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Intracellular Ca^{2+} pool content is linked to control of cell growth

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ABSTRACT **A close correlation was observed between intracellular Ca^{2+} pool depletion and refilling and the onset of DNA synthesis and proliferation of DDT_1MF-2 smooth muscle cells. The intracellular Ca^{2+} pump inhibitors 2,5-di-*tert*-butylhydroquinone (DBHQ) and thapsigargin (TG) specifically emptied identical inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive Ca^{2+} pools and both arrested cell growth at concentrations corresponding to Ca^{2+} pump blockade. However, an important distinction was observed between the two inhibitors with respect to their reversibility of action. Upon removal of DBHQ from DBHQ-arrested cells, Ca^{2+} pools immediately refilled, and 14 hr later cells entered S phase followed by normal cell proliferation; the time for entry into S phase was identical to that for cells released from confluence arrest. Although TG irreversibly blocked Ca^{2+} pumping and emptied Ca^{2+} pools, high serum treatment of TG-arrested cells induced recovery of functional Ca^{2+} pools in 6 hr (via probable synthesis of new pump); thereafter cells proceeded to S phase and normal cell proliferation within the same time period (14 hr) as that following release of DBHQ-arrested cells. The precise relationship between Ca^{2+} pump blockade and growth arrest indicates that Ca^{2+} pool emptying maintains cells in a G_0-like quiescent state; upon refilling of pools, normal progression into the cell cycle is resumed. It is possible that a specific cell cycle event necessary for G_0 to G_1 transition depends upon signals generated from the $InsP_3$-sensitive Ca^{2+} pool.**

The second messenger inositol 1,4,5-trisphosphate ($InsP_3$) generates cytosolic Ca^{2+} signals by activating Ca^{2+} release from Ca^{2+}-sequestering target organelles and, in so doing, mediates a multitude of cellular responses (1, 2). Although heterogeneous (2–5), the target Ca^{2+} pools released by $InsP_3$ are believed to include the endoplasmic reticulum (ER) and nuclear envelope (6–10). In response to signals mediated by hormones and growth factors, these pools of Ca^{2+} are transiently depleted by activation of the $InsP_3$-dependent Ca^{2+} channel in the ER membrane (11); upon termination of the $InsP_3$-generating signal the pools are able to refill via Ca^{2+} pumping activity (12). Ca^{2+} pools within cells comprise $InsP_3$-sensitive and -insensitive compartments (5–7); Ca^{2+} movement between the two compartments can be mediated by a specific guanine nucleotide-activated Ca^{2+} translocation process (3–6) that may control the size of the $InsP_3$-releasable Ca^{2+} pool (6, 7). Uptake of Ca^{2+} into both compartments is blocked by the potent, specific, and essentially irreversible Ca^{2+} pump inhibitor thapsigargin (TG) (13–17). In contrast to transient agonist-mediated Ca^{2+} pool depletion, Ca^{2+} pump blockade by TG induces a highly persistent depletion of $InsP_3$-sensitive Ca^{2+} pools (17, 18); it was also noted that cells treated with TG appear to cease cell division (18). Using TG and a distinct and fully reversible Ca^{2+} pump blocker, 2,5-di-*tert*-butylhydroquinone (DBHQ) (19), studies reported here reveal a precise correlation exists between the Ca^{2+} content of intracellular Ca^{2+} pools and the ability of cells to initiate DNA synthesis and divide.

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MATERIALS AND METHODS

Cell Culture. DDT_1MF-2 smooth muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2.5% serum (Calf-Plus; Inovar, Gaithersburg, MD) as described (3).

$^{45}Ca^{2+}$ Uptake into Permeabilized Cells. Cells were harvested, permeabilized with 0.005% saponin (5 min, 37°C, 2×10^6 cells per ml) in intracellular-like medium (ICM: 140 mM KCl/10 mM NaCl/2.5 mM $MgCl_2$/10 mM Hepes-KOH, pH 7.0), washed free of saponin, and resuspended in ICM. Uptake of $^{45}Ca^{2+}$ into permeabilized cells was measured in ICM containing 1 mM ATP, 50 μM $CaCl_2$ (with 150 Ci of $^{45}Ca^{2+}$ per mol; 1 Ci = 37 GBq) buffered to 0.1 μM with EGTA, and 3% polyethylene glycol at 37°C for the times shown. Uptake was quenched with ICM containing 1 mM $LaCl_3$, and cells were rapidly filtered on glass fiber filters and radioactivity was counted. These procedures were as described (20–23).

