Functional Identification and Quantitation of Three Intracellular Calcium Pools in GH_4C_1 Cells: Evidence That the Caffeine-Responsive Pool Is Coupled to a Thapsigargin-Resistant, ATP-Dependent Process†

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ABSTRACT: We have recently reported that basal oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in intact GH_4C_1 cells are dependent on a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. The purpose of the present study was to characterize the uptake and release pathways for intracellular Ca^{2+} in GH_4C_1 cells. We have used both permeabilized cells and microsome preparations, and we have monitored the change in ambient $[Ca^{2+}]$ using the dye, fluo 3. We find that there are three functionally distinct nonmitochondrial, ATP-dependent Ca^{2+} pools in these cells: Pool 1 is an inositol 1,4,5-trisphosphate ($InsP_3$) responsive pool which is filled by a thapsigargin (Tg) sensitive Ca^{2+}-ATPase; pool 2 is a second Tg-sensitive pool which is $InsP_3$-unresponsive; and pool 3 is a Tg-resistant pool, at least a part of which has the characteristics of a CICR mechanism. These pools were established as follows. Tg caused additional Ca^{2+} release after maximum release was induced by prior addition of $InsP_3$. In contrast, the $InsP_3$ response was abolished in a time-dependent manner after pretreatment with Tg. Ambient Ca^{2+}, added after maximum blockade by Tg, was still able to be sequestered. Ionomycin released Ca^{2+} even after maximum depletion by Tg. The ionomycin-releasable pool remaining after Tg treatment was also ATP-dependent, because this pool was completely discharged by ATP-depletion. Two additional inhibitors of intracellular Ca^{2+}-ATPases, 2,4-di(*tert*-butyl)hydroquinone and cyclopiazonic acid, which are structurally unrelated to Tg, acted on the same targets as Tg. To estimate accurately the distribution of Ca^{2+} among compartments, we developed a new approach based on the analysis of two equilibrium states of Ca^{2+} distribution. Using this method, the size of the Tg-sensitive pools (pools 1 + 2) was estimated to be 63 ± 2.5% of total non-mitochondrial Ca^{2+} in our preparation. Caffeine induced Ca^{2+} release, and this action was observed even after complete depletion of the Tg-sensitive pool, indicating that pool 3 had the characteristics of a CICR compartment. Because caffeine pretreatment caused an increase in the size of pools 1 + 2, the CICR-like mechanism operated primarily on pool 3. These new results strengthen our model, in which a distinct CICR-like pool is responsible for Ca^{2+} oscillations in GH_4C_1 cells, and also support the concept that different types of Ca^{2+} efflux pathways occur in Ca^{2+}-storing nonmitochondrial organelles containing different types of Ca^{2+}-ATPases.

A variety of signal transduction processes, such as those initiated by hormones, neurotransmitters, growth factors, and fertilization, are mediated by increases in cytosolic free calcium concentrations ($[Ca^{2+}]_i$)[1] (Rasmussen & Barrett, 1984; Berridge & Irvine, 1989; Berridge, 1993). After the initial Ca^{2+} increase, which is often mediated by inositol 1,4,5-trisphosphate ($InsP_3$) (Berridge & Irvine, 1989; Berridge, 1993), repetitive spikes in intracellular Ca^{2+} have been observed in a variety of cells (Berridge & Galione, 1988; Berridge, 1990; Meyer & Stryer, 1991; Stojilkovic & Catt, 1992). Although there is little direct experimental evidence for the functional significance of these oscillations, the concept that they constitute an intracellular digital regulatory signal derived from extracellular analogue information has arisen (Berridge & Galione, 1988; Meyer & Stryer, 1991).

Several mechanistic models have been proposed to explain the oscillatory behavior; these include $InsP_3$–Ca^{2+} cross-coupling (Meyer & Stryer, 1991) and Ca^{2+}-sensitized, $InsP_3$-induced Ca^{2+} release (CSIICR) (Iino, 1990; Finch et al., 1991; Miyazaki et al., 1992; DeLisle & Welsh, 1992). These models are based on a single type of intracellular Ca^{2+} pool. However, other studies have revealed that there are two Ca^{2+}-storing intracellular pools in certain cell types which appear to be responsible for Ca^{2+} oscillations, namely, an $InsP_3$-sensitive pool and an $InsP_3$-resistant pool. In some cell types (Busa et al., 1985; Iino et al., 1988), the latter pool has the characteristics of a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism, first described in the sarcoplasmic reticulum (SR) of skeletal muscle cells (Endo, 1977).

In the CICR model (Berridge & Galione, 1988; Berridge, 1990), the initial rise in $[Ca^{2+}]_i$ evoked by either $InsP_3$ or entry of extracellular Ca^{2+} triggers Ca^{2+} release from an

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[1] Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+} release; CSIICR, Ca^{2+}-sensitized, $InsP_3$-induced Ca^{2+} release; $InsP_3$, inositol 1,4,5-trisphosphate; Tg, thapsigargin; PMSF, phenylmethanesulfonyl fluoride; HKG, hexokinase + glucose; BHQ, 2,4-di(*tert*-butyl)hydroquinone; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; SERCA, sarcoendoplasmic type Ca^{2+}-ATPase; SR sarcoplasmic reticulum.

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adjacent intracellular Ca^{2+} pool, and this newly released Ca^{2+} causes additional Ca^{2+} release, and so on, to generate propagation of a Ca^{2+} wave (Berridge, 1993). The plant alkaloid ryanodine and caffeine modulate CICR in muscle cells (Endo, 1977), and the cloned receptor for ryanodine (Takeshima et al., 1989) has been shown to be a Ca^{2+} channel.

Rat pituitary GH_4C_1 cells, which secrete prolactin in response to thyrotropin-releasing hormone (TRH), have been used extensively as a model to investigate the relationships among agonist action, intracellular Ca^{2+} signaling, and hormone secretion (Albert & Tashjian, 1984, 1986; Tashjian et al., 1987; Law et al., 1990). Recently, we have shown that intact GH_4C_1 cells exhibit basal Ca^{2+} oscillations that are caused by the release of intracellular Ca^{2+} by a caffeine-sensitive CICR-like mechanism (Wagner et al., 1993). Interestingly, we also found that thapsigargin (Tg), an intracellular Ca^{2+}-ATPase inhibitor, does not abolish basal intracellular Ca^{2+} oscillations in GH_4C_1 cells, indicating that a Tg-resistant pool is responsible for the oscillations (Wagner et al., 1993).

The goal of the present study was to characterize the uptake and release pathways for intracellular Ca^{2+} in GH_4C_1 cells. Specifically, we have used both permeabilized cells and a microsome preparation to confirm the presence of a caffeine-sensitive CICR-like pool and to dissect the interrelationships among Tg sensitivity, $InsP_3$ response, and the CICR-like mechanism in these cells. In addition, we have developed a new method for the determination of relative Ca^{2+} pool sizes on the basis of the analysis of two equilibrium states for Ca^{2+}. Using these approaches, we have identified three functionally distinct ATP-dependent, nonmitochondrial Ca^{2+} pools in GH_4C_1 cells, and we have shown that the CICR-like mechanism in GH_4C_1 cells is coupled to a Tg-resistant, ATP-dependent pool.

MATERIALS AND METHODS

Materials. Fluo 3 pentaammonium salt and DTPA Polymetal Sponge B (DTPA polyacrylamide) were purchased from Molecular Probes Inc. (Eugene, OR). Ionomycin was from Calbiochem Corp. (La Jolla, CA), thapsigargin was from LC Services Corp. (Woburn, MA), MicroBCA (protein assay reagent kit) was from Pierce (Rockford, IL), and Chelex 100 resin was from Bio-Rad (Richmond, CA). Other chemicals were reagent grade and were from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted.

In general, buffers were treated at least once with Chelex 100 resin. For some experiments, buffers were treated further, where indicated, with Polymetal Sponge B (Meyer et al., 1990) to remove excess Ca^{2+} or heavy metal ions from solution. Free ion concentrations for Ca^{2+} and Mg^{2+} were calculated by the computer program Chelator ver. 1.0 (Schoenmakers et al., 1992). Statistical analyses (student's *t*-test, linear regression, and nonlinear curve fitting) were performed using Sigma Plot ver. 4.0 (Jandel Scientific, Corte Madera, CA). Each figure presents results which are representative of at least five independent experiments.

Cell Culture. GH_4C_1 cells were grown in either plastic culture dishes or roller bottles (Tashjian, 1979) in Ham's F10 medium supplemented with 15% (v/v) horse serum and 2.5% (v/v) fetal bovine serum ($F10^+$) at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Cultures were fed with fresh medium every 3–4 days.

Preparation of Permeabilized GH_4C_1 Cells. Permeabilization of GH_4C_1 cells was performed with digitonin as described previously (Koshiyama & Tashjian, 1991; Biden et al., 1986) with minor modifications. Briefly, cells [(1.5–2) $\times 10^7$ cells for each measurement] were detached from the culture surface using HEPES-buffered salt solution (HBSS: 118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, and 20 mM HEPES, pH 7.2) containing 0.02% EDTA and resuspended with 20 mL of $F10^+$ in a 50-mL conical tube. After incubation with gentle agitation at 37 °C for 1–3 h, cells were collected by centrifugation, washed once with 12 mL of HBSS containing 1 mM EGTA (low Ca-HBSS) and once with 12 mL of intracellular-like solution A (ISA: 125 mM KCl, 25 mM HEPES, 2 mM KH_2PO_4, 0.25 mM EGTA, 1 mg/mL bovine albumin, and 0.5 mM Mg-ATP), and resuspended in 4 mL of the same solution. After incubation at 37 °C for 5 min, 0.001–0.0015 vol of a 5 mM digitonin solution was added to the cells at 37 °C. Exactly 4 min later, 3× vol of ice-cold intracellular-like solution B (ISB: ISA without EGTA) was added, and the cells were immediately centrifuged at 160*g* for 3 min. After one wash with ISB and collection by centrifugation at 80*g* for 5 min, the permeabilized cells were resuspended in ISB containing antimycin A (0.2 μM) and oligomycin (2 μg/mL) and placed on ice until use. Cells treated in this way were 94 ± 1% permeabilized as assessed by trypan blue uptake (Koshiyama & Tashjian, 1991).

