Involvement of intact inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores in cell cycle progression at the G1/S boundary in serum-stimulated human fibroblasts

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Abstract Thapsigargin, a selective inhibitor of the endoplasmic reticulum Ca^{2+} pump, has been shown to deplete inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores. Here we report that when thapsigargin was introduced to serum-stimulated human fibroblasts at a time point just before the G1/S boundary, it completely inhibited expression of cyclin A, activation of $p33^{CDK2}$ cyclin-dependent kinase and initiation of DNA synthesis. In contrast, the Ca^{2+} mobilizing ionophore ionomycin was without effect. These findings indicate that Ca^{2+} inside the inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores plays a pivotal role for traverse across the G1/S transition point.

Key words: Thapsigargin; Ca^{2+} store; Cell cycle; Cyclin-dependent kinase

1. Introduction

It has been widely recognized that stimulation of quiescent cells with a variety of growth factors or mitogens induces a transient rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), through rapid Ca^{2+} mobilization from both inositol-1,4,5-trisphosphate ($InsP_3$)-sensitive intracellular stores and outside of cells [1–5]. The increase in $[Ca^{2+}]_i$ is postulated to serve as a mitogenic signalling for re-entry into the cell cycle at the G0/G1 border [6,7]. Recently, Gill and his colleagues [8–10] have demonstrated that thapsigargin, which is a selective inhibitor of endoplasmic reticulum Ca^{2+} pump [11] and depletes $InsP_3$-sensitive Ca^{2+} stores [12,13], potently inhibits cell proliferation. In their experiments, density-arrested DDT_1MF-2 smooth muscle cells were growth-stimulated by trypsinizing and replating into fresh growth media. When thapsigargin was added to cells at the time of plating, it completely inhibited the following DNA synthesis and cell division, without altering cell morphology or viability. The thapsigargin dose–response curve for inhibition of DNA synthesis was comparable to that for inhibition of Ca^{2+} pumping activity into $InsP_3$-sensitive Ca^{2+} stores [8,13]. The growth inhibitory effect of thapsigargin was relieved only after washout of thapsigargin and refilling of IP_3-sensitive Ca^{2+} stores [10]. In contrast, when thapsigargin was added to actively proliferating cells 24 h after growth stimulation, the growth inhibitory effect of thapsigargin was substantially reduced [8]. These findings led the authors to the conclusion that a specific cell cycle event necessary for the G0/G1 transition depends upon signals generated from functional $InsP_3$-sensitive Ca^{2+} stores [9,10]. However, a question whether or not IP_3-sensitive Ca^{2+} stores are involved in the other phases of cell cycle progression is not addressed in these studies. Also, molecular mechanisms underlying thapsigargin-induced inhibition of DNA synthesis are not well resolved yet.

In the present study, we explored a possibility that functional $InsP_3$-sensitive Ca^{2+}stores are required for cell cycle progression at points later than the G0/G1 border. Further, we tried to determine whether IP_3-sensitive Ca^{2+} stores play any role in the activation of a cyclin-dependent protein kinase which is critical for cell cycle progression. We found that thapsigargin completely inhibits DNA synthesis when it is added to cells just before the G1/S boundary. The results of the present study indicate that the mechanism of this thapsigargin-mediated G1/S block involves complete suppressions of cyclin A expression and $p33^{CDK2}$ kinase activation.

2. Materials and methods

Human foetal lung-derived diploid fibroblasts IMR-90 were maintained at subconfluent state in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum as described [14]. Before each experiment confluent cultures were deprived of serum for 2 days in DMEM containing 0.2% bovine serum albumin and made quiescent [14]. Cells were growth-stimulated by changing this medium to fresh growth media (0 time point). Thapsigargin (Sigma) was added to cells at indicated points. [^{3}H]Thymidine incorporation into DNA was measured by pulse-labeling of cells with 2 μCi/ml of [^{3}H-methyl]thymidine during the last 1 h of cell incubations, followed by counting of radioactivity incorporated into acid-precipitable material [14]. Measurement of histone H1 kinase activity associated with $p33^{CDK2}$ and Western blot analysis of $p33^{CDK2}$ were performed by using a polyclonal antibody raised against a synthetic peptide corresponding to the carboxyl terminal sequence of human $p33^{CDK2}$ as described in detail elsewhere [15]. Western blot analyses of cyclin A and cyclin D1 were performed by using rabbit polyclonal antibodies raised against the carboxyl terminal sequence of human cyclin A (UBI) and human cyclin D1 (Medical and Biological Laboratories, Nagoya, Japan), respectively.

