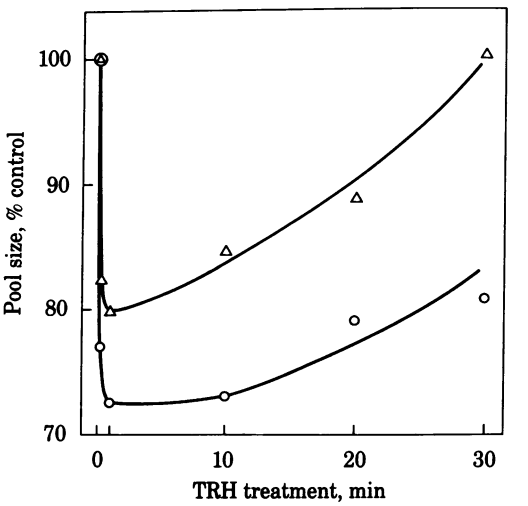

FIG. 3. Time course of 1 μM TRH-induced depletion of cellular $^{45}Ca^{2+}$ pools under $^{45}Ca^{2+}$ steady-state conditions at 37°C. Treatment was terminated by addition of 3 vol of ice-cold $^{45}Ca^{2+}$ labeling medium and immediate centrifugation. ○, Pool I; △, pool II.

induced by FCCP (Table 2, Exp. 1), TFP, and calcium antagonists (not shown) was significant only after 2 min of treatment. TRH-induced pool depletion was followed by a recovery phase (Fig. 3). Mitochondrial $^{45}Ca^{2+}$ content returned to control levels by 30 min after TRH addition. Recovery of ER $^{45}Ca^{2+}$ was slower; control levels were not achieved even by 60 min (not shown).

Previous treatment of steady-state $^{45}Ca^{2+}$-loaded cells with calcium uptake inhibitors (D600, nifedipine) did not alter the ability of TRH to promote net efflux of cellular $^{45}Ca^{2+}$ pools (not shown). However, treatment with FCCP or valinomycin prior to TRH addition diminished TRH-induced $^{45}Ca^{2+}$ depletion (Table 3). Valinomycin blunted TRH action on both pools while previous treatment with FCCP blocked only mitochondrial depletion by TRH.

DISCUSSION

Digitonin permeabilization has been used previously to selectively measure cytosolic and mitochondrial compartments of various metabolites (18). In addition, cells permeabilized with digitonin at low concentrations have been used to study Ca^{2+} uptake by intracellular organelles (8–10). These studies and the distribution of cholesterol in cellular membranes (7) suggested

Table 3. Effects of inhibitors on TRH-induced depletion of cellular $^{45}Ca^{2+}$

Exp.	Treatment	Pool I cpm	Pool I %	Pool II cpm	Pool II %	Total cpm
1	None (control)	8,800	100	6,600	100	17,600
	TRH	6,500	74	4,700	71	13,600
	FCCP	7,400	84	2,150	32.5	11,400
	TRH/FCCP	4,800	54.5	2,100	32	8,350
2	None (control)	10,200	100	6,050	100	18,500
	TRH	7,400	72.5	5,350	88	14,600
	Valinomycin	7,400	72.5	2,850	47	12,000
	TRH/valinomycin	6,800	67	2,500	41	11,100

Steady-state $^{45}Ca^{2+}$-labeled cells were incubated with various agents and $^{45}Ca^{2+}$ contents of pools I and II were determined. Concentrations and treatment times were TRH, 1 μM for 2 min; FCCP, 5 μM for 15 min; valinomycin, 10 μM for 15 min. TRH was added after 13 min of incubation with inhibitors.

the application of digitonin permeabilization for determining the *in situ* distribution of Ca^{2+} in ER and mitochondria, the two major Ca^{2+}-sequestering organelles (16). In this paper, we have reported that $^{45}Ca^{2+}$-labeled GH_3 cells contain two pools of Ca^{2+} (I and II) that have discrete digitonin sensitivities. Enzymes characteristic of ER and mitochondrial matrix were rendered accessible to substrates at detergent concentrations well resolved from each other and approximating the digitonin sensitivities of Ca^{2+} pool I and II, respectively.