Microsome Preparation from GH_4C_1 Cells. The microsome fraction was prepared by hypotonic lysis as described previously (Ghosh et al., 1988), with some modifications. In brief, GH_4C_1 cells were cultured and harvested exactly as described above. Harvested cells were washed once with low Ca-HBSS, once with ice-cold ISA, and once with ice-cold ISB. Washed cells were suspended in cold lysing buffer [25 mM sucrose and 10 mM HEPES/KOH (pH 7.0) containing 25 μg/mL leupeptin, 20 μg/mL aprotinin, 100 μg/mL soybean trypsin inhibitor, 1 mM DTT, 100 μM PMSF, and 0.5 mM Mg-ATP] and were stirred gently at 4 °C for 5–10 min. Swollen cells were then homogenized on ice using a Dounce-type glass homogenizer with pestle A for 30–40 strokes. The homogenate was diluted with an equal volume of 2× concentrated intracellular-like solution C (ISC: ISB without bovine albumin, but with 1 mM DTT, 100 μM PMSF, and 0.5 mM Mg-ATP) and was immediately centrifuged at 80*g* for 4 min. The supernatant was then centrifuged at 7000*g* for 13 min and centrifuged again at 35000*g* for 30 min at 4 °C. The pellet was gently dispersed with the same glass homogenizer for 8 strokes and resuspended at 1 mg of protein/mL in ISC. The protein concentration was measured using the Pierce MicroBCA method (protein assay reagent kit).

Measurement of Ca^{2+} Release and Sequestration. For measurement of Ca^{2+} release and sequestration, permeabilized cells were suspended in 3 mL of ISB containing 2 μM fluo 3, an ATP-regenerating system (10 units/mL creatine kinase, 10 mM sodium phosphocreatine, and 1 mM K^+ ATP), antimycin A (0.2 μM), and oligomycin (2 μg/mL), and placed in a stirred cuvette at 37 °C during the experiment. Permeabilized cells preincubated for 2 h under these conditions had essentially the same response to test compounds as freshly permeabilized cells without preincubation.

For measurement of Ca^{2+} release and sequestration in GH_4C_1 microsomes, the microsome fraction was suspended at 600 μg of protein/mL in ISC containing 2 μM fluo 3 and the ATP-regenerating system as described above, and the suspension was placed in a stirred cuvette at 37 °C. Microsomes preincubated for 2.5 h under these conditions gave essentially the same response to the test compounds as fresh microsomes without preincubation.

Fluorescence was measured with a Spex Fluorog F111A spectrofluorometer (excitation wavelength, 490 nm; emission wavelength, 535 nm). Each test compound was added to the reaction mixture from a 200–1000× concentrated stock solution.

The calibration of $[Ca^{2+}]$ was performed by two different methods. In method A, at the end of each trace (after 200 nM ionomycin was added), 2 mM $CaCl_2$ and 10 mM $MnCl_2$ were added sequentially, and F_{max} and F_{Mn} were obtained. $[Ca^{2+}]$ values were calculated by the following equation: $[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$, where K_d = 400 nM and $F_{min} = 1.25F_{Mn} - 0.25F_{max}$. In method B, at the end of each run (after ionomycin), 1 mM EGTA/Tris (pH 7.2) was added, as were increments of $CaCl_2$, sequentially (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 mM as final concentrations), and the F value after each addition was obtained. The concentration of free Ca^{2+} after each addition was calculated to be, respectively, 70, 157, 268, 416, 622, 927, and 1422 nM (in the presence of 1.5 mM ATP). The least-squares-fitted hyperbolic curve for F vs [free Ca^{2+}] was used to calculate $[Ca^{2+}]$ at points between actual additions. Using buffer alone, Ca^{2+}-depleted permeabilized cells, and Ca^{2+}-depleted microsomes (see below), both calibration methods were tested. When buffer was used without cells, the results from each method corresponded well, and both were in good agreement with $[Ca^{2+}]$ calculated from Ca^{2+}/EGTA buffer combinations within the range 70–1422 nM. In microsome suspensions, however, the results from method A were aberrant, whereas those from method B were accurate in comparison with the Ca^{2+}/EGTA buffer. The perturbation was presumably due to the presence of DTT in the microsome buffer, which caused a major shift in fluorescence in the crucial measurement range without affecting F_{max} and F_{min}. Therefore, method A was used for the calibration of permeabilized cell experiments, and method B was used for microsome experiments.

Control Experiments. Because some of the chemicals used in this study could cause an artifactual change in the fluorescence of the Ca^{2+} indicator dye, which might appear as Ca^{2+} release or sequestration, we measured the effects of test materials on fluo 3 fluorescence in Ca^{2+}-depleted cells and Ca^{2+}-depleted microsomes.

To prepare Ca^{2+}-depleted cells, permeabilized cells were treated with 200 nM ionomycin, antimycin (0.2 μM), and oligomycin (2 μg/mL) to deplete all intracellular Ca^{2+} pools. After incubation for 20 min at 37 °C, cells were washed once with ISB and resuspended with the ISB (pretreated with Polymetal Sponge B), the test materials were added, and the change in fluorescence was measured in the presence of fluo 3 (2 μM).

To prepare Ca^{2+}-depleted microsomes, the microsome suspension was treated with 200 nM ionomycin to deplete all intravesicular Ca^{2+}. After incubation for 20 min at 37 °C, 3 mL of the microsome suspension was mixed with 100 μL of Polymetal Sponge B, and the mixture was gently agitated for 5 min. After centrifugation at 80g for 2 min, the supernatant, which had low ambient $[Ca^{2+}]$ similar to the basal level in undepleted microsomes, was used as the Ca^{2+}-depleted microsome preparation. Direct effects of test materials on the fluorescence of fluo 3 (2 μM) were then examined.

These two Ca^{2+}-depleted preparations were also used to test the $[Ca^{2+}]$ calibration as described in the previous section.

RESULTS

Because permeabilized cells are closer to intact cells than are microsomes, we first performed experiments with permeabilized cells. Subsequently, microsomes were used because of improved precision, which enabled the development of a new estimation method for the determination of Ca^{2+} pool sizes. No major qualitative differences were noted between the results obtained in permeabilized cells and those from microsome preparations.

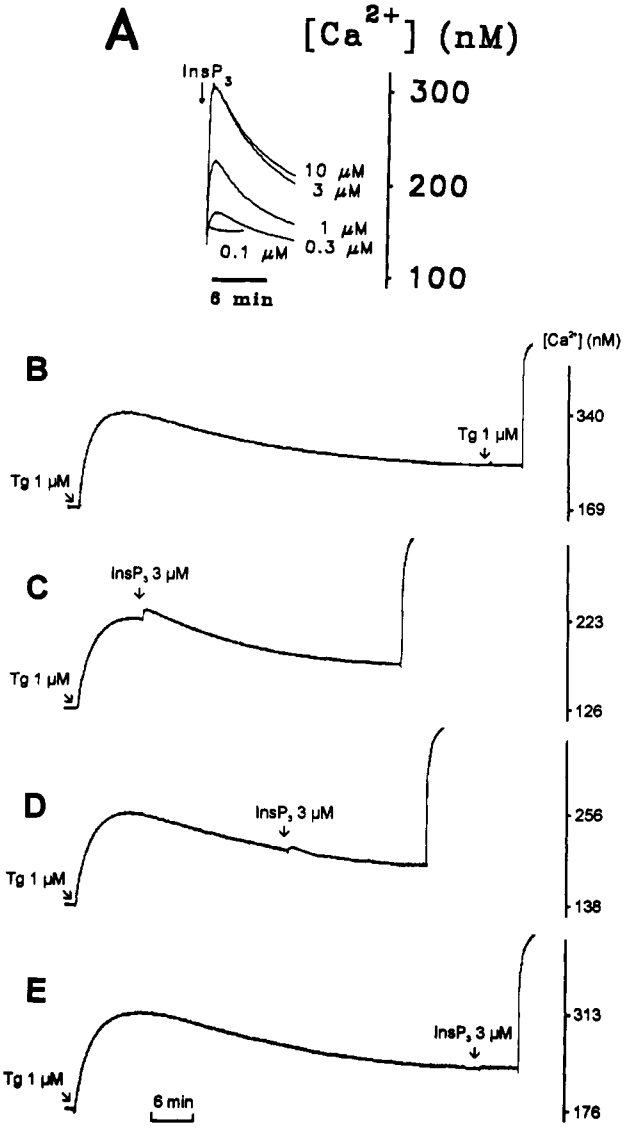

FIGURE 1: Actions of Tg and subsequent $InsP_3$ on Ca^{2+} release in permeabilized GH_4C_1 cells. Digitonin-permeabilized GH_4C_1 cells were suspended in ISC containing an ATP-regenerating system, oligomycin, and antimycin, and the ambient $[Ca^{2+}]$ was measured with 2 μM fluo 3. (A) $InsP_3$ caused a rapid (approximately 15–20 s to peak) concentration-dependent release of Ca^{2+}, which was followed by Ca^{2+} sequestration. (B) Tg (1 μM) caused a gradual release (10–15 min to peak) of Ca^{2+} followed by slow uptake. (C and D) $InsP_3$ (3 μM) added after Tg (C, 10 min; D, 30 min after Tg) could still release a small amount of Ca^{2+}. (E) $InsP_3$ added after longer treatment with Tg (55 min) did not cause Ca^{2+} release. Intracellular pools were still filled with Ca^{2+}, as shown by the abruptly rising curve induced by ionomycin at the end of each trace.