Intracellular Free Ca^{2+} Measurements. Details of these procedures have been described (18). Briefly, cells were harvested in Hepes-buffered Kreb's medium (HBKM: 107 mM NaCl/6 mM KCl/1.2 mM $MgSO_4$/1 mM $CaCl_2$/1.2 mM KH_2PO_4/11.5 mM glucose/0.1% bovine serum albumin/20 mM Hepes-KOH, pH 7.4) and loaded with 2 μM fura-2/acetoxymethyl ester (fura-2 AM) (45 min, 20°C, 3×10^6 cells per ml, in the dark). After three washes with HBKM, cells were resuspended at 5×10^5 cells per ml, and fluorescence emission at 505 nm was monitored at 25°C using a PTI dual-wavelength spectrofluorometer system, with excitation at 340 and 380 nm. Free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were calculated from ratios of fluorescence intensities obtained every 1 sec using the calculations of Grynkiewicz *et al.* (24) and a K_d of 135 nM. Dye was considered saturated upon lysis with 0.1% Triton X-100; minimum fluorescence was determined with 5 mM EGTA.

Incorporation of [^{3}H]Thymidine. Cells were plated in 24-well dishes (1×10^5 cells per well) and incubated with 1 μCi of [^{3}H]thymidine per ml in growth medium (DMEM with either 2.5% or 20% serum). At the times shown, medium was removed and cells were harvested, processed, and assayed for radioactivity as described (18).

Materials and Miscellaneous Procedures. $InsP_3$ was from Calbiochem. TG was from LC Services (Woburn, MA). Fura-2 AM was from Molecular Probes. The DDT_1MF-2 cell line was from James Norris and Lawrence Cornett (University of Arkansas).

Abbreviations: TG, thapsigargin; $InsP_3$, inositol 1,4,5-trisphosphate; DBHQ, 2,5-di-*tert*-butylhydroquinone; DMSO, dimethyl sulfoxide; $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; ER, endoplasmic reticulum.

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RESULTS AND DISCUSSION

The recently identified Ca^{2+} pump inhibitors TG (13–15) and DBHQ (24) specifically block Ca^{2+} pump isoforms of ER and sarcoplasmic reticulum but not the plasma membrane (13–16). As shown in Fig. 1, $^{45}Ca^{2+}$ flux experiments on permeabilized DDT_1MF-2 smooth muscle cells reveal that TG and DBHQ have identical specificity for intracellular Ca^{2+}-pumping pools yet differ with respect to their reversibility of action. Maximally effective concentrations of both agents blocked Ca^{2+} pumping into the same total $InsP_3$-releasable Ca^{2+} pool (3–9, 21–23) observed in the presence of $InsP_3$ and GTP† (Fig. 1*A*). The lack of any additivity of action of TG and DBHQ indicates emptying of entirely overlapping pools. If cells, cultured for 24 hr with 3 μM TG, were washed, cultured for a further 24 hr without TG, and then permeabilized, Ca^{2+} accumulation into the TG-sensitive Ca^{2+} pool remained completely blocked with no further effect of TG, DBHQ, or $InsP_3$ with GTP (Fig. 1*B*). In contrast, using cells pretreated with 30 μM DBHQ instead of TG followed by washing and permeabilization, Ca^{2+} uptake returned to normal and was again sensitive to Ca^{2+} pump blockers and $InsP_3$ (Fig. 1*C*). Even when added directly to cells after permeabilization, the inhibitory effect of DBHQ was completely reversed by simple washing (not shown).

