Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 551-558

EVIDENCE FOR MULTIPLE INTRACELLULAR CALCIUM POOLS IN GH_4C_1 CELLS: INVESTIGATIONS USING THAPSIGARGIN*

Hiroyuki Koshiyama[1] and Armen H. Tashjian, Jr.[2]

Laboratory of Toxicology, Harvard School of Public Health,
and the Department of Biological Chemistry and Molecular
Pharmacology, Harvard Medical School, Boston, MA 02115

Received April 25, 1991

SUMMARY The actions of thapsigargin (Tg), a plant sesquiterpene lactone, on Ca^{2+} homeostasis were investigated in digitonin-permeabilized GH_4C_1 rat pituitary cells. Tg (1 μM) caused a rapid and sustained increase in ambient Ca^{2+} concentration ($[Ca^{2+}]$) and inhibited the rise in $[Ca^{2+}]$ induced by subsequent addition of TRH (100 nM), inositol 1,4,5-trisphosphate (IP_3, 10 μM), or the nonhydrolyzable GTP analogue guanosine 5'-0-(3-thiotriphosphate) (GTPγS, 10 μM). However, neither IP_3 nor GTPγS pretreatment, which themselves release sequestered Ca^{2+}, prevented the Ca^{2+} accumulation induced by Tg. Pretreatment with heparin (100 μg/ml, 10 min), an IP_3 receptor antagonist, did not affect Ca^{2+} accumulation induced by Tg, although it abolished the rise in $[Ca^{2+}]$ induced by IP_3. The ability of Tg to increase $[Ca^{2+}]$ was dependent on added ATP. We conclude that, in GH_4C_1 cells, Tg acts, in part, on TRH-, IP_3- and GTPγS-sensitive Ca^{2+} pools; however, Tg also acts on an ATP-dependent pool of intracellular Ca^{2+} which is not sensitive to TRH, IP_3 or GTPγS, indicating a complexity of intracellular Ca^{2+} pools not previously appreciated in these cells.

Inositol 1,4,5-trisphosphate (IP_3) is one of the mediators that mobilizes intracellular Ca^{2+} in response to agonists which act via cell surface receptors (1). However, the IP_3-sensitive intracellular Ca^{2+} pool accounts for only a portion of the total sequestered intracellular Ca^{2+} (1). Gill and his colleagues have proposed that there are two intracellular Ca^{2+} pools, distinguishable by IP_3 sensitivity and oxalate permeability, in several non-pituitary cell types (2). In their model, GTP mediates the direct transfer of Ca^{2+} from an IP_3-insensitive to an IP_3-responsive pool. GTPγS does not mimic the action of GTP in this transfer process. Recently, we have described the involvement of a putative G protein in the translocation of Ca^{2+} from an IP_3-insensitive to an IP_3-responsive pool by movement of the ion indirectly through the cytosol

*This investigation was supported in part by a grant from the National Institute of Diabetes, Digestive and Kidney Diseases (DK 11011).

[1]Supported in part by the Lilly International Fellowship Program.

[2]To whom all correspondence should be addressed.

0006-291X/91 $1.50

in GH_4C_1 cells (3). In these cells, GTP was ineffective, suggesting that different intracellular Ca^{2+} redistribution systems exist in different cell types.

Thapsigargin (Tg), a sesquiterpene lactone from the roots of the plant *Thapsia garganica L.* (4), is a nonphorbol ester tumor promoter (5). Tg causes elevation of cytosolic free Ca^{2+} in several cell types (6-12). Tg prevents the rise in intracellular Ca^{2+} by agents that stimulate the formation of IP_3 (7,8,10,12), indicating that it acts, at least in part, on an IP_3-sensitive target pool. However, Tg is not a Ca^{2+} ionophore (13), and it does not affect the generation of inositol polyphosphates (7,10). Tg is a potent inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase in rat hepatocytes (11), suggesting that Tg causes the rise in cytosolic free Ca^{2+} by preventing the ATP-dependent sequestration of Ca^{2+} into intracellular stores. In the present study, we investigated the actions of Tg in GH_4C_1 cells permeabilized with digitonin and found that Tg acts on multiple intracellular Ca^{2+} pools, including those responsive to TRH, IP_3 and GTPγS as well as an additional ATP-dependent pool.