The $InsP_3$-Responsive Ca^{2+} Pool Is Tg-Sensitive in Permeabilized GH_4C_1 Cells. $InsP_3$ caused a concentration-dependent rapid Ca^{2+} release followed by resequestration in permeabilized cells (Figure 1A). At 3 μM $InsP_3$, the effect was maximal. The acute response of permeabilized cells to $InsP_3$ after preincubation for 120 min at 37 °C was essentially the same as that shown in Figure 1A (data not shown). In contrast, as shown in Figure 1B, 1 μM Tg, a supramaximum concentration (Bian et al., 1991; Ely et al., 1991; Verma et

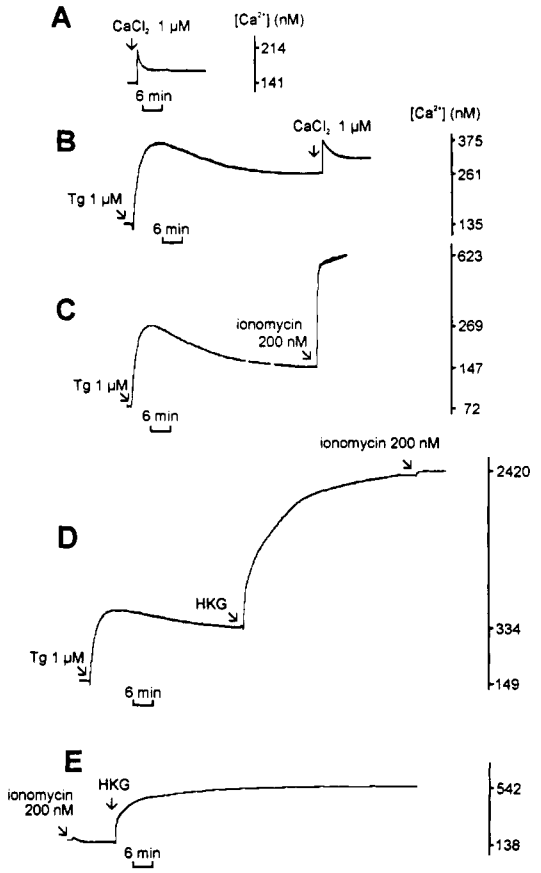

FIGURE 2: Actions of Tg and ATP depletion on Ca^{2+} release in permeabilized GH_4C_1 cells. For A–D, experimental conditions were as described in the legend for Figure 1. For E, Ca^{2+}-depleted cells were prepared (see Materials and Methods) from permeabilized cells. (A) Ca^{2+} sequestration after the addition of 1 μM $CaCl_2$. (B) Tg (1 μM) induced Ca^{2+} release. $CaCl_2$ added 30 min after Tg was resequestered. (C) Ionomycin (200 nM) released a large amount of Ca^{2+} after the maximum effect of Tg. (D) The ionomycin response was essentially abolished by prior treatment with hexokinase (6 units/mL) and glucose (25 mM) (HKG). (E) The addition of HKG to Ca^{2+}-depleted, permeabilized cells caused a transient change in $[Ca^{2+}]$, presumably because HKG-induced ATP depletion resulted in the loss of a major Ca^{2+} chelator in the incubation medium.

al., 1990), caused a much slower Ca^{2+} release followed by slow uptake. The decrease in $[Ca^{2+}]$ beginning 10–15 min after addition of Tg was not due to loss of the activity of Tg, because the addition of fresh Tg (1 μM) 55 min after the first addition had no effect (Figure 1B). The action of $InsP_3$ (3 μM) was attenuated progressively by preincubation with Tg in a time-dependent manner (Figure 1C–E). As shown in Figure 1C,D, $InsP_3$ added 10 and 30 min after Tg caused only a small and progressively smaller Ca^{2+} release, respectively, whereas $InsP_3$ added 60 min or longer after Tg did not release Ca^{2+} at all (Figure 1E). These results indicate that the $InsP_3$-responsive pool in GH_4C_1 cells is dependent on a Tg-sensitive mechanism for filling and that it takes 30 min or longer for Tg to deplete this Tg-sensitive pool completely in permeabilized cells.

A Tg-Resistant, but ATP-Dependent Ca^{2+} Pool Is Present in Permeabilized GH_4C_1 Cells. Ca^{2+} added to the permeabilized cell preparation was sequestered as shown in Figure 2A. Interestingly, Ca^{2+} added after prolonged incubation (>50 min) with Tg was still able to be sequestered as shown in Figure 2B. Furthermore, ionomycin (200 nM) had marked Ca^{2+}-releasing activity after prolonged preincubation with Tg (Figure 2C). These results indicate that there is a compartment for Ca^{2+} uptake that is insensitive to Tg. To

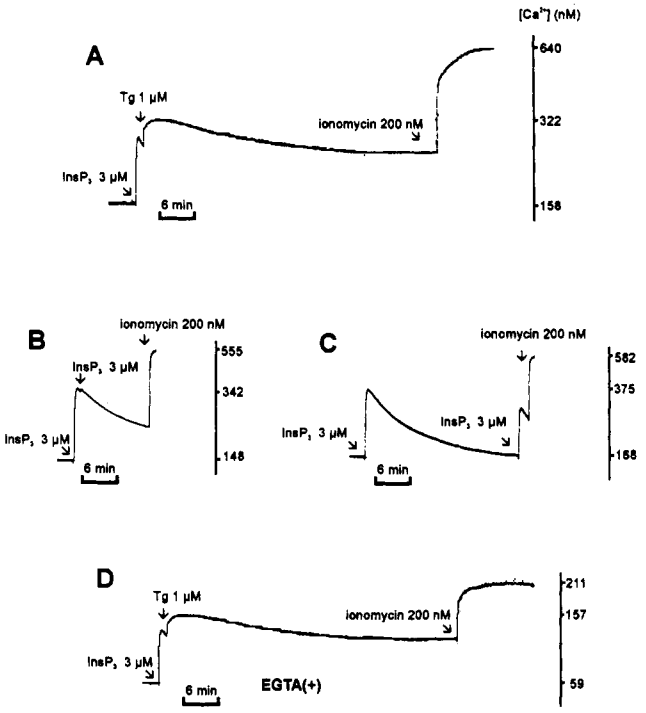

FIGURE 3: Actions of $InsP_3$ and subsequent Tg on Ca^{2+} release from permeabilized GH_4C_1 cells. Experimental conditions were as described in the legend for Figure 1. (A) $InsP_3$ (3 μM) caused a rapid Ca^{2+} release, and Tg (1 μM) added at the peak of $InsP_3$ action caused further Ca^{2+} release. (B and C) A second addition of $InsP_3$ was made at 1 min (B) and at 25 min (C) after the first addition. C shows that the $InsP_3$-responsive pool can be essentially refilled within 25 min. (D) To maintain the ambient $[Ca^{2+}]$ below 300 nM, 20 μM EGTA/Tris (pH 7.0) was added 10 min before the trace shown. At this low $[Ca^{2+}]$, $InsP_3$ again released only a portion of the Tg-sensitive pool.

test whether this Tg-resistant pool was ATP-dependent, we used hexokinase and glucose (HKG) to deplete ATP. This procedure caused a large increase in $[Ca^{2+}]$, and no subsequent release was induced by ionomycin (Figure 2D). Because depletion of ATP alone could cause an increase in $[Ca^{2+}]$ by eliminating the chelation of Ca^{2+} by ATP, we examined the effect of HKG in Ca^{2+}-depleted, permeabilized cells (see Materials and Methods). As anticipated, the non-Ca^{2+} pool effect of HKG was confirmed by the increase in $[Ca^{2+}]$ observed in response to HKG in the absence of stored Ca^{2+} in ATP-dependent vesicular pools (Figure 2E). However, the lack of ionomycin-induced Ca^{2+} release after HKG addition (Figure 2D) clearly demonstrates that the Tg-resistant pool is ATP-dependent.

Action of Tg or $InsP_3$ (Second Addition) after Preincubation with $InsP_3$. Tg (1 μM) added at the peak of the $InsP_3$-induced Ca^{2+} response could release additional Ca^{2+} (Figure 3A). A second addition of $InsP_3$, 1 min after the first addition, caused no further release of Ca^{2+} (Figure 3B), whereas a second addition 25 min after the first addition caused Ca^{2+} release that was nearly comparable to that induced by the first addition (Figure 3C). These results indicate that, in contrast to persistent inhibition of Ca^{2+} uptake caused by Tg, the $InsP_3$-sensitive pool was largely refilled within the time course observed, presumably because of the degradation of $InsP_3$. It could be argued that $InsP_3$ did not release all of the Ca^{2+} from the $InsP_3$ receptor-containing pool, because the elevated ambient $[Ca^{2+}]$ would close the $InsP_3$-responsive Ca^{2+} channel (Finch et al., 1991; Iino, 1990; Parker & Ivorra, 1990; Bezprozvanny et al., 1991). However, this was not the case for GH_4C_1 cells. By the addition of 20 μM EGTA, ambient $[Ca^{2+}]$ was kept below 300 nM (as shown in Figure 3D), a