The actions of TG and DBHQ on Ca^{2+} fluxes in permeabilized cells were consistent with their effects on $[Ca^{2+}]_i$ levels measured in intact fura-2-loaded cells (Fig. 2). TG and DBHQ induced the same rapid increases in $[Ca^{2+}]_i$, reaching a maximum of over twice resting levels within 45 sec (Fig. 2 *A* and *B*). The transient nature of increased $[Ca^{2+}]_i$ reflects leak and emptying of Ca^{2+} from internal pools (17); $[Ca^{2+}]_i$ decreases thereafter as a result of continued efflux across the plasma membrane. Clearly, TG and DBHQ readily enter intact cells and deplete intracellular pools. Following pool depletion by either agent, a second addition of Ca^{2+} pump inhibitor induced little further release, indicating again that both inhibitors empty a single overlapping pool (Fig. 2 *A* and *B*). Ionomycin caused some further release likely from a TG/DBHQ-insensitive Ca^{2+} pool, as revealed in Fig. 1 and previous studies (17, 18). After pretreatment of cells with TG for 24 hr in culture followed by washing and fura-2-loading, subsequent addition of TG induced no change in $[Ca^{2+}]_i$, indicating that the pool remained entirely empty (Fig. 2*C*; the slightly lower apparent free $[Ca^{2+}]_i$ may reflect loss of signal from fura-2 loaded within internal organelles that became depleted of Ca^{2+}). In contrast, cells pretreated with DBHQ followed by the same washing and loading displayed intracellular Ca^{2+} release the same as untreated cells. Thus, DBHQ, while present, maintains the same intracellular Ca^{2+} pool as TG in a depleted state; however, the effect of DBHQ is reversible and this pool rapidly refills after removal of DBHQ. TG and DBHQ block the same intracellular Ca^{2+} pumps (7, 15–19); the pool sensitive to DBHQ or TG is exactly the total $InsP_3$-releasable Ca^{2+} pool within these cells (3–9). In DDT_1MF-2 cells, this Ca^{2+} pool can be rapidly mobilized by $InsP_3$-generating agonists, including bradykinin, histamine, epinephrine, and ATP (6–9, 18); however, in the presence of DBHQ or any time after TG-treatment, the cells are entirely devoid of Ca^{2+} signals in response to agonists (unpublished data). In spite of Ca^{2+} pool absence, cells still maintain resting cytosolic Ca^{2+} levels and retain normal morphology, viability, and metabolic activity for several days (18).

†We have shown that two pools of Ca^{2+} exist in cells, only one of which is directly releasable by $InsP_3$; in the presence of GTP, transfer of Ca^{2+} between the two pools is permitted, allowing Ca^{2+} to be released from both pools.

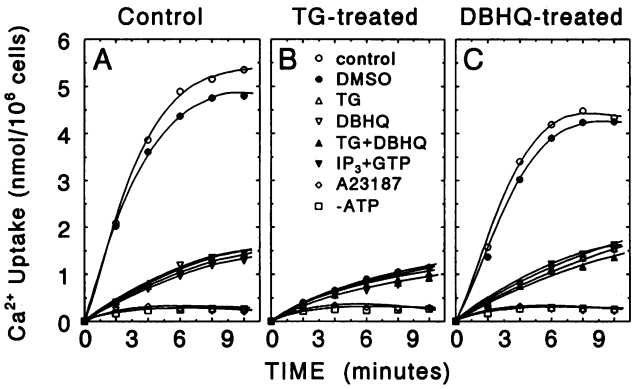

FIG. 1. Specificity and reversibility of TG- and DBHQ-induced inhibition of Ca^{2+} pumping into intracellular Ca^{2+} pools within permeabilized DDT_1MF-2 cells. On day 6 after passaging, cells were treated with either control vehicle [0.3% (vol/vol) dimethyl sulfoxide (DMSO)] (*A*), 3 μM TG (*B*), or 30 μM DBHQ (*C*). After 24 hr of treatment, cells were washed three times with fresh growth medium free of inhibitors and cultured for a further 24 hr. $^{45}Ca^{2+}$ uptake took place either without other additions (○), with 0.3% DMSO (●), 3 μM TG (△), 30 μM DBHQ (▽), 3 μM TG with 30 μM DBHQ (▲), 10 μM $InsP_3$ with 20 μM GTP (▼), or 1 μM A23187 (◇), or without ATP (□). Results are typical of eight identical experiments.