MATERIALS AND METHODS

Materials: TRH was obtained from Peninsula Laboratories (Belmont, Ca). Heparin (H3125, grade I), IP_3, ATP and GTPγS were purchased from Sigma Chemical Co. (St. Louis, MO). Digitonin, Tg and fura-2 acetoxymethyl ester (fura-2/AM) were obtained from Fisher Scientific Co. (Pittsburgh, PA), LC Services Co. (Woburn, MA) and Molecular Probes Inc. (Eugene, OR), respectively. All other chemicals used were of reagent grade.

Cell Culture: GH_4C_1 cells were grown as monolayers in 100 mm culture dishes in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum ($F\text{-}10^+$) in a water-saturated atmosphere of 5% $C0_2$ and 95% air at 37°C, as described previously (14-16). The cells were grown for 7-10 days and refed with fresh $F\text{-}10^+$ 24 h prior to each experiment.

Permeabilized Cell Studies: Cells were permeabilized with digitonin as described previously (3,17). The permeabilized cells were incubated with 3 μM fura-2/AM for 35 min at 37°C. They were washed twice, then incubated for 5 min at room temperature, and washed once again. The cells were then added to a cuvette, kept at 37°C, and stirred continuously throughout the experiment. A preincubation period of 10 min was used after adding cells to the cuvette before starting each experiment. This maneuver reduced the variation in response to Tg. Fluorescence was measured in a Spex Fluorog F 111A spectrophotometer (excitation wavelength, 342 nm; emission wavelength, 492 nm) (3,16). The ambient free Ca^{2+} concentration ($[Ca^{2+}]$) was calculated using the equation: $[Ca^{2+}] = K_d\,(F\text{-}F_{min})/(F_{max}\text{-}F)$, where K_d = 224 nM, and F is the fluorescence signal in arbitrary units. F_{max} was obtained with 2 mM $CaCl_2$ in the presence of 25 μM digitonin, and F_{min} was obtained with 5 mM EGTA (pH was adjusted to > 8.3 with 1 mM Tris-base). The determined $[Ca^{2+}]$ in this system is a measure of the total free Ca^{2+} in the permeabilized preparation and, therefore, reflects the balance between Ca^{2+} release from and Ca^{2+} uptake into sequestered pools. Because the volume of medium in the cuvette vastly exceeds the intracellular cytosolic volume, the measured changes in $[Ca^{2+}]$ are relatively small (3).

We found no qualitative differences between results obtained with fura-2/AM and those using fura-2 free acid (3). Cells were shown to be 94 ± 1% permeabilized and they did not reseal under the conditions used (3). In addition, fura-2 free acid, not fura-2/AM, responded to the change in $[Ca^{2+}]$ (3). Therefore, the $[Ca^{2+}]$ measurements reported here do not represent those made on resealed cells which might have taken up fura-2/AM.

Statistical Evaluation: Statistical differences were calculated by analysis of variance in combination with Student's *t*-test.

RESULTS

Tg increases $[Ca^{2+}]$ in permeabilized GH_4C_1 cells and prevents the increase in $[Ca^{2+}]$ induced by TRH: Tg caused an increase in $[Ca^{2+}]$ in permeabilized GH_4C_1 cells. The rise in $[Ca^{2+}]$ reached a peak within < 1 min and showed a sustained elevation for more than 5 min (Fig. 1A, *right panel*). TRH caused a smaller sustained increase in $[Ca^{2+}]$ (Fig. 1A, *left panel*), as reported previously (3). Pretreatment with Tg for 5 min blocked the increase of $[Ca^{2+}]$ induced by TRH (Fig. 1B). In contrast, TRH pretreatment did not block the Ca^{2+} response to subsequent addition of Tg (data not shown). Taken together, these results suggest that Tg and TRH act on overlapping, but nonidentical, Ca^{2+} pools.