3. Results and discussion

Stimulation of quiescent IMR-90 cells with 10% foetal calf serum resulted in the initiation of DNA synthesis after the G1 period of 12–14 h [14]. As shown in Fig. 1A, when thapsigargin was added just prior to the addition of serum, it dose-dependently inhibited DNA synthesis with the EC_{50} value of approximately 10 nM, which is comparable to that reported for DDT_1MF-2 smooth muscle cells (20 nM) [8]. Thapsigargin had no effect on cell viability or morphology. To examine whether or not thapsigargin blocks cell cycle progression at steps later than the G0/G1 border, we added thapsigargin at various time points after serum addition (= zero time point) and measured DNA synthesis at 22 h (Fig. 1B). Thapsigargin similarly and

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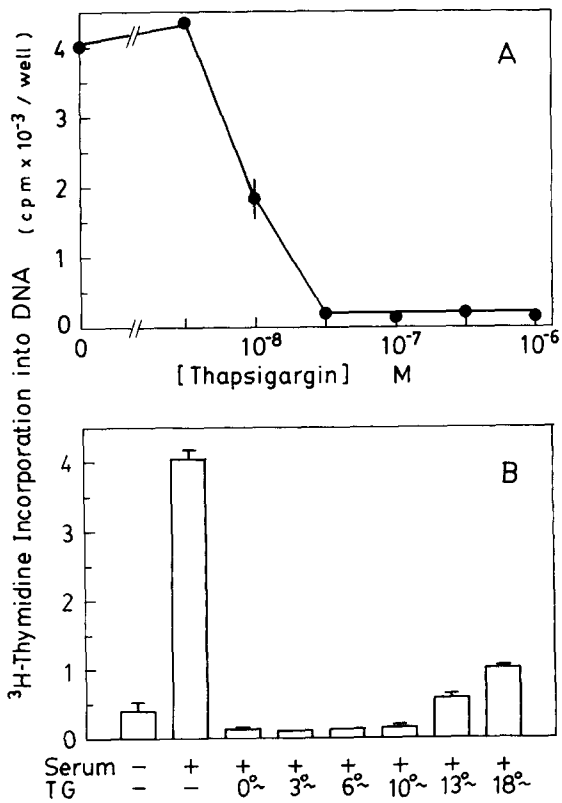

Fig. 1. A. The thapsigargin dose–response curve for inhibition of DNA synthesis in IMR-90 human diploid fibroblasts. Various concentrations of thapsigargin were added just before serum stimulation, and [^{3}H]thymidine incorporation into DNA was measured after 22 h. B. Thapsigargin completely inhibits DNA synthesis when it is added to serum-stimulated cells at any time point in the G1 phase and even just before the G1/S boundary. Thapsigargin (0.1 μM) (TG) was added to cells at indicated times (h) after serum stimulation and [^{3}H]thymidine incorporation was measured at 22 h. Data represent the mean ± S.E. of three determinations. Bars are absent where the S.E. value is smaller than half the size of the symbol (A) or the width of the line (B).

completely inhibited DNA synthesis when it was introduced to cultures at either 0, 3, 6 or 10 h after serum stimulation. When thapsigargin was added to cells either 13 or 18 h after serum stimulation, i.e. the time points that correspond to the G1/S boundary and the early S phase, respectively, it still very strongly inhibited serum-stimulated DNA synthesis, though to lesser extents as compared to earlier additions. These results clearly indicate that thapsigargin completely blocks entry into the S phase when it is added to cells at any time point in the G1 phase and, most notably, even just before the initiation of DNA synthesis.

Recent studies have revealed that cell cycle is driven by sequential activation of a family of cyclin-dependent kinases (CDKs) [16]. CDK2/cyclin A complex has been demonstrated to be indispensable for entry into and progression through the S phase. Thus, p33^{CDK2}-associated kinase activity starts to rise just prior to the G1/S boundary and continues to increase throughout the S phase [15,17]. p33^{CDK2} protein is readily detectable in quiescent cells and moderately increases as cells progress into the S phase [15,17]. In contrast, cyclin A is undetectable in the G0 or the G1 phase, and is induced to be expressed at the G1/S border, progressively accumulating along with the S phase progression [18]. The activation of CDK2 as an active kinase depends on both the complex formation with a cyclin subunit and phosphorylation at Thr160 by CDK activating kinase (CAK) [19–21]. Microinjection of antisense cyclin A cDNA or antibodies against either cyclin A or p33^{CDK2} has been shown to block completely the initiation of DNA synthesis [17,18,22,23]. In addition, it is reported that cyclin A and CDK2 colocalize at the site of DNA replication in S phase nucleus [24].