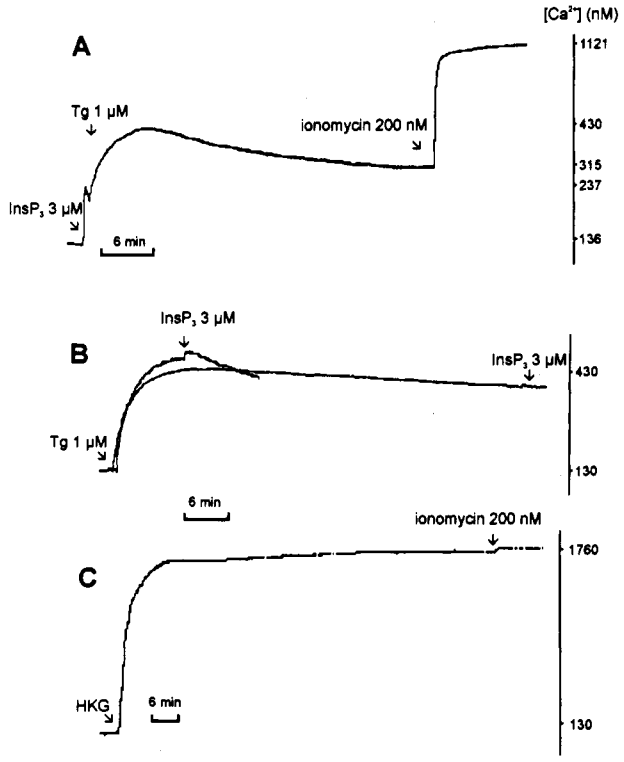

FIGURE 4: Ca^{2+} release and resequestration in GH_4C_1 microsomes. Microsomes from GH_4C_1 cells were suspended in ISC buffer containing an ATP-regenerating system, and the change in $[Ca^{2+}]$ was measured with 2 μM fluo 3. (A) $InsP_3$ (3 μM) caused rapid (approximately 12 s to peak) release of Ca^{2+} followed by sequestration. Tg (1 μM) added at the peak of the $InsP_3$ action caused further Ca^{2+} release. (B) Tg (1 μM) caused a gradual release (10–20 min to peak) of Ca^{2+}. $InsP_3$ (3 μM) added at the peak (10 min) of the Tg-induced effect released a small amount of Ca^{2+}, while $InsP_3$ added after longer pretreatment (>50 min) with Tg did not cause Ca^{2+} release. (C) The ionomycin-releasable Ca^{2+} in microsomes was essentially abolished by prior treatment with HKG.

concentration which does not affect the open state of the $InsP_3$-responsive Ca^{2+} channel (Finch et al., 1991; Iino, 1990; Parker & Ivorra, 1990). Even at this low ambient $[Ca^{2+}]$, maximum $InsP_3$ did not prevent Tg-induced Ca^{2+} release (Figure 3D). Because Tg, which releases Ca^{2+} much more slowly than $InsP_3$, can release additional Ca^{2+} when added at the peak of the $InsP_3$-induced $[Ca^{2+}]$ rise (Figure 3A), it is clear that there is another compartment for Ca^{2+} storage that is Tg-sensitive but not responsive to $InsP_3$.

Therefore, we conclude that there are at least three functionally distinct ATP-dependent, nonmitochondrial Ca^{2+} pools in permeabilized GH_4C_1 cells: pool 1, an $InsP_3$-responsive, Tg-sensitive pool; pool 2, an $InsP_3$-unresponsive, Tg-sensitive pool; and pool 3, a Tg-resistant pool. We also demonstrate that all of the ionomycin-releasable Ca^{2+} in these cells, which is presumably endoplasmic reticulum (ER) related (Rossier & Putney, 1991), is maintained by ATP-dependent mechanisms.

Comparison of the Results of Microsome Preparation and Permeabilized Cells. The microsome fraction will be the starting material for experiments to separate physically each Ca^{2+} pool, the next logical step in these long-term studies. Therefore, we compared results using microsomes with those obtained with permeabilized cells. Because results using the microsome preparation were not affected by preincubation with antimycin and oligomycin (data not shown), functional contamination with mitochondria was considered to be insignificant. As shown in Figure 4, the key findings described for permeabilized cells were also observed in microsome preparations. $InsP_3$ caused rapid Ca^{2+} release, and subsequent Tg caused a gradual, but further, Ca^{2+} release (Figure 4A). The action of $InsP_3$ on Ca^{2+} release was maximal at 3 μM (data not shown), which was similar to the value in permeabilized cells. Pretreatment of microsomes with Tg for longer than 30 min totally abolished the $InsP_3$ response (Figure 4B). Ionomycin was able to release Ca^{2+} even after the maximum effect of Tg (Figure 4A), but this Tg-resistant pool was ATP-dependent as defined by the action of HKG, as shown in Figure 4C. Thus, permeabilized cells and microsomes gave similar results. Because of ease, reproducibility, and ability to remove cytosolic factors that might affect the following analysis, we used the microsome preparation for subsequent experiments.

Actions of Ca^{2+}-ATPase Inhibitors on Microsomes from GH_4C_1 Cells. Recently, two non-Tg inhibitors of intracellular Ca^{2+}-ATPases, BHQ and CPA, were shown to abolish agonist-induced increases in $[Ca^{2+}]_i$ (Mason et al., 1991; Demaurex et al., 1992; Muallem et al., 1991; Kass et al., 1989; Schilling et al., 1992). They have been studied in intact cells (Demaurex et al., 1992), and the intracellular target has not been demonstrated. Because BHQ and CPA are structurally unrelated to Tg, we investigated whether they could inhibit the Tg-resistant ATP-dependent uptake of Ca^{2+}. As shown in Figure 5A, BHQ alone caused Ca^{2+} discharge, with a time course similar to that of Tg. After microsomes were incubated with 10 μM BHQ for 20 min, Tg had no further effect on Ca^{2+} release (Figure 5A). Likewise, BHQ added 30 min after a maximum concentration of Tg did not cause further Ca^{2+} release (Figure 5B). CPA alone also caused Ca^{2+} discharge (Figure 5C). CPA added after Tg (Figure 5D) and Tg added after CPA (Figure 5C) did not release additional Ca^{2+}. These results demonstrate that the intracellular targets, presumably Ca^{2+}-ATPases, in GH_4C_1 cell microsomes for BHQ and CPA are the same as those for Tg. These results also indicate that the effect of Tg is complete, persistent, and not desensitized. If the actions of Tg were incomplete or desensitized, it is likely that additional ATPase inhibition would have been observed with BHQ or CPA, which are structurally distinct from Tg but act on the same target.

Estimation of the Size of the Tg-Sensitive Ca^{2+} Pool in GH_4C_1 Cells. It is essential to define quantitatively the intracellular distribution of Ca^{2+} in order to understand intracellular Ca^{2+} homeostasis. However, it has been difficult to do so in intact cells, because any increase in $[Ca^{2+}]_i$ may simultaneously activate Ca^{2+}-handling mechanisms such as Ca^{2+}-ATPases (either on the ER or plasma membrane), the Na^+–Ca^{2+} exchanger, mitochondrial uptake, and so on. It has been argued that the maximum $\Delta[Ca^{2+}]_i$ in intact cells or $\Delta[Ca^{2+}]$ in permeabilized cells induced by test materials gives an estimate of the size of the sequestered intracellular Ca^{2+} pool which is sensitive to that agent (Ely et al., 1991; Law et al., 1990; Takemura et al., 1989). However, it is likely that this approach underestimates the size of any particular pool (such as the Tg-sensitive pool), because Ca^{2+} released from a pool may be resequestered during the time course of the measurement. In the present study, we have developed a new method to estimate more accurately the distribution of Ca^{2+} among intracellular pools.

Equilibration of Ca^{2+} across the membrane of intracellular pools can be generally described as follows (Jencks, 1989):

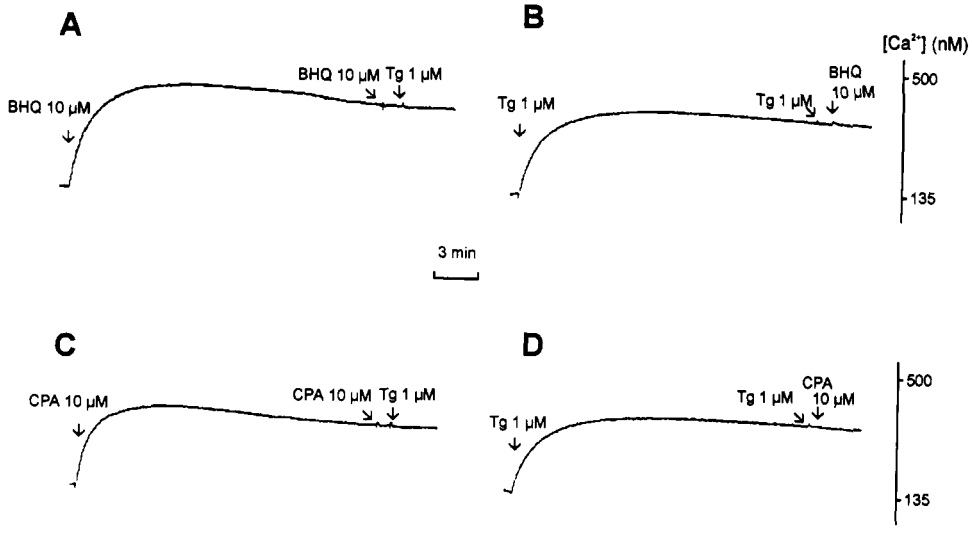

FIGURE 5: Actions of Tg, BHQ, and CPA on Ca^{2+} release from GH_4C_1 microsomes. Experimental conditions were as described in the legend of Figure 4. (A) BHQ (10 μM) caused Ca^{2+} release, which had a similar time course as that of Tg. Tg added 20 min after BHQ caused no Ca^{2+} release. (B) BHQ added 20 min after Tg caused no Ca^{2+} release. (C) CPA (10 μM) caused Ca^{2+} release, which had a similar time course as that of Tg. Tg added 20 min after CPA caused no Ca^{2+} release. (D) CPA added 20 min after Tg caused no Ca^{2+} release.

$$2Ca^{2+}_{out} + ATP \rightleftarrows 2Ca^{2+}_{in} + ADP + P_i$$

$$K = \left(\frac{([Ca^{2+}]_{out})^2}{([Ca^{2+}]_{in})^2}\right)\left(\frac{[ATP]}{[ADP][P_i]}\right)$$

where $[Ca^{2+}]_{out}$ is the extravesicular $[Ca^{2+}]$, and $[Ca^{2+}]_{in}$ is the intravesicular $[Ca^{2+}]$. The following scheme (Figure 6) is based on results from our microsome experiments (Figure 4). The subscript "s" designates a Tg-*sensitive* pool(s), and "r" indicates a Tg-*resistant* pool(s). For example, $[Ca^{2+}]_{r,basal}$ is the intravesicular $[Ca^{2+}]$ in the Tg-resistant pool in the basal state. The equilibrium state for each pool in the basal situation (Figure 6A) is described as follows:

$$K_s = \left(\frac{([Ca^{2+}]_{out,basal})^2}{([Ca^{2+}]_{s,basal})^2}\right) C \quad \text{or} \quad [Ca^{2+}]_{s,basal} = \left(\sqrt{\frac{C}{K_s}}\right)[Ca^{2+}]_{out,basal}$$

$$K_r = \left(\frac{([Ca^{2+}]_{out,basal})^2}{([Ca^{2+}]_{r,basal})^2}\right) C \quad \text{or} \quad [Ca^{2+}]_{r,basal} = \left(\sqrt{\frac{C}{K_r}}\right)[Ca^{2+}]_{out,basal} \quad (1)$$

where K_s is the equilibrium constant for the Tg-sensitive pool, K_r is the equilibrium constant for the Tg-resistant pool, and $C = [ATP]/[ADP][P_i]$, which is kept constant by use of the ATP-regenerating system.