Tg pretreatment prevents the Ca^{2+}-releasing actions of IP_3 and GTPγS: When Tg was added 5 min before IP_3, Tg prevented the increase in $[Ca^{2+}]$ induced by IP_3 (Fig. 2A). However, when Tg was added 5 min after IP_3, IP_3 pretreatment did not affect the action of Tg (Fig. 2A). Serial addition of IP_3, which resulted in attenuation of the response to IP_3, did not inhibit the action of Tg (Fig. 2B). As in the case with TRH, these results suggest that Tg and IP_3 also act on overlapping, but nonidentical, intracellular Ca^{2+} pools. Tg pretreatment also

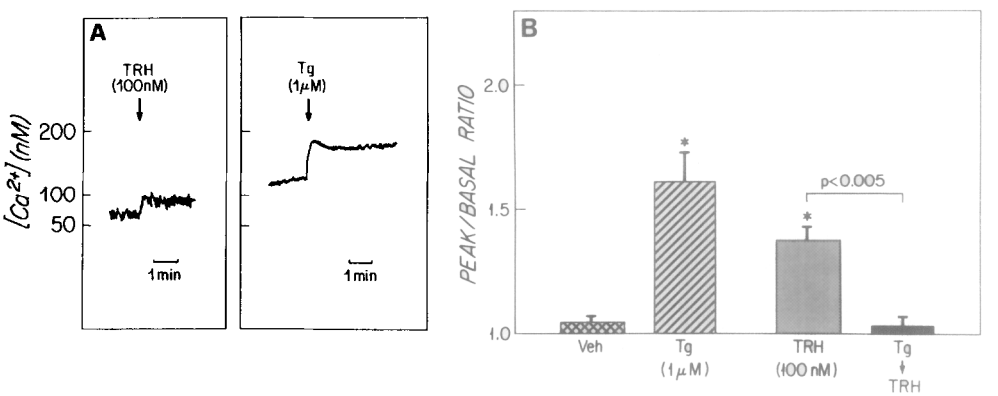


Fig. 1A. Actions of Tg and TRH on $[Ca^{2+}]$ in permeabilized GH_4C_1 cells. Permeabilized cells were treated with 1 μM Tg (*right panel*) or 100 nM TRH (*left panel*). These traces are representative of results obtained in many experiments. Basal $[Ca^{2+}]$ varied about two-fold between experiments with this permeabilization procedure; however, the responses shown here were highly reproducible as summarized in Fig. 1B.

Fig. 1B. Summary of 3-14 independent experiments showing the actions of Tg and TRH on $[Ca^{2+}]$ in permeabilized GH_4C_1 cells. Each *bar* gives the mean value of the peak/basal ratio of $[Ca^{2+}]$ and the *brackets* give the SE. Peak $[Ca^{2+}]$ was taken as the maximum $[Ca^{2+}]$ achieved within the first 30 sec after addition of test compound. Basal $[Ca^{2+}]$ was that measured before any treatment. In the fourth column to the right, the effect of pretreatment with Tg (1 μM) on the Ca^{2+} response to the subsequent addition of TRH (100 nM) is shown. Basal $[Ca^{2+}]$, under these conditions, was taken as the $[Ca^{2+}]$ immediately before the addition of TRH. * $P < 0.05$, difference from vehicle (Veh) control.