An intriguing feature of the actions of the two Ca^{2+} pump inhibitors is the precise correlation between their distinct effects on Ca^{2+} pool emptying and on cell proliferation. As shown in Table 1, the proliferation of DDT_1MF-2 cells is abolished by TG and DBHQ. During a 72-hr treatment of newly passaged cells in culture with either 30 μM DBHQ or 3 μM TG, the number of cells remained essentially unchanged as compared to control cells, which increased by 7-

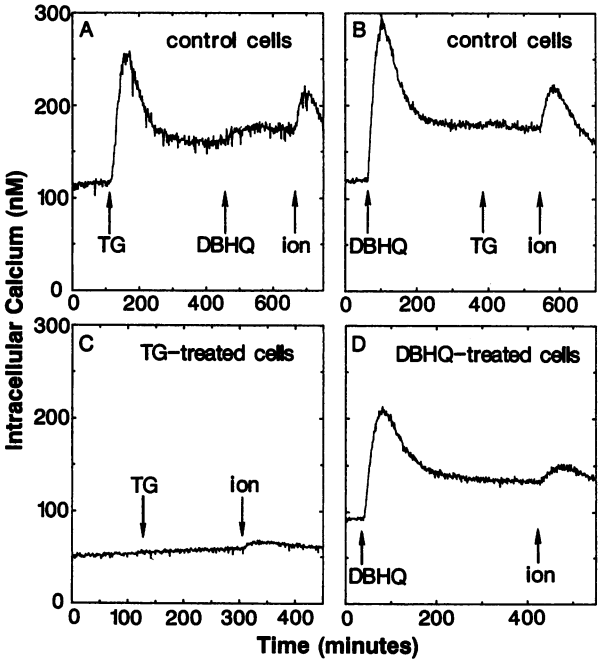

FIG. 2. Influence of TG and DBHQ on cytosolic free Ca^{2+} levels in DDT_1MF-2 cells. Cells were grown either for 4 days under standard growth conditions (*A* and *B*) or for 3 days under standard conditions followed by 1 day in the presence of either 3 μM TG (*C*) or 30 μM DBHQ (*D*). Cells were harvested in HBKM (without pump inhibitors), loaded with fura-2 AM, and used in fluorescence studies to determine free cytosolic Ca^{2+}. Additions of either control DMSO (0.1%), 3 μM TG, 30 μM DBHQ, or 2 μM ionomycin (ion) were made to cells in the cuvettes at the times shown. The results are representative of four similar experiments.

Table 1. Comparison of the effects of DBHQ and TG on proliferation of DDT_1MF-2 cells in culture

Condition	Cell number ($\times 10^{-4}$)	Fold change in cell number
Start	5.42 ± 0.52	1.00
Control	43.44 ± 5.17	8.01
DMSO	41.09 ± 6.68	7.58
30 μM DBHQ	6.31 ± 0.99	1.16
30 μM DBHQ; 24-hr wash	31.38 ± 3.04	5.79
3 μM TG	4.69 ± 0.12	0.87
3 μM TG; 24-hr wash	5.38 ± 1.13	0.99

Cells were passaged into standard growth medium containing inhibitors or vehicle as shown; numbers of cells (mean ± SD) were determined after 3 days and compared to numbers originally plated (start). Where indicated, DBHQ- or TG-treated cells were washed at 24 hr and growth was continued in inhibitor-free medium for a further 2 days. Results are typical of five similar experiments. Cell viability was >95%.

to 8-fold. Importantly, if the DBHQ-treated cells were washed free of DBHQ after the first 24 hr, the cells resumed rapid proliferation and grew to >5-fold the original number during the 48-hr period following washing. In contrast, after a similar 24-hr treatment with TG followed by washing, there was no resumption of growth. This sharp distinction between the reversibility of DBHQ and irreversibility of the action of TG correlates with the effects of the two agents on Ca^{2+} pool emptying. The EC_{50} values for DBHQ for Ca^{2+} pool emptying and growth arrest were both ≈5 μM (data not shown). We previously measured the EC_{50} of TG for inhibition of Ca^{2+}-pumping activity as 30 nM (17), which corresponds well with the EC_{50} of 20 nM for growth inhibition (18).