At equilibrium, after incubation with Tg (Figure 6C),

$$[Ca^{2+}]_{s,postTg} = [Ca^{2+}]_{out,postTg}$$

$$K_r = \left(\frac{([Ca^{2+}]_{out,postTg})^2}{([Ca^{2+}]_{r,postTg})^2}\right) C \quad \text{or} \quad [Ca^{2+}]_{r,postTg} = \left(\sqrt{\frac{C}{K_r}}\right)[Ca^{2+}]_{out,postTg} \quad (2)$$

Ionomycin discharges Ca^{2+} from all pools. Because $[Ca^{2+}]_{s,postTg}$ is equal to $[Ca^{2+}]_{out,postTg}$, and the volume of each Ca^{2+}-storing pool is much smaller than the total incubation volume, the total amount of Ca in the reaction mixture is described as follows:

$$\frac{V_s[Ca^{2+}]_{s,basal}}{F_s} + \frac{V_r[Ca^{2+}]_{r,basal}}{F_r} + \frac{V_{out}[Ca^{2+}]_{out,basal}}{F_{out}} = \frac{V_{out}[Ca^{2+}]_{iono}}{F_{out}} \quad (3)$$

$$\frac{V_r[Ca^{2+}]_{r,postTg}}{F_r} + \frac{V_{out}[Ca^{2+}]_{out,postTg}}{F_{out}} = \frac{V_{out}[Ca^{2+}]_{iono}}{F_{out}} \quad (4)$$

where V_s is the volume of the Tg-sensitive pool, V_r is the volume of the Tg-resistant pool, V_{out} is the volume of the reaction mixture in the cuvette, F_s is the fraction of Ca present as free Ca^{2+} in the Tg-sensitive pool, F_r is the fraction of Ca present as free Ca^{2+} in the Tg-resistant pool, and F_{out} is the fraction of Ca present as free Ca^{2+} in the extravesicular medium.

In order for eq 3 and 4 to hold, F_r and F_{out} must be constant over the measurement range. In other words, F_r (F_{out}) in eq 3 should be the same as that in eq 4. The Ca^{2+}-buffering activity in the extravesicular fluid is principally by ATP, which is kept constant. Because the range of $[Ca^{2+}]$ in our measurements is much lower than K_d of Ca^{2+} for ATP, F_{out} should be constant, indicating that [total Ca] in the incubation mixture parallels $[Ca^{2+}]_{iono}$. Figure 7 shows the linear correlation ($R = 0.70$) between measured $[Ca^{2+}]_{out,postTg}$ and $[Ca^{2+}]_{iono}$. Because $[Ca^{2+}]_{r,postTg}$ parallels $[Ca^{2+}]_{out,postTg}$ (eq 2), $[Ca^{2+}]_{r,postTg}$ also parallels [total Ca] in the incubation mixture. Therefore, it is likely that F_r is constant over the measurement range used.

Substitution of eq 1 in eq 3 gives eq 5, and eq 2 in eq 4 gives eq 6.

$$\frac{F_{out}V_s}{F_sV_{out}}\sqrt{\frac{C}{K_s}} + \frac{F_{out}V_r}{F_rV_{out}}\sqrt{\frac{C}{K_r}} + 1 = \frac{[Ca^{2+}]_{iono}}{[Ca^{2+}]_{out,basal}} \quad (5)$$

$$\frac{F_{out}V_r}{F_rV_{out}}\sqrt{\frac{C}{K_r}} + 1 = \frac{[Ca^{2+}]_{iono}}{[Ca^{2+}]_{out,postTg}} \quad (6)$$

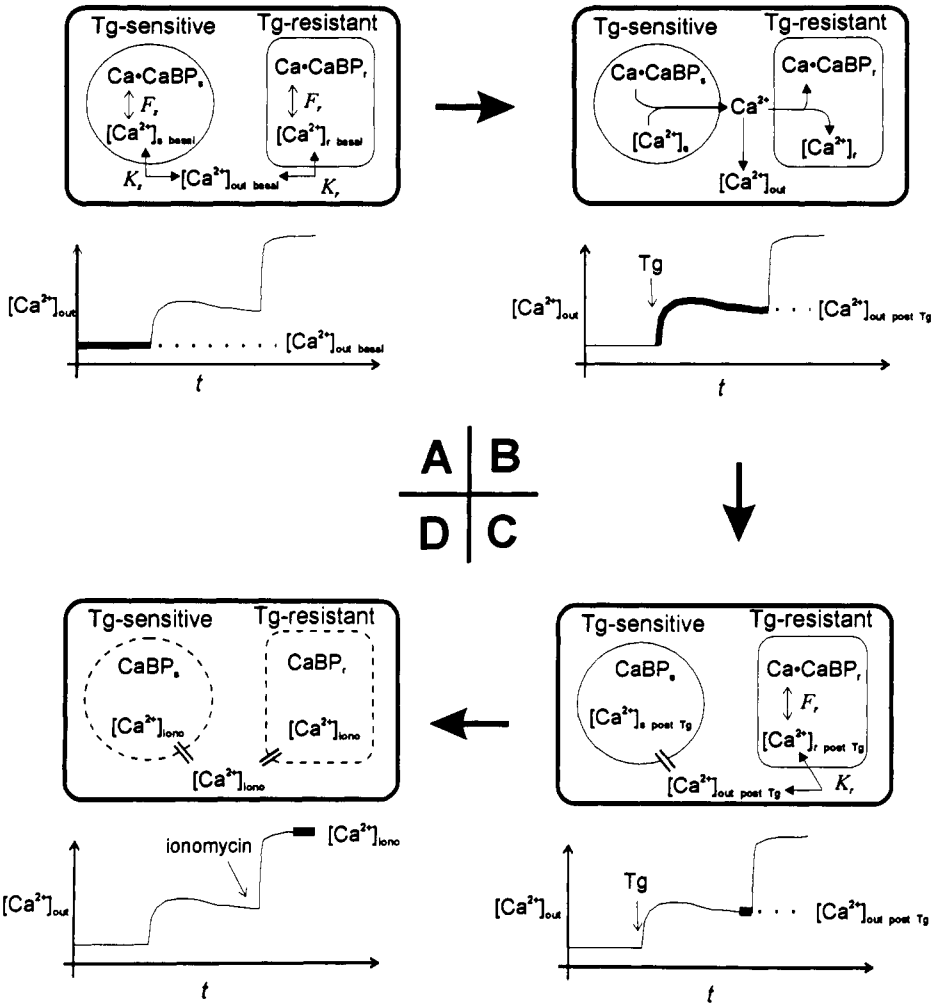

FIGURE 6: Schematic presentation of the time course of the actions of Tg and ionomycin on extravesicular $[Ca^{2+}]$ in a microsome preparation from GH_4C_1 cells. These figures depict the time course (A → B → C → D) of the actions of Tg and ionomycin on extravesicular $[Ca^{2+}]$. Definitions are given in the text. Lower figures in each panel show typical changes in $[Ca^{2+}]$. The bold line in each trace shows the $[Ca^{2+}]$ during that particular phase of the experiment. (A) In the basal state, most of the intravesicular Ca^{2+} is bound to Ca-binding proteins (CaBP); only a part of the Ca^{2+} is present as free Ca^{2+}. Free Ca^{2+} is in equilibrium with $[Ca^{2+}]_{out}$ as a result of Ca^{2+} pumping into the vesicle by Ca^{2+}-ATPase and the passive leak from the vesicle (eq 1). (B) After the addition of Tg, the Tg-sensitive pool begins to discharge Ca^{2+}. Most of discharged Ca^{2+} is resequestered by the Tg-resistant pool until that pool reaches a new steady state. Even when the ambient $[Ca^{2+}]$ is at an initial plateau value, Ca^{2+} is still being discharged from the Tg-sensitive pool, because it requires more than 30 min to discharge Ca^{2+} completely from the Tg-sensitive pool (see Figures 1 and 4). (C) After incubation for more than 30 min, Ca^{2+} is completely discharged from the Tg-sensitive pool and $[Ca^{2+}]_{s,postTg}$ approaches $[Ca^{2+}]_{out,postTg}$. In this state, the amount of Ca^{2+} in the Tg-resistant pool is larger than that in the basal state, because only the Tg-resistant pool has Ca^{2+} uptake activity (eq 2). (D) Ionomycin discharges Ca^{2+} from all Ca^{2+}-storing pools. Two equations (eqs 3 and 4) were derived to describe the amount of Ca^{2+} in the reaction mixture at A, C, and D (see text).