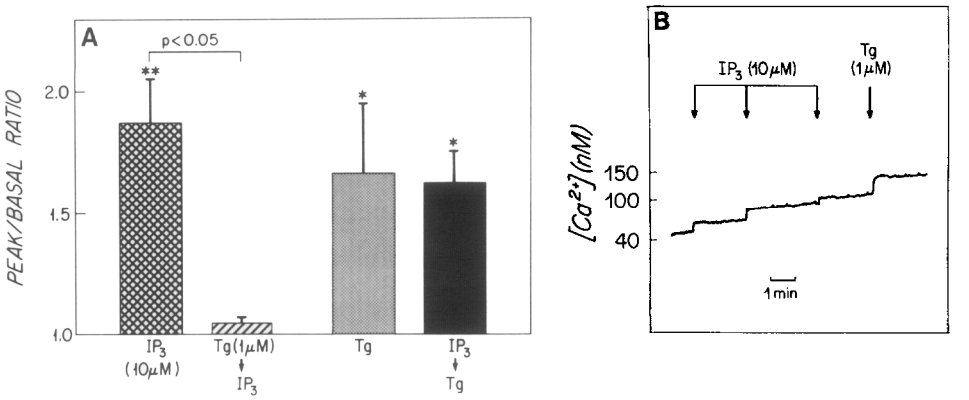


Fig. 2A. Interactions of Tg and IP_3 on $[Ca^{2+}]$ in permeabilized GH_4C_1 cells. IP_3 (10 μM) or Tg (1 μM) was added 5 min before the addition of the second agent. Each *bar* gives the mean value of the peak/basal ratio of $[Ca^{2+}]$, and the *brackets* give the SE of 3-17 independent experiments. Basal $[Ca^{2+}]$ was taken as the $[Ca^{2+}]$ immediately before addition of the second agent when two agents were added sequentially. * $P < 0.05$, difference from vehicle control. ** $P < 0.01$, difference from vehicle control.

Fig. 2B. Repeated addition of IP_3 (10 μM each addition) caused attenuation of the rise in $[Ca^{2+}]$ in permeabilized GH_4C_1 cells. Subsequent addition of Tg (1 μM) caused an additional highly significant increase in $[Ca^{2+}]$. This experiment has been repeated several times with similar results.

blocked the Ca^{2+}-releasing action of GTPγS, but GTPγS did not prevent the rise in $[Ca^{2+}]$ induced by Tg (Fig. 3). In aggregate, these data indicate that Tg acts not only on intracellular Ca^{2+} pools which are targets for TRH, IP_3, and GTPγS, but also on some additional pool or pools which are not depleted by repeated exposure to IP_3 and are not responsive to GTPγS.

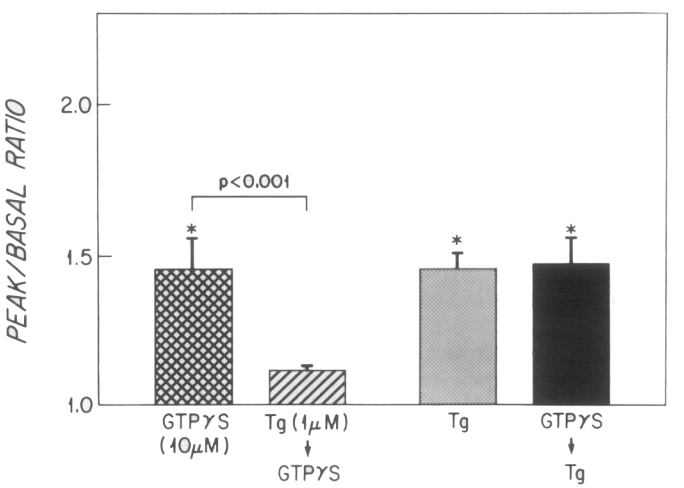
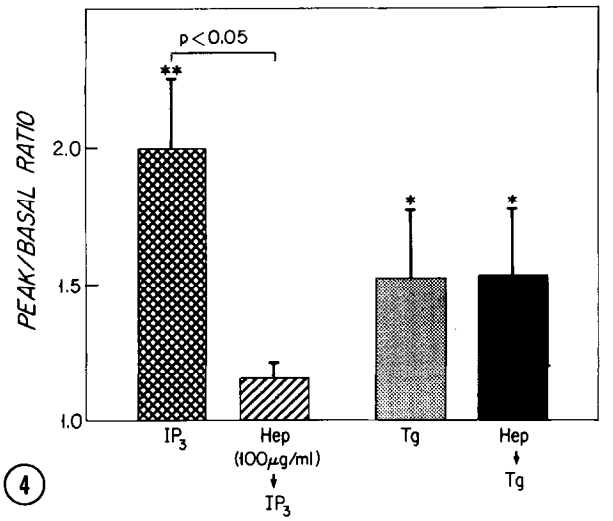


Fig. 3. Interactions of Tg and GTPγS on $[Ca^{2+}]$ in permeabilized GH_4C_1 cells. GTPγS (10 μM) or Tg (1 μM) was added 5 min before the addition of the second agent. Each *bar* gives the mean value of the peak/basal ratio of $[Ca^{2+}]$, and the *brackets* give the SE of 3-16 independent experiments. * $P < 0.05$ difference from vehicle control.