Based on these current understandings, we tested the possibility that thapsigargin might affect the activation of cyclin A/CDK2 complex as an underlying molecular mechanism for cell cycle block at the G1/S border. Indeed, as shown in Fig. 2, thapsigargin completely inhibited serum-induced activation of CDK2 kinase, with a dose-response relationship similar to that obtained for inhibition of DNA synthesis (see Fig. 1A). Importantly, thapsigargin completely abolished the CDK2 kinase activation when it was added to cells as late as 10 h after serum stimulation, just like DNA synthesis. The inhibitory effect of thapsigargin was slightly attenuated when it was added at 13 h, i.e. at the beginning of the S phase. Thapsigargin did not at all directly inhibit the CDK2 kinase activity in vitro when it was introduced into kinase assay tubes, indicating that the target(s) of thapsigargin action resides in one or more steps of CDK2 activation process in vivo. We then examined protein levels of p33^{CDK2} and cyclin A by Western blot analysis (Fig. 3). In serum-stimulated cells, as cells progressed into the S phase the protein level of p33^{CDK2} gradually increased and the Thr160-phosphorylated active form emerged, the latter being detected as a band with a higher mobility on electrophoresis [19]. Thapsigargin-mediated inhibition of p33^{CDK2} kinase activation was associated with total disappearance of the Thr160-phosphorylated form of p33^{CDK2}, but not detectable decrease in the amount of the major p33^{CDK2} band as compared to serum-stimulated control cells (Fig. 3, upper panel). More significantly, thapsigargin completely abolished the expression of cyclin A in serum-stimulated cells (Fig. 3, lower panel). Induction of cyclin A was similarly abolished whether thapsigargin was added to cells at the G0/G1 border (0 time point) or at later

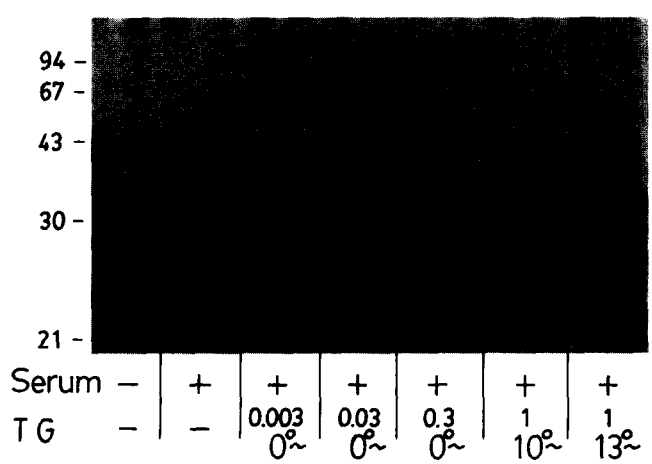

Fig. 2. Thapsigargin completely inhibits the activation of CDK2 kinase when it is added to cells just before the G1/S boundary. Thapsigargin (0.003–1 μM) was added to cells at indicated time points (h) after serum stimulation and p33^{CDK2}-associated histone H1 kinase activity was measured at 22 h. Thapsigargin up to 1 μM did not inhibit CDK2 kinase activity in vitro when introduced into kinase assay tubes.

time points, even 10 h after serum stimulation that is just before the G1/S boundary. From these observations it was concluded that thapsigargin suppressed serum-induced activation of CDK2 kinase through mechanisms involving inhibitions of cyclin A expression and CAK-mediated phosphorylation of $p33^{CDK2}$ at Thr160. In addition, thapsigargin inhibited serum-induced increase in the amount of a G1 cyclin, cyclin D1, to a basal unstimulated level (Fig. 3, lower panel), raising a possibility that thapsigargin inhibits activation of other members of CDKs including CDK4 [16].

It is generally known that thapsigargin induces an increase in $[Ca^{2+}]_i$. Therefore, it might be possible that the inhibitory effects of thapsigargin on G1/S progression were mediated through an increase in $[Ca^{2+}]_i$. To test this possibility, we studied whether or not the Ca^{2+} ionophore ionomycin mimicked the effects of thapsigargin. Ionomycin (0.1 μM), when added 5 h after serum stimulation, barely affected [^{3}H]thymidine incorporation into DNA (4029 ± 188 vs. 3833 ± 75 cpm per well for serum-stimulated control vs. serum-stimulated, ionomycin-treated cells, mean ± S.D. from three determinations). Under the same experimental condition, ionomycin did not inhibit the expression level of either cyclin A or cyclin D1 (Fig. 4). The results clearly indicate that depletion of $InsP_3$-sensitive Ca^{2+} stores, but not mobilization of Ca^{2+} itself, is responsible for thapsigargin-induced cell cycle arrest at the G1/S boundary.