Inhibitors whose actions are both specific and well understood are not available for studies of Ca^{2+} metabolism. Nonetheless, studies with several inhibitors indicated at least partial selectivity in effects on pool I or II Ca^{2+}, implying that these pools have characteristics distinct from one another. The monovalent ionophores used as mitochondrial uncouplers principally affected pool II, with a smaller effect on pool I. The latter could be the indirect result of mitochondrial inhibition (e.g., altered ATP levels) or, alternatively, the result of direct effects on ER to release Ca^{2+} as suggested by Shoshan *et al.* for sarcoplasmic reticulum (19). Trifluoperazine acted selectively to deplete Ca^{2+} from pool I. Ca^{2+} ATPases have been correlated with Ca^{2+} pump activity (20) and these may be TFP sensitive (21). A TFP-sensitive Ca^{2+} ATPase activity is present in GH_3 microsomes and ATP-dependent Ca^{2+} uptake into pool I in GH_3 cells permeabilized with 0.003% digitonin is TFP sensitive as well (unpublished results). These results are consistent with an assignment of pool I with ER.

In these studies, we have shown that TRH treatment of cells equilibrated with $^{45}Ca^{2+}$ led to rapid loss of $^{45}Ca^{2+}$ from both ER and mitochondria. Since our experiments were carried out in the continued presence of $^{45}Ca^{2+}$, loss of $^{45}Ca^{2+}$ represents net loss of cellúlar Ca^{2+}. The fact that prior treatment with either valinomycin or FCCP blocked TRH-promoted $^{45}Ca^{2+}$ pool depletion is consistent with the interpretation that TRH acts on the same Ca^{2+} pools (ER and mitochondria) as these agents. Our findings may be compared with several recent studies that have examined the effect of TRH on GH cellular calcium. Tan and Tashjian (2), using $^{45}Ca^{2+}$ steady-state-labeled monolayer cultures, found that TRH depleted 50% of cell-associated $^{45}Ca^{2+}$ by 1 min. Although these authors postulated, on the basis of indirect studies, that the Ca^{2+} mobilized by TRH was surface-associated rather than intracellular, our studies show that TRH mobilizes Ca^{2+} from organelles on a similar time scale. In contrast, Moriarty and Leuschen (4), using atomic absorption spectrophotometry, reported no effect of a 20-min TRH treatment on cellular calcium. However, this result may be due to the relatively long treatment time used. Rebecchi *et al.* (5) recently communicated that brief TRH treatment (1.5 min) led to a 25% loss of cell calcium as measured with arsenazo III. Taken together, these results are consistent with the transient nature of the TRH effect reported here.

TRH-stimulated mobilization of Ca^{2+} from organelles might result in at least transiently elevated cytosolic Ca^{2+} levels. Recent studies in our laboratory have shown that TRH transiently stimulates the Ca^{2+}-dependent phosphorylation of a major cytosolic phosphoprotein (22). Stimulated phosphorylation is detected at 15 sec, a time scale consistent with the organelle Ca^{2+} efflux described here. Although our data do not exclude the possibility that TRH may stimulate influx of extracellular Ca^{2+}, the observation that calcium channel blockers fail to inhibit TRH-induced Ca^{2+} pool depletion is consistent with other studies that indicated a nonobligatory role for Ca^{2+} influx in TRH stimulation of Ca^{2+} efflux and hormone secretion (3–5).

The rapid onset of TRH-induced Ca^{2+} efflux reported here (<10 sec) as well as the cell surface localization of TRH receptors shown previously (23) motivate the search for an intracellular mediator that can couple TRH receptor occupancy with mobilization of intracellular Ca^{2+}. The rapid stimulation of phosphatidylinositol turnover promoted by TRH in GH_3 cells was recently reported (ref. 24; see also refs. 25, 26). Such effects on phospholipid metabolism have been suggested as a general mechanism underlying hormonal effects on Ca^{2+} translocation (27). To test such a model for TRH action, it will be necessary to identify the mechanism by which Ca^{2+} efflux from ER and mitochondria is concomitantly elicited.

Finally, the digitonin-permeabilization method described here should be generally applicable to studies of hormone-stimulated Ca^{2+} translocation in suspension cells, especially where the limitations inherent in homogenization and fractionation preclude an unambiguous assessment of *in situ* Ca^{2+} distribution.

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