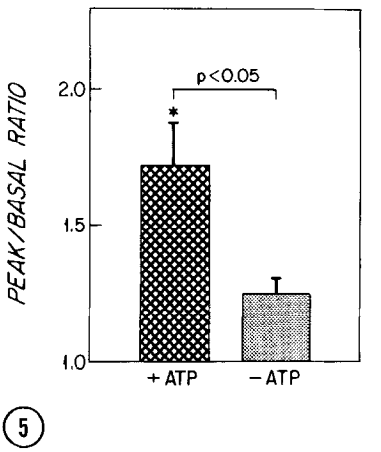


Fig. 4. Effect of heparin on the $[Ca^{2+}]$ increase induced by IP_3 and Tg. Permeabilized GH_4C_1 cells were preincubated with heparin (Hep, 100 μg/ml) for 10 min at 37°C before addition of IP_3 (10 μM) or Tg (1 μM). Each *bar* gives the mean value of the peak/basal ratio of $[Ca^{2+}]$, and the *brackets* give the SE of 3-10 independent experiments. * and ** give $P < 0.05$ or $P < 0.01$, respectively, for difference from vehicle control.

Fig. 5. Effect of Tg on $[Ca^{2+}]$ in permeabilized GH_4C_1 cells in the presence or absence of added ATP. In the -ATP experiments, ATP was excluded from all buffers, and the effect of Tg (1 μM) was examined in permeabilized cells. In the +ATP experiments, the concentration of added ATP was 0.5 mM. Each *bar* gives the mean value of the peak/basal ratio of $[Ca^{2+}]$, and the *brackets* give the SE of 3-8 independent experiments. * $P < 0.05$ difference from vehicle control.

Roles of the IP_3 receptor and ATP in the actions of Tg: Heparin binds to the IP_3 receptor (18) and inhibits IP_3-activated Ca^{2+} release (19). Preincubation of permeabilized GH_4C_1 cells with heparin (100 μg/ml, 37°C for 10 min), which blocks the action of IP_3 in this preparation (3, and Fig. 4), did not inhibit the effect of Tg (Fig. 4), indicating that Tg does not act via stimulation of the IP_3 receptor by IP_3.

In order to investigate the role of ATP in the action of Tg, we examined the effect of Tg on $[Ca^{2+}]$ in permeabilized GH_4C_1 cells incubated in the medium lacking ATP. In the absence of added ATP, the action of Tg was greatly diminished (Fig. 5).

DISCUSSION

In the present study in permeabilized GH_4C_1 cells, Tg increased $[Ca^{2+}]$ and inhibited the TRH-induced the rise in $[Ca^{2+}]$. These results are consistent with findings previously reported in intact GH_4C_1 cells (10); these authors suggested that the TRH-responsive compartment is included within the Tg-releasable pool of sequestered calcium. However, unlike the results of Law *et al.* in intact cells (10), we did not detect an inhibitory effect of TRH on the rise in $[Ca^{2+}]$ induced by Tg in permeabilized GH_4C_1 cells. In other cell systems, $PGF_{2\alpha}$ did not

prevent the rise in $[Ca^{2+}]$ induced by Tg, although Tg abolished the response to $PGF_{2\alpha}$ (12). Jackson *et al.* (7) have offered several possible explanations why Tg produces a further rise in $[Ca^{2+}]$ after maximal stimulation by a Ca^{2+}-mobilizing agonist: (a) Tg acts, at least in part, on a separate or different Ca^{2+} store; (b) Tg acts on the same store but after resequestration of Ca^{2+} into that store; (c) Tg acts on a subpopulation of cells which is insensitive to the agonist. The second hypothesis seems unlikely in permeabilized GH_4C_1 cells, because TRH induced a sustained increase in $[Ca^{2+}]$ which is not be compatible with extensive resequestration within the time intervals studied. It is difficult to rule out the third hypothesis, but there is no direct evidence to support it at this time. We favor the first proposal for the reasons given below.