Thus, the relative size of the Tg-sensitive pool in the basal state (Figure 6A) can be calculated as follows:

$$\frac{\text{Tg-sensitive pool}}{\text{total ionomycin-releasable pool}} = \frac{V_s[Ca^{2+}]_{s,basal}/F_s}{V_s[Ca^{2+}]_{s,basal}/F_s + V_r[Ca^{2+}]_{r,basal}/F_r} = \frac{1}{1 + (F_sV_r)/(F_rV_s)\sqrt{K_s/K_r}} = \left(\frac{[Ca^{2+}]_{iono}}{[Ca^{2+}]_{postTg}}\right)\left(\frac{[Ca^{2+}]_{postTg} - [Ca^{2+}]_{basal}}{[Ca^{2+}]_{iono} - [Ca^{2+}]_{basal}}\right) \quad (7)$$

We present one example of the application of the new estimation method using a set of representative values from microsome experiments (Figure 8). Using eq 7, the size of the Tg-sensitive pool in this experiment was calculated to be 61.5% of the total ionomycin-releasable pool. The value would have been calculated to be 31.5% by comparison of the ratio of maximum $\Delta[Ca^{2+}]_{Tg\text{-induced}}$ to the total ionomycin-releasable $[Ca^{2+}]$, as has been done by previous investigators. In 10 independent experiments, the size of the Tg-sensitive pool was found to be 63 ± 2.5%, which would have been estimated to be 33 ± 2.3% using the conventional method. Thus, we believe the conventional method underestimates the size of the Tg-sensitive pool by a considerable margin.

Caffeine Induces Ca^{2+} Release from a Tg-Resistant Pool. Caffeine, which has been widely used as a modulator of the CICR channel (Endo, 1977), caused a gradual concentration-dependent release of Ca^{2+} in the microsome preparation (Figure 9A). Because caffeine caused a rapid increase in dye fluorescence which might be artifactual, we tested the action of caffeine on Ca^{2+}-depleted microsomes (see Materials and Methods). As shown in Figure 9B, the addition of caffeine caused only the rapid increase in fluorescence and no subsequent gradual change in the fluorescence, which was observed in Ca^{2+}-loaded microsomes (Figure 9A). Because

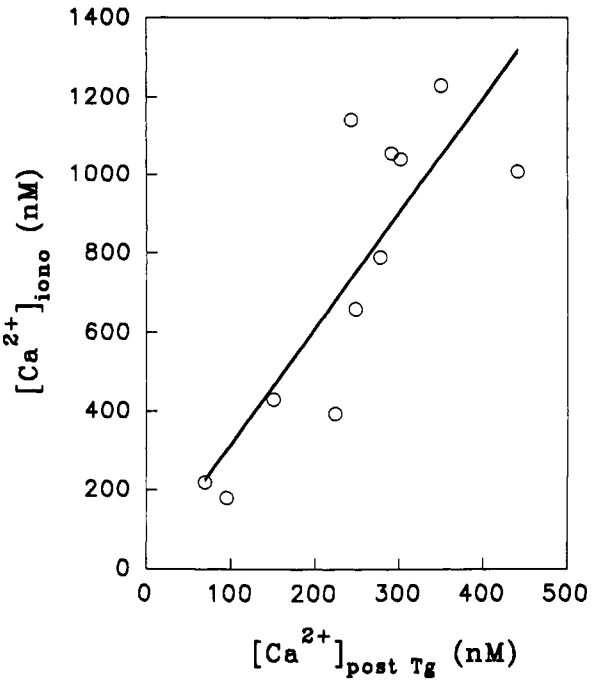

FIGURE 7: Relationship between $[Ca^{2+}]_{out,postTg}$ and $[Ca^{2+}]_{iono}$. Ca^{2+} release was measured using GH_4C_1 microsomes as described in the legend for Figure 4. Additions of Tg (1 μM) and ionomycin (200 μM) were made, and $[Ca^{2+}]_{out,postTg}$ and $[Ca^{2+}]_{iono}$ were determined as shown in Figure 6. Data from several independent microsome preparations are plotted in the same graphs as $[Ca^{2+}]_{out,postTg}$ vs $[Ca^{2+}]_{iono}$. In order to achieve several different $[Ca^{2+}]$ concentrations, some data points were acquired either using the prior addition of 1–5 μM $CaCl_2$ or by removal of excess Ca^{2+} from the microsome suspension. For the latter purpose, the microsome suspension was mixed with DTPA resin (Polymetal Sponge B) for 5–10 min, and the resin was removed by centrifugation at 80*g* for 2 min. The solid line gives a least-squares-fitted line.

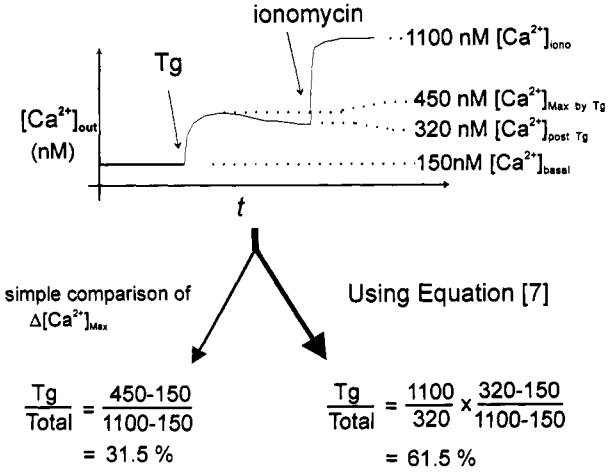

FIGURE 8: Schematic example of our estimation method. Shown here is a representative example of our estimation method for the distribution of Ca^{2+} in each microsome compartment. The basal $[Ca^{2+}]$ level was 150 nM. After the addition of Tg (1 μM), $[Ca^{2+}]$ rose to 450 nM at 15 min and to 320 nM at 40 min. Ionomycin discharged all stored Ca, and then ambient $[Ca^{2+}]$ reached 1100 nM. According to eq 7, the size of the Tg-sensitive pool in this example was calculated to be 61.5% of the total ionomycin-releasable pool. The estimated value would have been 31.5% by comparison of the maximum $\Delta[Ca^{2+}]_{Tg\text{-}induced}$/total ionomycin-releasable Ca^{2+}.

the Ca^{2+}-depleted control microsome suspension contains exactly the same cellular components as the experimental microsome suspension, except stored Ca^{2+}, the rapid increase was considered to be an artifactual increase in fluorescence, and the gradual increase was the true increase in Ca^{2+} release.

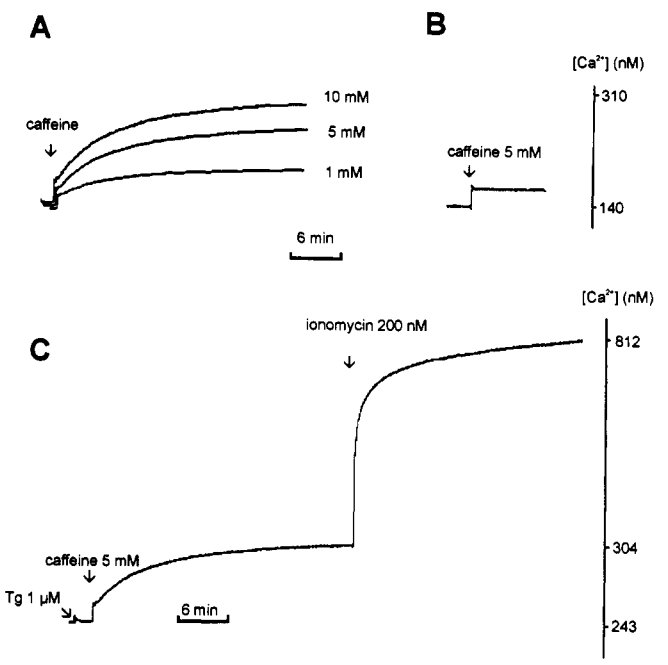

FIGURE 9: Action of caffeine on Ca^{2+} release from GH_4C_1 microsomes. Either intact (A and C) or Ca^{2+}-depleted microsomes (B) from GH_4C_1 cells were suspended in ISC buffer containing an ATP-regenerating system, and the change in $[Ca^{2+}]$ was measured with 2 μM fluo 3. (A) Caffeine (1–10 mM) caused an immediate rise in the fluorescence intensity and a subsequent slow Ca^{2+} release. (B) In Ca^{2+}-depleted microsomes (see Materials and Methods), caffeine only caused the rapid rise in the fluorescence intensity; no subsequent change was observed. (C) The microsome suspension was pretreated with Tg (1 μM) for 20 min. The whole mixture was then mixed with the Ca^{2+}-chelating resin (DTPA Polymetal Sponge B) for 2 min, and the resin was removed by centrifugation at 80*g* for 2 min. The microsomes were transferred to a cuvette, and ambient $[Ca^{2+}]$ was measured. The lack of an effect of Tg added at the beginning of the trace shows that the Tg pretreatment had completely emptied the Tg-sensitive pool. Addition of caffeine (5 mM) caused a gradual Ca^{2+} release similar to that observed in non-Tg-treated microsomes (see A above).

To investigate the source of the true Ca^{2+} release induced by caffeine, microsomes were preincubated with Tg. To keep $[Ca^{2+}]_{postTg}$ as close as possible to $[Ca^{2+}]_{basal}$, the Tg-pretreated microsome suspension was exposed briefly (see figure caption) to the DTPA Polymetal Sponge, and then ambient $[Ca^{2+}]$ was monitored. As shown in Figure 9C, addition of Tg had no effect, whereas caffeine caused a gradual release of Ca^{2+}. This result indicates that at least a part of the Tg-resistant pool has the characteristics of a caffeine-responsive CICR mechanism.