The close correlation between the concentration dependence and reversibility of action of the two different Ca^{2+} pump inhibitors on pool emptying and growth suggests a causal relationship between the two events. This conclusion is reinforced by the actions of the Ca^{2+} pump inhibitors on the onset of DNA synthesis in DDT_1MF-2 cells in culture. As shown in Fig. 3*A*, in the presence of either 30 μM DBHQ or 3 μM TG, DNA synthesis was completely abolished. If after the first 24 hr of cell culture with DBHQ the growth medium was replaced with DBHQ-free medium, a sharp increase in DNA synthesis was observed ≈14 hr later as a proportion of cells entered S phase (Fig. 3*A*). This was in stark contrast to TG-treated cells, in which DNA synthesis remained completely blocked at all times after washing with TG-free medium. The time of onset of increased DNA synthesis after removal of DBHQ was very close to the initial time of onset of DNA synthesis (14–16 hr) observed for control cells after passaging (Fig. 3*A*). This suggests that DBHQ-treated cells are maintained in the same state as confluence-arrested cells. Confluent cells are assumed to be arrested prior to entry into G_1 in a G_0-like quiescent phase; the interval before the start of DNA synthesis reflects their reentry into the cell cycle and progression through a protracted G_1 phase before the onset of S phase (25). If TG or DBHQ was added to newly passaged confluence-arrested cells up to 12 hr following passaging, onset of DNA synthesis was still prevented (data not shown), indicating that the filled Ca^{2+} pool was required through much of the period before onset of S phase. When DBHQ was added to cells actively synthesizing DNA (e.g., 24 hr after passaging), DNA synthesis continued but declined to zero after several hours (unpublished data), indicating that DNA synthesis *per se* was not inhibited by DBHQ. Identical results were observed upon TG addition to cells actively synthesizing DNA (18). Whatever the cell cycle state of Ca^{2+} pump inhibitor-arrested cells, it is clear that cells can be maintained in this state for several days with DBHQ; even after prolonged DBHQ treatment (1–3 days), DNA synthesis resumed

