A Novel Ca\textsuperscript{2+} Entry Mechanism Is Turned On during Growth Arrest Induced by Ca\textsuperscript{2+} Pool Depletion\textsuperscript{*}

(Received for publication, August 14, 1995, and in revised form, September 13, 1995)

Carmen A. Ufret-Vincenty, Alison D. Short, Amparo Alfonso\textsuperscript{§}, and Donald L. Gill

From the Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201

Ca\textsuperscript{2+} pool depletion with Ca\textsuperscript{2+} pump blockers induces growth arrest of rapidly dividing DDT\textsuperscript{MF-2} smooth muscle cells. When Ca\textsuperscript{2+} pumps are inhibited by thapsigargin, Ca\textsuperscript{2+} channel blockers SKF96365 or verapamil, and is independent of the Ca\textsuperscript{2+} pump blocker, thapsigargin, is correlated with the appearance of a novel caffeine-activated Ca\textsuperscript{2+} influx mechanism. Ca\textsuperscript{2+} influx through this mechanism is clearly distinct from and additive with Ca\textsuperscript{2+} entry through store-operated channels (SOCs). Whereas SOC-mediated entry is activated seconds after Ca\textsuperscript{2+} pool release, caffeine-sensitive influx requires at least 30 min of pool emptying. Although activated in the 1–10 mM caffeine range, this mechanism has clearly distinct methylxanthine specificity from ryanodine receptors and is not modified by ryanodine. It is also unaffected by the Ca\textsuperscript{2+} channel blockers SKF96365 or verapamil and is independent of modifiers of cyclic nucleotide levels. Growth arrest of rapidly dividing DDT\textsuperscript{MF-2} smooth muscle cells was as described previously (6). Untreated cells were exposed to the same conditions except without thapsigargin. Treatment of Ca\textsuperscript{2+} pool-depleted cells with high serum or arachidonic acid causes the methylxanthine-sensitive Ca\textsuperscript{2+} influx mechanism to be turned off, normal receptor-operated Ca\textsuperscript{2+} signaling to resume, and re-entry of cells into the cell cycle. The function of this novel Ca\textsuperscript{2+} influx mechanism and its precise turning on and off may be important events in the relationship between Ca\textsuperscript{2+} signaling and the growth state of cells.

EXPERIMENTAL PROCEDURES

Growth of Cells and Thapsigargin Treatment—Cultures of DDT\textsuperscript{MF-2} smooth muscle cells was as described previously (6). Thapsigargin-inhibited, Ca\textsuperscript{2+} pool-depleted cells were transferred into HKM (107 mM NaCl, 6 mM KCl, 12 mM MgSO\textsubscript{4}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1 mM CaCl\textsubscript{2}, 11.5 mM glucose, 0.1% bovine serum albumin, 20 mM Hepes-KOH, pH 7.3) and loaded with 2 \muM fura-2 acetoxy-methyl ester for 10 min at 20°C in the dark. After deesterification in fresh loading medium for 15 min at 20°C, coverslips were inserted into a Dvorak-Stotler chamber. Groups of 5–10 fura-2-loaded cells were viewed through a Nikon 40\times\ UV-fluor objective. Excitation at 340, 358, or 380 nm was generated using a PTI D103 light source, and fluorescence emission at 505 nm was monitored at 24°C using a PTI D104 photometer. Free intracellular Ca\textsuperscript{2+} concentrations were calculated from either 340/380 or 358/380 ratios of fluorescence intensities using the method of Grynkiewicz et al. (10) and a Kd of 135 nM. Rates of Mn\textsuperscript{2+} uptake were estimated by measuring Mn\textsuperscript{2+}-dependent fluorescence quenching at 358 nm.

Materials and Miscellaneous Procedures—Thapsigargin was from LC Services Corp. Fura-2 acetoxy-methyl ester was from Molecular Probes. Other compounds were from Sigma. The DDT\textsuperscript{MF-2} cell line was originally obtained from Drs. James Norris and Lawrence Cornell, University of Arkansas.