It has been reported that Tg abolishes the Ca^{2+}-releasing activity of GTP and IP_3 in rat liver microsomes (11). Addition of Tg together with IP_3 does not give an additive effect on $[Ca^{2+}]$ accumulation in permeabilized rat parotid acinar cells (8) or rat brain microsomes (20). These results suggest that Tg causes accumulation of free Ca^{2+} from the same or overlapping pools of sequestered Ca^{2+} that are targets for IP_3 and GTP. In the present study, Tg inhibited Ca^{2+} mobilization induced by IP_3 and GTPγS. In permeabilized GH_4C_1 cells, GTP is ineffective in raising $[Ca^{2+}]$, but GTPγS does raise $[Ca^{2+}]$ and causes refilling of an IP_3-responsive pool (3). Thus, we have proposed that a putative G protein, which does not require GTP hydrolysis for its action, is involved in the translocation of intracellular Ca^{2+} from an IP_3-insensitive pool to an IP_3-sensitive pool (3). Although it has been reported that treatment with GTP or IP_3 can decrease the amount of Tg-releasable Ca^{2+} in rat liver microsomes (11), neither GTPγS nor IP_3 affected Tg-induced Ca^{2+} accumulation in permeabilized GH_4C_1 cells. Because the responses to TRH, IP_3 and GTPγS were all abolished by Tg, the Ca^{2+} pools sensitive to TRH, IP_3 and GTPγS must be included in the Tg-sensitive pool or pools. However, the failure of TRH, IP_3 and GTPγS to prevent the Tg response suggests the existence of a Ca^{2+} pool which is insensitive to TRH, IP_3 and GTPγS, but is responsive to Tg. Furthermore, results with rat brain microsomes (20) suggest that some Ca^{2+} pools are insensitive to Tg, and some are sensitive to both Tg and IP_3, indicating marked differences between cell types. Therefore, we conclude that Tg acts, in part, on Ca^{2+} pools that are responsive to TRH, IP_3 and GTPγS; however, Tg also acts on a Ca^{2+} pool which is not sensitive to these three agents.

Tg does not act on the IP_3 receptor in GH_4C_1 cells because heparin, which blocked the Ca^{2+} release induced by IP_3 (3), did not inhibit Tg action. A similar result has been observed in rat brain microsomes (20). The absence of ATP greatly diminished or prevented the action of Tg on Ca^{2+} accumulation in our experiments. This effect is probably due to the lack of

Ca^{2+} sequestration in target pools produced by the ATP-dependent Ca^{2+} pump. In addition, because Tg has been reported to act by inhibiting endoplasmic reticulum Ca^{2+} pumps (11) thereby preventing Ca^{2+} sequestration, it is likely that the lack of effect of Tg, in the absence of ATP, is due to little or no pumping by the Tg-sensitive Ca^{2+}-ATPase. We have reported that ATP is necessary for the immediate action of GTPγS to release Ca^{2+}, but not for the acute actions of IP_3 and TRH in permeabilized GH_4C_1 cells (3), suggesting a greater dependency on ATP of the rapid actions of Tg and GTPγS than for the actions of IP_3 and TRH. In some other cell systems, studies indicate a requirement for ATP in IP_3-induced Ca^{2+} mobilization (21,22); however, others do not (23,24). In addition, it is clear that ATP acts elsewhere in addition to its role to power the Ca^{2+}-ATPase, for example in protein phosphorylation (21,23).

In summary, Tg acts in permeabilized GH_4C_1 cells, at least in part, on Ca^{2+} pools which are sensitive to TRH, IP_3 and GTPγS, but in addition Tg may have another Ca^{2+} pool as its target. The additional Tg-responsive pool or pools is insensitive to IP_3, GTPγS and TRH, suggesting a complexity in intracellular Ca^{2+} homeostasis not heretofore recognized.

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