Caffeine Pretreatment Increased the Size of the Tg-Sensitive Pool. To test whether caffeine released Ca^{2+} from the Tg-sensitive pool, quantitation of the size of the Tg-sensitive pool was performed before and after pretreatment with caffeine by use of eq 7. First, Tg added after caffeine could still release Ca^{2+} (Figure 10B) with a time course comparable to that in the control experiment without caffeine (Figure 10A). Use of eq 7 revealed that a significant ($p < 0.05$) increase in the size of the Tg-sensitive pool occurred after pretreatment with caffeine from 983 ± 38 to 1232 ± 57 pmol of Ca^{2+}/mg of protein, indicating the transfer of Ca^{2+} from the Tg-resistant pool to the Tg-sensitive pool during treatment with caffeine. This result strongly suggests that caffeine-induced release of Ca^{2+} principally occurred in the Tg-resistant pool.

It has been reported in *Xenopus* oocytes (Parker & Ivorra, 1991) and in rat cerebellum (Brown et al., 1992) that caffeine inhibits $InsP_3$-induced Ca^{2+} release. It is interesting, however,

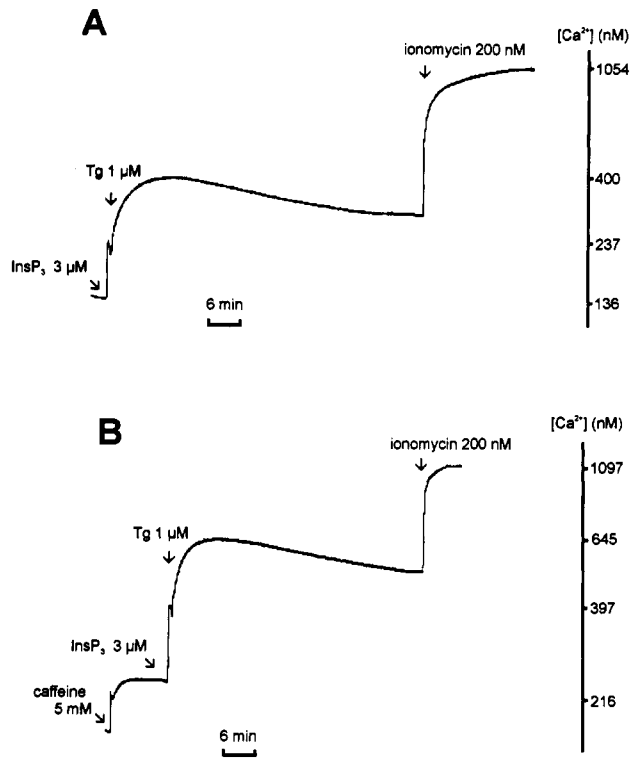

FIGURE 10: Effect of caffeine pretreatment on the distribution of Ca^{2+} in GH_4C_1 microsomes. Ca^{2+} release was measured in the microsome suspension exactly as described in the legend for Figure 4. (A) Control trace of the actions of $InsP_3$, Tg, and ionomycin. (B) $InsP_3$ (3 μM) and Tg (1 μM) added after caffeine (5 mM) each could still release Ca^{2+}. Compared to A, caffeine pretreatment caused an increase in $\Delta[Ca^{2+}]$ induced by both $InsP_3$ and Tg. Calculation by eq 7 showed a significant ($p < 0.05$) increase in the size of the Tg-sensitive pool by pretreatment with caffeine (see text).

as shown in Figure 10A,B, that caffeine pretreatment did not inhibit $InsP_3$-induced Ca^{2+} release in GH_4C_1 microsome preparations.

DISCUSSION

From the results presented in this report, we conclude that there are at least three functionally distinct ATP-dependent nonmitochondrial Ca^{2+}-storing pools in permeabilized GH_4C_1 cells: pool 1, an $InsP_3$-responsive, Tg-sensitive pool; pool 2, an $InsP_3$-unresponsive, Tg-sensitive pool; and pool 3, a Tg-resistant pool, at least a part of which has the Ca^{2+} release characteristics of a caffeine-sensitive CICR mechanism. Using a new approach to calculate the size of these Ca^{2+} pools in broken cell preparations (eq 7), we have determined that approximately 63% of the total ionomycin-releasable Ca^{2+} is stored in Tg-sensitive pools in the basal state. Most strikingly, a CICR mechanism was found to operate principally in a Tg-resistant pool. Because a specific inhibitor of Ca^{2+} uptake into the ATP-dependent, Tg-resistant pool is not yet available, our conclusion depends on accurate quantitation of the total ionomycin-releasable Ca^{2+} and the size of the Tg-sensitive pools before and after incubation with caffeine. Until the CICR-like compartment is isolated biochemically or a specific inhibitor of Ca^{2+} uptake into that pool is in hand, our evidence must be considered indirect. Nevertheless, the data and conclusion presented here are consistent with prior observations on the mechanism of cytosolic Ca^{2+} oscillations in single intact GH_4C_1 cells, in which the fluctuations were shown to depend on a CICR-like mechanism that was resistant to preincubation with Tg (Wagner et al., 1993), and with a population cell study with intact GH_4C_1 cells (Law et al., 1990).

As shown in Figure 9C, 5 mM (and even 10 mM, data not shown) caffeine, added after the maximum effect of Tg, did not totally abolish pool 3. It is not clear yet whether this is because 10 mM is not a maximum concentration of caffeine, because caffeine cannot discharge all Ca^{2+} from its responsive pool, or because there is a fourth compartment for Ca^{2+} storage which is Tg-resistant and unrelated to the caffeine-sensitive CICR mechanism. The dose response for caffeine on the CICR mechanism is known to differ from one cell type to another. In some cells, such as rat chromaffin cells (Malgaroni et al., 1990), 2 mM caffeine is stimulatory, whereas 10 mM is inhibitory for Ca^{2+} oscillations. However, in other cells, such as sea urchin eggs (Galione et al., 1991), the action of caffeine on the CICR mechanism is simply stimulatory over the entire concentration range up to 10 mM. Although the action of caffeine (up to 10 mM) on CICR was only stimulatory in our microsome preparations, it could have been inhibitory at still higher concentrations of caffeine. However, we did not test higher concentrations of caffeine, because of a major disturbance of the fluorescence measurement at higher concentrations and because of possible nonspecific effects of higher concentrations of caffeine, making it impossible to interpret the result precisely.

We have also tested the action of ryanodine on broken cell preparations of GH_4C_1 cells, because caffeine is thought to act via a ryanodine receptor Ca^{2+} channel (Endo, 1977; Takeshima et al., 1989). However, ryanodine had no effect on caffeine-induced Ca^{2+} release in our preparations (data not shown). Likewise, ryanodine did not affect oscillations induced by caffeine in nonoscillating intact GH_4C_1 cells (Wagner et al., 1993). The reason for the absence of a ryanodine response has not been determined. However, it has been shown in some studies, such as in rat chromaffin cells (D'Andrea et al., 1993), that ryanodine sensitivity does not necessarily correlate with caffeine sensitivity or Ca^{2+} release induced by ryanodine. Whether this result is due to the presence of subtypes of the ryanodine receptor or ryanodine receptor-like molecules remains to be elucidated.

It has been proposed that some cell types that exhibit Ca^{2+} oscillations contain two nonmitochondrial Ca^{2+} compartments. This proposal is based largely on responsiveness to $InsP_3$, i.e., an $InsP_3$-sensitive pool and an $InsP_3$-insensitive pool (Berridge & Irvine, 1989; Berridge, 1990). After the discovery of Tg as an inhibitor of intracellular Ca^{2+}-ATPase, two types of Ca^{2+} pools were defined from the standpoint of Tg sensitivity (Foskett & Wong, 1991; Bian et al., 1991). However, interrelationships among Tg sensitivity, $InsP_3$ sensitivity, and a CICR mechanism appear to differ from one cell type to another. For instance, in adrenal chromaffin cells (Liu et al., 1991) and PC12 cells (Zacchetti et al., 1991), overlapping of the caffeine-responsive and $InsP_3$-responsive pools has been reported. In nasal exocrine cells (Shuttleworth & Thompson, 1992), the size of the $InsP_3$-responsive pool is almost the same as that of the Tg-sensitive pool. In a population cell study with intact GH_4C_1 cells, Tg, but not caffeine, blocked TRH-induced Ca^{2+} release (Law et al., 1990). In smooth muscle cells (Bian et al., 1991), a Ca^{2+} pool has been described that is insensitive to $InsP_3$ or GTP and contains a Tg-resistant Ca^{2+} pump. Foskett and Wong (1991) have reported that caffeine-induced oscillations in salivary acinar cells were not abolished by pretreatment with Tg. In lacrimal acinar cells (Bird et al., 1992), all rapidly exchangeable Ca^{2+} is contained within a nonmitochondrial, Tg-sensitive Ca^{2+} pool, which appears to be wholly and homogeneously sensitive to $InsP_3$.

Among the above studies reviewed, Ca^{2+} distribution in adrenal chromaffin cells (Liu et al., 1991), PC12 cells (Zacchetti et al., 1991), and lacrimal acinar cells (Bird et al., 1992) is distinctly different from that in GH_4C_1 cells, while the results from population studies with intact GH_4C_1 cells (Law et al., 1990), salivary acinar cells (Foskett & Wong, 1991), and smooth muscle cells (Bian et al., 1991) are in reasonable agreement with our findings. Differences may be due in part to the method of cell preparation used. In fact, considerable differences between intact cells and permeabilized cells have been reported (Bird et al., 1992). However, because our results from single intact GH_4C_1 cells (Wagner et al., 1993), results from population studies with intact GH_4C_1 cells (Law et al., 1990), and our permeabilized cell/microsome preparations agree so well, we believe that the broken cell preparations used in the present study are likely to prove to be useful for further dissection of the multiple intracellular Ca^{2+} pools present in intact GH_4C_1 cells. In addition, our results clearly show that the caffeine-sensitive CICR mechanism does not operate in an $InsP_3$-sensitive pool in GH_4C_1 cells. Presumably the CICR-like mechanism observed by others in $InsP_3$-sensitive pools, in other words CSIICR, which is sensitive to oxidizing agents (Missiaen et al., 1991; Miyazaki et al., 1992), is different from the caffeine-sensitive CICR mechanism characterized in muscle cells and GH_4C_1 cells.