\textsuperscript{*} This work was supported by National Institutes of Health Grants NS19304 and GM15407, by National Science Foundation Grant DMB 9307746, and by the award of a postdoctoral fellowship from the Mary-land Heart Association, Maryland Affiliate (to A.D.S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Pharmacology, Cambridge University, Tennis Court Road, Cambridge CB2 1QJ, United Kingdom.

‡ Recipient of a postdoctoral fellowship from Formacio´n del Personal Investigador en el Extranjero, Ministerio de Educacio´n y Ciencia, Spain.

§ To whom correspondence should be addressed: Dept. of Biological Chemistry, University of Maryland School of Medicine, 108 North Greene St., Baltimore, MD 21201. Office Tel.: 410-706-2593; Laboratory Tel.: 410-706-7247; Fax: 410-706-6676.

\textsuperscript{1} The abbreviations used are: SOC, store-operated channel; InsP\textsubscript{3}, inositol 1,4,5-trisphosphate; 3,7-DMPX, 3,7-dimethyl-1-propargylxanthine; IBMX, 3-isobutyl-1-methylxanthine; 1,7-DMX, 1,7-dimethylxanthine; HKM, Hepes-buffered Kreb’s medium.

\textsuperscript{2} Whereas discrete channel proteins have yet to be identified, the convention, SOC, was adopted in May 1995 at the International Con-vention on Receiver-regulated Calcium Influx, Pacific Grove, CA, to provide a general description of Ca\textsuperscript{2+} store-activated influx channels variously described as capacitative Ca\textsuperscript{2+} entry, deplet-iation-activated channels, and I\textsubscript{CRAC}.

26790
FIG. 1. Caffeine-induced and SOC-induced Ca\(^{2+}\) influx in normal and Ca\(^{2+}\)-pool-depleted DDT,MF-2 cells. [Ca\(^{2+}\)]\(_i\) was measured in normal cells (Untreated) or cells treated with thapsigargin to empty Ca\(^{2+}\) pools and induce growth arrest (TG-treated). A, untreated and thapsigargin-treated cells were treated with nominally Ca\(^{2+}\)-free HKM for 6 min and then returned to normal HKM. 3 min later, 10 mM caffeine was added to both cell types. B, 10 mM caffeine was added to untreated and thapsigargin-treated cells at 30 s in normal HKM. C, thapsigargin-treated cells were treated at 30 s with Ca\(^{2+}\)-free HKM and at 90 s with 10 mM caffeine; after 5 min in Ca\(^{2+}\)-free HKM cells were returned to normal HKM.

RESULTS AND DISCUSSION

The Ca\(^{2+}\) pump blocker, thapsigargin, empties intracellular Ca\(^{2+}\) pools (11) and rapidly activates Ca\(^{2+}\) entry through SOCs (2). In many cells including DDT,MF-2 cells this entry becomes efficiently deactivated a few minutes after pool emptying (7). SOCs can be transiently reactivated by brief removal of Ca\(^{2+}\)_o (12). As shown in Fig. 1A, in DDT,MF-2 cells, even 24 h after thapsigargin-induced Ca\(^{2+}\) pool depletion and the establishment of growth arrest, Ca\(^{2+}\)_o removal results in an immediate decrease in resting cytosolic Ca\(^{2+}\), presumably reflecting the contribution of some residual non-deactivated SOC activity. During the absence of Ca\(^{2+}\)_o, SOC activity becomes reactivated as reflected by the large "overshoot" in cytosolic Ca\(^{2+}\) observed upon readdition of Ca\(^{2+}\)_o, the transient nature of the overshoot reflects a temporary activation of SOCs, which again undergo rapid deactivation. Normal DDT,MF-2 cells with filled Ca\(^{2+}\) pools and presumably without any activated SOCs exhibit little change in cytosolic Ca\(^{2+}\) as a result of transient Ca\(^{2+}\)_o removal. Interestingly, addition of 10 mM caffeine to the pool-depleted cells induces a further rapid and substantial increase in Ca\(^{2+}\), which also appears to undergo deactivation (Fig. 1A). This caffeine effect is not dependent on brief Ca\(^{2+}\)_o removal since, added directly to thapsigargin-arrested cells, caffeine induces the same large transient Ca\(^{2+}\)_i increase (Fig. 1B). Cells treated with the alternative Ca\(^{2+}\) pump blocker, 2,5-di-tert-butylhydroquinone, which also induces pool emptying and growth arrest (6), develop the same caffeine-induced Ca\(^{2+}\) response (data not shown). Importantly, normal cells (that is untreated with Ca\(^{2+}\) pump blockers) are devoid of any caffeine response (Fig. 1, A and B).