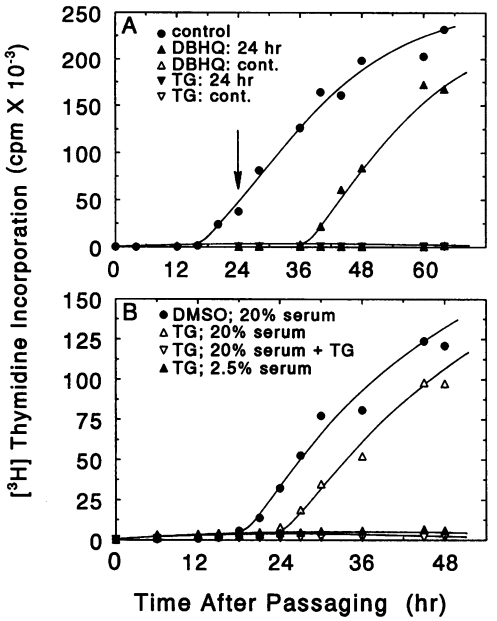

FIG. 3. TG- and DBHQ-induced inhibition of DNA synthesis in cultured DDT_1MF-2 cells. Cells plated in 24-well dishes were incubated with 1 μCi of [^{3}H]thymidine per ml in growth medium (DMEM with either 2.5% or 20% serum) for the times shown. (*A*) After the first 24 hr, growth medium was replaced with fresh medium containing the same concentration of [^{3}H]thymidine and serum (2.5%). Wells contained either no other additions before and after 24 hr (●), 30 μM DBHQ (△) or 3 μM TG (▽) continuously (cont.) before and after 24 hr, or 30 μM DBHQ (▲) or 3 μM TG (▼) for the first 24 hr only followed by inhibitor-free medium after 24 hr. (*B*) Prior to passaging, confluent dishes of cells were treated in culture for 24 hr either with 3 μM TG (△, ▽, ▲) or control DMSO (●) in growth medium containing 2.5% serum. Cells were passaged into 24-well plates containing growth medium with [^{3}H]thymidine and either 20% serum (●, △), 20% serum with 3 μM TG (▽), or 2.5% serum (▲). At the times shown, cells were harvested, processed, and assayed for radioactivity. Results are representative of four similar experiments.

after the same lag period (14–16 hr) following DBHQ withdrawal from the medium (data not shown). Thus, refilling of Ca^{2+} pools following DBHQ removal correlates with the reentry of cells into the cell cycle.

A surprising observation was that even TG-arrested cells can be induced to reenter the cell cycle and that such reentry again closely correlates with the reappearance of Ca^{2+} pools. As shown in Table 2, if cells were treated with 3 μM TG for

Table 2. Reversal of TG-induced suppression of DDT_1MF-2 cell proliferation by high serum

Pretreatment of cells	Cell number ($\times 10^{-4}$)	Fold change in cell number
DMSO		
Start	9.03 ± 1.28	1.00
2.5% serum	61.30 ± 2.24	6.79
20% serum	82.24 ± 6.16	9.11
TG		
Start	12.27 ± 1.18	1.00
2.5% serum + DMSO	13.34 ± 1.06	1.09
2.5% serum + 3 μM TG	12.53 ± 1.03	1.02
20% serum + DMSO	95.56 ± 4.50	7.79
20% serum + 3 μM TG	10.97 ± 0.72	0.89

Confluent cells in standard growth medium (2.5% serum) were treated with either 3 μM TG or control DMSO prior to passaging into dishes containing either 2.5% or 20% serum together with TG or vehicle as shown. Cell numbers (mean ± SD) were determined at the start and 3 days after passaging. Cell viability was >95%. Results are typical of three similar experiments.

24 hr immediately prior to passaging into new medium containing standard 2.5% serum,‡ they were unable to proliferate whether or not TG was included in the new medium, consistent with the irreversible action of TG described above. However, if the new medium contained high (20%) serum, then cells were able to resume proliferation at a rate almost as rapid as cells that had not been exposed to TG (almost 8-fold increase in cells in 72 hr). High serum was therefore able to overcome the otherwise persistent action of TG on cell proliferation.§ However, high serum-induced recovery of cell division was still TG sensitive since it was completely blocked if TG was added along with high serum. In other words, the only way TG-induced inhibition could be reversed was with a combination of high serum and the absence of TG, indicating that induction of cell growth is still dependent on a filled TG-sensitive Ca^{2+} pool.

The time course of onset of DNA synthesis occurring after high serum-induced reversal of TG-inhibited cells revealed that an additional period of time was required before cells were able to begin entering S phase. As shown in Fig. 3*B*, with cells exposed to TG for 24 hr prior to passaging into 20% serum, DNA synthesis did not begin to occur until ≈22 hr after passaging. In contrast, cells not preexposed to TG (DMSO control) and passaged under identical conditions began to synthesize DNA at ≈16 hr. DNA synthesis progressed at the same rate for both types of cells; however, there was a clear 6-hr time lag in the progression of DNA synthesis in the TG-pretreated cells. It is also clear that, as for cell proliferation, the continued presence of TG entirely blocked the stimulatory action of high serum on DNA synthesis. Thus, the mechanism of high serum-induced recovery of growth still involves a TG-sensitive step.