Ghosh et al. [8] previously reported that the addition of 3 μM thapsigargin to DDT_1 MF-2 cells at the G0/G1 border reduced

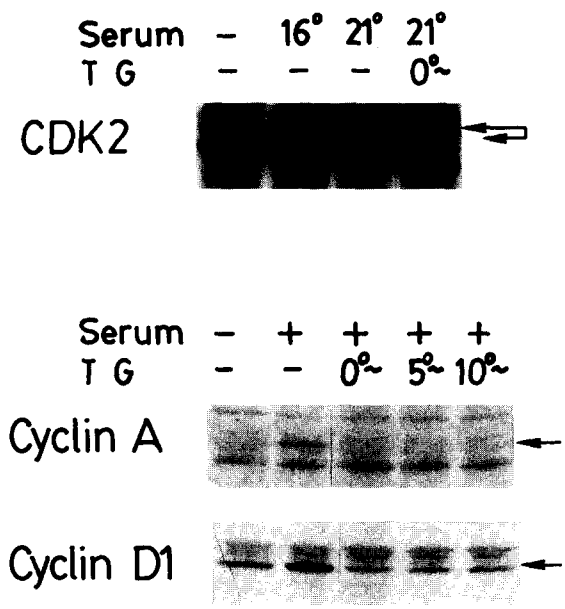

Fig. 3. (Upper panel) Thapsigargin does not suppress an increase in the protein level of CDK2, but completely inhibits CDK2 phosphorylation at Thr160 in serum-stimulated cells. Cells were incubated with serum for indicated time periods (h). Thapsigargin (0.1 μM) was added together with serum where indicated. Anti-$p33^{CDK2}$ immunoprecipitate was resolved by electrophoresis, followed by Western blotting. Positions of the major 33 kDa band (a long arrow) and the Thr160-phosphorylated form (a short arrow and a dot) are indicated. (Lower panel) Thapsigargin completely inhibits induction of cyclins A and D1 in serum-stimulated cells. Cells were incubated in the presence or absence of serum for 21 h. Thapsigargin (0.1 μM) was added at indicated time points (h) after serum stimulation. Seventy microgram of cellular protein was analyzed by Western blotting for cellular content of cyclins A and D1, the positions of which are indicated by arrows.

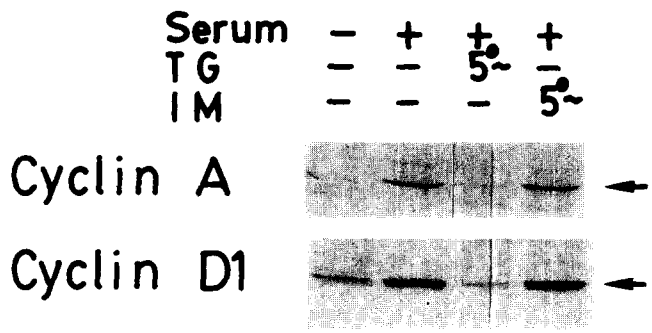

Fig. 4. Ca^{2+} mobilizing ionomycin does not inhibit serum-induced expression of cyclins A and D1. Thapsigargin (TG) or ionomycin (IM) at 0.1 μM each was added 5 h after serum stimulation and cell lysate was obtained at 21 h for Western blot analysis.

the incorporation of [^{3}H]methionine to cellular proteins by 70% after 56 h. We have also observed that 0.1 to 1 μM of thapsigargin suppressed serum-induced increase in the cellular protein content by approximately 60%. It is indeed suggested that Ca^{2+} inside endoplasmic reticulum plays a role in translation, processing and trafficking of certain proteins [25]. However, it is remarkable to note that, in a T lymphocyte cell line Jurkat cells, thapsigargin, in combination with a phorbol ester, induced a prominent *increase* in the synthesis of interleukin-2, in the face of depletion of $InsP_3$-sensitive Ca^{2+} stores [26]. Nevertheless, thapsigargin plus a phorbol ester failed to stimulate proliferation in this case. These observations strongly suggest that Ca^{2+} filling inside $InsP_3$-sensitive Ca^{2+} stores, which include endoplasmic reticulum, is critical for growth factor-induced expression of a subset of cellular proteins, but not others. Our findings clearly show that cyclins A and D1, but not CDK2, are included in the former category.