The effect of caffeine is clearly on influx of Ca\(^{2+}\) since it induces no change in the absence of Ca\(^{2+}\)_o (Fig. 1C). When Ca\(^{2+}\) is added back in the presence of caffeine, a rapid and even larger transient of Ca\(^{2+}\)_i is observed (Fig. 1C). The most exact doubling in the size of the transient after readdition of Ca\(^{2+}\) reflects clear additivity of SOC- and caffeine-mediated Ca\(^{2+}\) entry, providing further evidence that the two mechanisms are independent. The Ca\(^{2+}\)_i entry blocker SKF96365 at 50 \(\mu M\) completely blocks SOC-mediated Ca\(^{2+}\)_i entry but even as high as 2 \(\mu M\) has no effect on caffeine-mediated Ca\(^{2+}\)_i entry (data not shown). These data indicate that caffeine induces an influx of Ca\(^{2+}\) distinct from that mediated by SOCs but only in Ca\(^{2+}\)-pool-depleted growth-arrested cells.

Even though both depend on Ca\(^{2+}\)_i pool emptying, an interesting divergence between the two influx mechanisms is their time dependence of activation following pool depletion. SOC-mediated Ca\(^{2+}\)_i influx becomes activated rapidly after thapsigargin-induced pool depletion but within 5 min has become almost completely deactivated (Fig. 2A). Indeed, the efficient turn-off of SOC-mediated entry prevents long-term increased cytosolic Ca\(^{2+}\) levels following pool depletion and may be important in the survival of DDT,MF-2 cells, albeit in a growth-arrested state, following pool emptying (13, 14); cells in which SOC-mediated entry is not deactivated can enter an irreversible apoptotic cycle (13, 14). Reactivation of SOCs can occur by transient Ca\(^{2+}\)_o removal as early as a few minutes after pool emptying (Fig. 2A). At this time addition of caffeine has no effect (Fig. 2B), further supporting an SOC-independent action of caffeine. The time dependence of pool emptying and appearance of caffeine-induced influx is shown in Fig. 2, C–E. After 10 min of thapsigargin-induced pool emptying no caffeine effect can be detected. The shortest period of thapsigargin treatment after which a significant caffeine-induced Ca\(^{2+}\)_i influx can occur is 30 min. However, immediately following this minimally effective period of thapsigargin treatment, the caffeine response is small; during a further period of 30–60 min after removal of thapsigargin the caffeine response becomes larger. In Fig. 2, C–E, after treatment with thapsigargin for the times shown, cells were incubated a further 60 min without thapsigargin. Also, with the shorter thapsigargin treatment times, onset of influx following caffeine addition is more variable in cells, some cells exhibiting a lag of 1–2 min prior to activation of influx (this is apparent in Fig. 2D in which the data are an average of
to be much less potent on InsP₃ receptors than caffeine (15).

The entry of Ca²⁺ induced by caffeine is clearly transient and becomes deactivated within a few minutes. Readdition of caffeine without washing induces no further effect; however, a 5-min wash of caffeine-treated cells followed by caffeine reapplication results in return of the full Ca²⁺ entry effect indicating that the entry mechanism becomes reactivated. More recent experiments have assessed cation selectivity and specificity of the caffeine-activated entry mechanism. These studies reveal that the entry mechanism is readily permeable to Mn²⁺. By assessing quench of cytosolic fura-2 in pool-depleted DDT₁MF-2 cells, rates of Mn²⁺ entry activated by different methylxanthines have been shown to correlate well with the effects on cytosolic Ca²⁺ shown in Fig. 3. Also, we have observed that caffeine-activated Ca²⁺ entry is blocked by other cations including Gd³⁺, La³⁺, and Co²⁺ but is not blocked by Mn²⁺. In contrast, while SOC activation induces a modest influx of Mn²⁺, the entry of Ca²⁺ through SOCs is substantially blocked by Mn²⁺, in keeping with the observations of others (20, 21). These results provide evidence of yet another distinction between the methylxanthine- and SOC-mediated Ca²⁺ entry mechanisms.