An important question to address was whether the onset of DNA synthesis and cell proliferation after high serum-induced reversal of action of TG was related to a reversal of Ca^{2+} pump blockade or whether the effect of high serum was to induce an increase in growth independent of Ca^{2+} accumulation into pools. Experiments measuring intracellular Ca^{2+} responses in TG- and high serum-treated cells strongly suggest the former postulate. As shown in Fig. 4*A*, after pretreatment of cells with TG for 24 hr followed by a further 24-hr treatment with 20% serum without TG, cells exhibited a Ca^{2+} release response to reapplied TG the same as cells not preexposed to TG, indicating that the TG-sensitive pool had indeed refilled. In contrast, if TG was present with the high serum, the TG-sensitive Ca^{2+} pool remained entirely empty. As expected, there was no pool recovery with 2.5% serum. Most significantly, the time of recovery of the TG-sensitive Ca^{2+} pool after high serum treatment closely corresponded to the delay in onset of DNA synthesis. As shown in Fig. 4*B*, using cells pretreated for 24 hr with TG, a significant TG-releasable Ca^{2+} pool could be observed after a 3-hr treatment with 20% serum; after 6 hr, the releasable pool had recovered to an extent comparable to normal, untreated, cells. These results provide excellent correlation between the onset of renewed growth in cells and the refilling of intracellular Ca^{2+} pools. It appears that only after pool refilling has taken place can cell cycle arrest be overcome. We would speculate that the 6-hr period during which Ca^{2+} pools recover reflects serum-activated *de novo* synthesis of Ca^{2+} pump protein

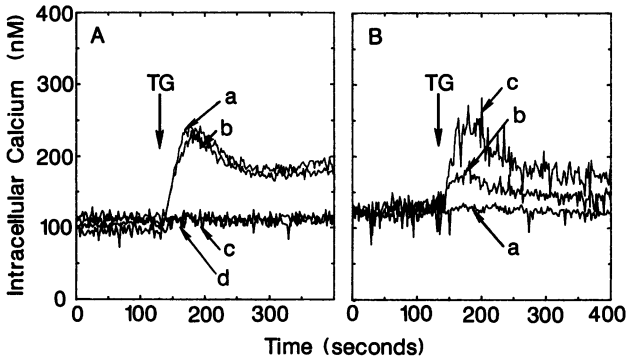

FIG. 4. Serum-induced reappearance of the TG-sensitive Ca^{2+} pool in fura-2-loaded DDT_1MF-2 cells. (*A*) Cells grown to confluency in standard growth medium (DMEM, 2.5% serum) were treated for 24 hr in culture without TG (a) or with 3 μM TG (b–d) prior to passaging. Cells were washed three times and passaged into new dishes containing growth medium either with 20% serum without TG (a and b), 2.5% serum without TG (c), or 20% serum with 3 μM TG (d). After 24 hr of culture, cells were harvested and free $[Ca^{2+}]_i$ was determined with fura-2. In all traces, 3 μM TG was added at the time shown. (*B*) Confluent cells pretreated with 3 μM TG for 24 hr as above were passaged into new dishes containing growth medium with 20% serum. Cells were harvested either immediately (a) or 3 hr (b) or 6 hr (c) after passaging. TG was added at the time shown. Fura-2 loading, fluorescence measurements, and calculation of free Ca^{2+} levels were as described in the text. Results are each typical of three similar experiments. Increased noise in Ca^{2+} measurements was typically encountered with short-term cultured cells (24 hr or less after passaging).

and/or functional assembly of Ca^{2+}-pumping organelles. As long as TG is absent, this new protein will be functional; if TG is present, then even newly synthesized pump will be blocked.