In summary, the present study demonstrates crucial involvement of Ca^{2+}-filled $InsP_3$-sensitive stores in cell cycle progression throughout the G1 phase and especially just before the G1/S transition point. In addition, Ca^{2+} inside nuclear envelope might also play a role in cell cycle progression, since the Ca^{2+} pump on the outer leaflet of nuclear envelope is reportedly identical to the endoplasmic reticulum Ca^{2+} pump [27].

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References

[1] McNeil, P.L., McKenna, M.P. and Taylor, D.L. (1985) J. Cell Biol. 101, 372–379.
[2] Metcalfe, J.C., Moore, J.P., Smith, G.A. and Hesketh, T.R. (1986) Br. Med. Bull. 42, 405–412.
[3] Takuwa, N., Takuwa, Y., Bollag, W.E. and Rasmussen, H. (1987) J. Biol. Chem. 262, 182–188.
[4] Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K. and Masaki, T. (1989) J. Biol. Chem. 264, 7856–7861.
[5] Gardner, P. (1989) Cell 59, 15–20.
[6] Clipstone, N.A. and Crabtree, G.R. (1992) Nature 357, 695–697.
[7] O'Keefe, S.J., Tamura, J., Kincaid, R., Tocci, M.J. and O'Neil, E.A. (1992) Nature 357, 692–694.
[8] Ghosh, T.K., Bian, J., Short, A.D., Rybak, S.L. and Gill, D.L. (1991) J. Biol. Chem. 266, 24690–24697.

[9] Short, A.D., Bian, J., Gosh, T.K., Waldron, R., Rybak, S.L. and Gill, D.L. (1993) Proc. Natl. Acad. Sci. USA 90, 4986–4990.
[10] Waldron, R.T., Short, A.D., Meadows, J.J., Ghosh, T.K. and Gill, D.L. (1994) J. Biol. Chem. 269, 11927–11933.
[11] Thastrup, O., Dawson, A.P., Scharff, O., Foder, B., Bjerrum, P.J. and Hanley, M.R. (1989) Agents Actions 27, 17–23.
[12] Takemura, H., Hughes, A.R., Thastrup, O. and Putney, J.W. Jr. (1989) J. Biol. Chem. 264, 12266–12271.
[13] Bian, J., Ghosh, T.K., Whang, G.-C. and Gill, D.L. (1991) J. Biol. Chem. 266, 8801–8806.
[14] Takuwa, N., Zhou, W., Kumada, M. and Takuwa, Y. (1993) J. Biol. Chem. 268, 138–145.
[15] Zhou, W., Takuwa, N., Kumada, M. and Takuwa, Y. (1993) J. Biol. Chem. 268, 23041–23048.
[16] Sherr, C.J. (1993) Cell 73, 1059–1065.
[17] Tasi, L.-H., Lees, E., Faha, B., Harlow, E. and Riabowol, K. (1993) Oncogene 8, 1593–1602.
[18] Girard, F., Strausfeld, U., Fernandez, A. and Lamb, N.J.C. (1991) Cell 67, 1169–1179.
[19] Fesquet, D., Labbé, J.-C., Derancourt, J., Capony, J.-P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Dorée, M. and Cavadore, J.-C. (1993) EMBO J. 12, 3111–3121.
[20] Poon, R.Y.C., Yamashita, K., Adamczewski, J.P., Hunt, T. and Shuttleworth, J. (1993) EMBO J. 12, 3123–3132.
[21] Solomon, M.J., Harper, J.W. and Shuttleworth, J. (1993) EMBO J. 12, 3133–3142.
[22] Fang, F. and Newport, J.W. (1991) Cell 66, 731–742.
[23] Pagano, M., Pepperkok, R., Verde, F., Ansorge, W. and Draetta, G. (1992) EMBO J. 11, 961–971.
[24] Cardoso, M.C., Leonhardt, H. and Nadal-Ginard, B. (1993) Cell 74, 979–992.
[25] Sambrook, J.F. (1990) Cell 61, 197–199.
[26] Breittmayer, J.-P., Ticchioni, M., Ferrua, B., Bernard, A. and Aussel, C. (1993) Cell. Immunol. 149, 248–257.
[27] Lanini, L., Bachs, O. and Carafoli, E. (1992) J. Biol. Chem. 267, 11548–11552.