Further reinforcement of the correlation between Ca²⁺ pool content, growth stage, and operation of caffeine-induced Ca²⁺ influx is derived from experiments in which thapsigargin-treated, growth-arrested cells are induced to reenter the growth cycle. We previously revealed that a 40-min treatment of thapsigargin-arrested cells with 20% serum induces synthesis of new pump protein, return of functional Ca²⁺ pools, and resumption of normal growth (7). Recent studies reveal that 10 μM arachidonic acid induces an identical recovery of Ca²⁺ pools and cell growth. As shown in Fig. 4, the reappearance of a functional bradykinin-activated Ca²⁺ pool is directly correlated with the disappearance of the caffeine-sensitive Ca²⁺ entry mechanism. In thapsigargin-arrested cells, the absence of an InsP₃-sensitive Ca²⁺ pool is reflected by the lack of response to receptor agonists such as bradykinin (Fig. 4A); in these cells caffeine always activates a substantial influx of Ca²⁺ (Fig. 4C). Thapsigargin-arrested cells treated with 20% serum recover from growth arrest and when examined 16 h later show normal bradykinin-induced Ca²⁺ signals (Fig. 4B) and a complete absence of the caffeine-induced Ca²⁺ response (Fig. 4D). Thus, cells have returned to the pregrowth-arrested state in which the caffeine-sensitive influx mechanism is turned off and normal agonist-sensitive Ca²⁺ pools are functional. Significantly, further experiments have shown that the time of disappearance of caffeine-induced influx closely correlates with that for reappearance of Ca²⁺ pools following high serum-induced recovery. 3 h after thapsigargin-arrested cells are induced to recover with high serum, a significant bradykinin-sensitive Ca²⁺ signal is observable in groups of cells as well as a still measurable caffeine response (although at a single cell with both activities has not been observed). At 6 h after induction, the response to caffeine has completely disappeared and the bradykinin response is the same as a normal cell. We have shown that following serum induction of thapsigargin-arrested cells, new Ca²⁺ pump protein appears as early as 1 h and pools become fully operational at 6 h (7); thereafter cells progress through G₂ and begin to enter G₁ 16 h later (6, 7). This entire sequence of events, including cessation of the caffeine response, is identically activated by 10 μM arachidonic acid as opposed to 20% serum. From these experiments it is clear that function of the caffeine-sensitive Ca²⁺ influx mechanism is restricted only

³ C. A. Ufret-Vincenty, A. Alfonso, A. D. Short, and D. L. Gill, manuscript in preparation.

⁴ M. N. Graber, A. Alfonso, and D. L. Gill, submitted for publication.
to Ca\(^{2+}\) pool-depleted, growth-arrested cells.

The studies described here demonstrate specific activation of a novel and distinct Ca\(^{2+}\) entry mechanism in pool-depleted growth-arrested DDT MF-2 cells that permits a transient but substantial entry of Ca\(^{2+}\). Indeed, the levels of intracellular Ca\(^{2+}\) achieved by activation of this mechanism are comparable with those attained by complete Ca\(^{2+}\) pool emptying or activation of SOC-mediated entry. A Ca\(^{2+}\)-conducting caffeine-sensitive influx channel was recently reported in adult gastric smooth muscle cells (22); however, in this case Ca\(^{2+}\) influx was not rapidly deactivated and methylxanthine specificity was not examined. Whereas functional ryanodine receptors in the same cells precluded absolute proof that Ca\(^{2+}\) influx was independent of ryanodine receptor function (23), it is intriguing that methylxanthine-sensitive Ca\(^{2+}\) entry channels might be expressed in nondividing smooth muscle cells. Our results are the first to provide evidence for a pharmacologically defined and apparently unique methylxanthine-sensitive Ca\(^{2+}\) entry pathway. Since caffeine has been widely used as a means of probing the action of Ca\(^{2+}\) release channels in many cell types (24), the present results are significant in providing awareness of the existence of a distinct caffeine-activated Ca\(^{2+}\) influx pathway. It is possible that this mechanism retains certain structural and/or functional similarities with ryanodine receptors. At this stage we do not know whether protein synthesis is required for turning on the entry mechanism. Another intriguing area of investigation will be to determine the means by which deactivation occurs. It is likely that operation of the influx pathway is transient within the cell cycle and/or restricted to discrete cell growth states.