The results establish a clear link between Ca^{2+} pool content and cell growth. The question of how this occurs is intriguing. There are two hypotheses. The first stems from the premise succinctly described by Sambrook (26) that Ca^{2+} levels inside the ER may control ER functions; without Ca^{2+}, those specific functions of the ER such as synthesis, processing, and trafficking of proteins could be compromised, resulting in cell growth arrest. Rather little information exists on the role and significance of Ca^{2+} inside ER mainly because of the paucity of means to specifically alter only intraluminal Ca^{2+}. Depletion of internal Ca^{2+} pools by EGTA and ionophores can impair translation of proteins (27); however, these agents cause substantial changes in cytosolic Ca^{2+}, which can directly inhibit initiation of translation (28). We noted a 70% reduction in total protein synthesis in DDT_1MF-2 cells during TG treatment (18); however, the remaining activity was sustained for several days and probably represents that level of protein synthesis expected of quiescent cells. Events within ER including translocation, folding, and processing of proteins could be altered by changed intraluminal Ca^{2+}. Ionophore-induced decreases in intraluminal Ca^{2+} can impair processing of proteins in ER (29, 30) and enhance expression of resident ER proteins such as BiP (Grp78) believed to associate with misfolded proteins (26). Recent studies (31) indicate that although TG-induced ER Ca^{2+} depletion can prevent correct folding of a specific Ca^{2+}-binding subunit of the asialoglycoprotein receptor, folding and secretion of other proteins such as albumin or transferrin are unaffected by TG. TG also does not detectably alter either synthesis or covalent post-translational modification of secreted proteins (31). With regard to trafficking and intervesicular transport of ER proteins, although *cytosolic* Ca^{2+} levels may modify these events (32), TG-induced depletion of ER Ca^{2+} appears to have no general effects on transport of proteins between

‡Cells were grown in 2.5% Calf-Plus, which is newborn calf serum supplemented with growth factors. Essentially identical results were obtained using fetal calf serum instead of Calf-Plus.

§The effect of high serum was not simply to bind or mask the action of TG. If cells were treated for 24 hr prior to passaging with 20% serum together with TG, followed by passaging and growth in medium with 2.5% serum without TG, then growth was still completely inhibited. Thus, TG still functions to inhibit proliferation in high serum and does so with a dose dependency similar to that in low serum.

ER and Golgi or post-Golgi secretory events (31). In general, whereas TG-treated cells are growth arrested, we noted previously (18) that they are otherwise functional since they (*i*) remain intact and viable for days, (*ii*) retain normal cellular and subcellular morphology, (*iii*) contain metabolically functional mitochondria, (*iv*) continue protein synthesis, albeit at a reduced rate, and (*v*) maintain physiologically normal $[Ca^{2+}]_i$.

The second and perhaps more likely hypothesis is that depletion of Ca^{2+} from ER eliminates the source of Ca^{2+} signals required as a stimulus for growth at one or more critical points in the cell cycle. Much evidence supports the view that intracellular Ca^{2+} exerts important control over cell cycle events (33) and that growth-regulating Ca^{2+} signals can be generated by growth factor-induced inositol phospholipid breakdown (1, 34). Although there is considerable uncertainty regarding how Ca^{2+} signals are precisely coordinated within the cell cycle (33), it appears that intracellular Ca^{2+} signals can mediate nuclear envelope breakdown and mitotic progression in dividing cells (35, 36) and that such signals can be generated through InsP_3-induced release of intracellular Ca^{2+} stores (37). A variety of growth factors mediate Ca^{2+} signals generated through activation of phospholipase C, inositol phospholipid breakdown, and InsP_3-activated channel opening in ER (1, 34). There is evidence that such Ca^{2+} signals play an important role in activating the transition from G_0 to G_1 during growth factor-induced onset of growth in quiescent cells (34, 38). Our results show that cells treated with Ca^{2+} pump inhibitors are devoid only of that Ca^{2+} store that is specifically releasable by InsP_3 (17, 18). The absence of Ca^{2+} from this store would obviously preclude the generation of Ca^{2+} signals mediated through inositol phospholipid breakdown and required for growth activation. Since Ca^{2+} pump-arrested cells appear to be held in a state equivalent to confluence-arrested cells and can be released from this state at any time upon refilling of the intracellular Ca^{2+} pool, it is tempting to consider that essential Ca^{2+} signals required for the transition between G_0 and G_1, and perhaps also required for maintenance of the G_1 state, can only be generated when the pool is filled. The InsP_3- and Ca^{2+}-pump blocker-sensitive Ca^{2+} pool almost certainly includes the nuclear envelope (6–10) and hence is proximal to the genetic machinery and nuclear events likely to be important for cell cycle control. The spatial and temporal nature of Ca^{2+} signals generated from this pool as well as the nature of the effectors mediating such Ca^{2+} signaling events will be important areas to address.

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