Other pituitary cells, such as normal somatotrophs (Holl et al., 1988) and gonadotrophs (Stojilkovic et al., 1992; Stojilkovic & Catt, 1992), also exhibit oscillations in intracellular $[Ca^{2+}]$. In gonadotrophs, it has been shown that oscillations involve the release of Ca^{2+} from an intracellular Ca^{2+} pool(s) (Shangold et al., 1988; Stojilkovic et al., 1992), like oscillations in GH_4C_1 cells (Wagner et al., 1993). Interestingly, however, involvement of the CSIICR mechanism has been postulated in gonadotrophs (Tse & Hille, 1993; Stojilkovic et al., 1993). The CSIICR mechanism is based on one Ca^{2+} pool, whereas our hypothesis and/or the CICR model requires the existence of two or more pools. It is interesting that two different mechanisms appear to operate in two different pituitary cell types. The coexistence of a CSIICR and a CICR mechanism has been demonstrated recently in sea urchin eggs (Galione et al., 1993).

Although several subtypes of intracellular Ca^{2+}-ATPase (sarcoendoplasmic reticulum type Ca^{2+}-ATPase, SERCA) have been cloned from muscle and non-muscle cells (Lytton & MacLennan, 1988; Gunteski-Hamblin et al., 1988), all of those tested are sensitive to Tg (Lytton et al., 1991). Papp et al. (1991) have described an unidentified isoform of Ca^{2+}-ATPase in platelet membrane vesicles, which is relatively insensitive to Tg in terms of phosphoenzyme formation. They have shown that Tg specifically inhibited the autophosphorylation of a 100-kDa Ca^{2+}-ATPase in the submicromolar range without inhibiting a 97-kDa Ca^{2+}-ATPase. There is other evidence for the existence of a Tg-resistant intracellular Ca^{2+} compartment. Bian et al. (1991) have proposed that the Ca^{2+} pool insensitive to $InsP_3$ (or GTP) contains a Tg-insensitive Ca^{2+} pump in DDT_1MF-2 smooth muscle cells. Takemura et al. (1989) have reported that, after pretreatment with Tg, there are additional pools of Ca^{2+} that can be released by ionomycin in parotid acinar cells. Our results clearly show that GH_4C_1 cells contain an ATP-dependent Ca^{2+} uptake compartment which is not inhibited by Tg, implying the presence of a subtype of Ca^{2+}-ATPase that is resistant to Tg in this compartment. Alternatively, there could be an ATP-dependent, non-ATPase mechanism. One could argue that Tg might not completely inhibit the Tg-sensitive Ca^{2+}-ATPase or that the effect of Tg was desensitized during the long time course of our experiments. However, there was no additional inhibition of Ca^{2+} uptake induced by BHQ or CPA over and above that induced by Tg (Figure 5). BHA and CPA are structurally different from Tg, but are believed to act on the same uptake mechanism, increasing the likelihood that inhibition of the Tg-sensitive Ca^{2+}-ATPase by Tg was complete and persistent (not desensitized), at least during the time course observed.

Our findings indicate that the caffeine-sensitive channel is coupled to a Ca^{2+}-ATPase different from that coupled to the $InsP_3$ receptor. Similar observations, in which different Ca^{2+} release channels couple to different types of Ca^{2+}-ATPase-like proteins, have been reported in adrenal chromaffin cells (Burgoyne et al., 1989). In these cells, the $InsP_3$ receptor is closely linked to a 140-kDa Ca^{2+}-ATPase-like protein, whereas a 100-kDa Ca^{2+}-ATPase-like protein shows a distribution similar to that of the caffeine-responsive pool. Although Tg sensitivity was not examined in that study, the finding that different types of Ca^{2+}-ATPase couple to different types of Ca^{2+} release channels is consistent with our findings.

It has been reported, in *Xenopus* oocytes (Parker & Ivorra, 1991) and in rat cerebellum (Brown et al., 1992), that caffeine inhibits $InsP_3$-induced Ca^{2+} release. It is interesting, however, as shown in Figure 10A,B, that caffeine pretreatment did not inhibit $InsP_3$-induced Ca^{2+} release in our microsome preparations. Rather, it appeared to modestly enhance the $\Delta[Ca^{2+}]_{InsP_3\text{-induced}}$ presumably because of the transfer of Ca^{2+} from the Tg-resistant pool to the $InsP_3$-responsive, Tg-sensitive pool. We have recently observed a similar lack of inhibition of $InsP_3$ channels by caffeine in sea urchin egg homogenates (Y. Tanaka and A. H. Tashjian, Jr., manuscript in preparation).

There is uncertainty as to the intracellular localization of Ca^{2+} pools (Rossier & Putney, 1991; Meldolesi et al., 1990). The concept of the "calciosome" was postulated originally to represent the $InsP_3$-responsive pool (Volpe et al., 1988), but recent studies have revealed that the ryanodine receptor containing pool might also fulfill this definition (Volpe et al., 1991). The current consensus appears to be that highly specialized portions of the endoplasmic reticulum are sites where Ca^{2+} is stored and that each Ca^{2+} pool needs to be composed of a Ca^{2+} release channel, a Ca^{2+}-ATPase or other Ca^{2+} pump, and contains Ca^{2+}-binding protein(s) (Meldolesi et al., 1990). Although we have separated intracellular Ca^{2+} pools from the pharmacological and functional standpoints, it can be argued that the organelles might not be separated anatomically. However, the functional coupling of a Ca^{2+} release channel to a Ca^{2+}-ATPase in two different cases (Tg-sensitive Ca^{2+}-ATPase to the $InsP_3$ receptor, and Tg-resistant Ca^{2+}-ATPase to caffeine sensitivity) strongly suggests that there are at least two highly specialized organelles for Ca^{2+} storage in GH_4C_1 cells. In other words, pools 1 and 3 are likely to be anatomically distinct.

The physiological importance of pools 1 and 3 in the generation of oscillation is clearly established. Pool 3, in the basal state, releases Ca^{2+} either by being triggered by Ca^{2+} influx through voltage-operated Ca^{2+} channels or by a spontaneous mechanism, exhibiting basal oscillations (Wagner et al., 1993). On agonist stimulation, $InsP_3$ liberated from phosphatidylinositol 4,5-bisphosphate releases Ca^{2+} from pool 1, forming the initial $[Ca^{2+}]_i$ spike. Ca^{2+} released from pool 1, in turn, causes agonist-induced oscillation either by overloading pool 3 or by directly triggering Ca^{2+} release from pool 3. The anatomical independence of pool 2 is not clear

at the present time, because there still remains a possibility that the Ca^{2+} stored in the $InsP_3$-responsive pool cannot be fully released by $InsP_3$ in a short period of time. In fact, in some cell types (Shuttleworth & Thompson, 1992; Bird et al., 1992), the Tg-sensitive pool is, functionally, mostly $InsP_3$-sensitive. However, because of the functional presence of pool 2 (Figures 3 and 4), we speculate that this pool may act as a Ca^{2+} reservoir for the filling of pool 1 and/or pool 3 in GH_4C_1 cells. A study estimating the size of the $InsP_3$-sensitive pool by analyzing the kinetics of Ca^{2+} release induced by $InsP_3$ is now being undertaken. Our preliminary results suggest that more than 70% of Ca^{2+} stored in the Tg-sensitive pool is released by $InsP_3$ (data not shown).

Many estimates of the size of intracellular Ca^{2+} pools have been reported. In most cases, estimation of the size of each pool has been made by using either ^{45}Ca or a Ca^{2+} indicator dye. In the case of dye studies, the maximum $\Delta[Ca^{2+}]$ induced by each compound has been used as an index of the size of that particular compartment (Ely et al., 1991; Law et al., 1990; Takemura et al., 1989; Cheek & Thastrup, 1989). However, there could well be misestimation of pool size, because Ca^{2+} released from one pool (such as the Tg-sensitive pool) will be readily sequestered by other pools (such as the Tg-resistant pool) or extruded from the cell, resulting in underestimation of the former pool. Such underestimation will occur especially with slowly acting agents such as Tg. Our new estimation method, applied in microsome experiments, has revealed that the conventional method underestimates the size of the Tg-sensitive pool by a considerable margin. We believe that such underestimation of relative Ca^{2+} pool sizes also occurs in studies with intact cells. In fact, Ca^{2+} extrusion mechanisms that are operating in intact cells, such as plasma membrane Ca^{2+}-ATPase(s) and Na^+–Ca^{2+} exchanger(s) on plasma membrane, could make the underestimation even more severe. Therefore, caution should be used in interpreting the results of previous studies.

One can argue that the *in vivo* distribution of Ca^{2+} might be altered during the microsome preparation and, therefore, that our estimation, which is performed under special *in vitro* conditions, might not represent the true intracellular distribution in intact cells. However, we believe that our results and approach will be useful in understanding the intact cell situation for the following reasons. First, the basal $[Ca^{2+}]$ in our experiment was quite close to the basal $[Ca^{2+}]_i$ in intact cells. This indicates that, in terms of the equilibrium of Ca^{2+} across ER membranes in the basal state, the microsome suspension and intact cells behave quite similarly. Second, although other Ca^{2+} handling mechanisms are present in intact cells (e.g., plasma membrane extrusion), the basis of our new method in microsomes is not a kinetic analysis, but an analysis of two equilibrium states achieved independently of other Ca^{2+} handling mechanisms. Therefore, we conclude that our approach to estimating the distribution of Ca^{2+} in intracellular compartments has a smaller error than previous estimation methods, which do not take Ca^{2+} uptake factors into account. Although not directly applicable, we believe the results from microsome studies will be useful in interpreting estimates of Ca^{2+} pool sizes in intact cells.

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