Most significantly, although the physiological activation of the caffeine-sensitive Ca\(^{2+}\) entry mechanism has yet to be characterized, the appearance of this clearly defined and substantial Ca\(^{2+}\) entry mechanism under such specific conditions of pool emptying and growth arrest indicates a potentially important role in mediating Ca\(^{2+}\) signals during transition into and out of the cell cycle or during cell division when Ca\(^{2+}\) pools undergo substantial reorganization. As such, pharmacological modification of this channel may provide an important means for controlling cell growth.

Acknowledgments—We thank Dr. Tarun K. Ghosh, 3M Pharmaceuticals, for helpful advice and discussions and Thuyly Nguyen for expert technical assistance.

REFERENCES

1. Berridge, M. J. (1993) Nature 361, 315–325
2. Putney, J. W., Jr., and Bird, G. St. J. (1993) Endor. Rev. 14, 610–631
3. Sambrook, J. F. (1990) Cell 61, 197–199
4. Gill, D. L., Ghosh, T. K., Bian, J., Short, A. D., Waldron, R. T., and Rybak, S. L. (1992) Adv. Second Messenger Phosphoprotein Res. 26, 255–308
5. Ghosh, T. K., Bian, J., Short, A. D., Rybak, S. L., and Gill, D. L. (1991) J. Biol. Chem. 266, 24690–24697
6. Short, A. D., Bian, J., Ghosh, T. K., Waldron, R. T., Rybak, S. L., and Gill, D. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4986–4990
7. Waldron, R. T., Short, A. D., Meadows, J. J., Ghosh, T. K., and Gill, D. L. (1994) J. Biol. Chem. 269, 11927–11933
8. Ghosh, T. K., Mullaney, J. M., Tarazi, F. I., and Gill, D. L. (1989) Nature 340, 236–239
9. Short, A. D., Klein, M. G., Schneider, M. F., and Gill, D. L. (1993) J. Biol. Chem. 268, 25887–25893
10. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 261, 3440–3450
11. Bian, J., Ghosh, T. K., Wang, J. C., and Gill, D. L. (1991) J. Biol. Chem. 266, 8801–8806
12. Missiaen, L., De Smedt, H., Parys, J. B., Oike, M., and Casteels, R. (1994) J. Biol. Chem. 269, 5811–5823
13. Waldron, R. T., Short, A. D., and Gill, D. L. (1995) J. Biol. Chem. 270, 11955–11961
14. Furuya, Y., Lindmo, P., Short, A. D., Gill, D. L., and Isacs, J. T. (1994) Cancer Res. 54, 6167–6175
15. Müller, C. E., and Daly, J. W. (1993) Biochem. Pharmacol. 46, 1825–1829
16. Rousseau, E., LaDine, J., Liu, Q-Y., and Melssen, G. (1988) Arch. Biochem. Biophys. 267, 75–80
17. Daly, J. W., Hide, L., Müller, C. E., and Shamin, M. (1991) Pharmacology 42, 309–321
18. Parker, I., and Ivorra, I. (1991) J. Physiol. (Lond.) 433, 229–240
19. Missiaen, L., Taylor, C. W., and Berridge, M. J. (1992) J. Physiol. (Lond.) 455, 623–640
20. Hoth, M., and Penner, R. (1992) J. Physiol. (Lond.) 465, 359–386
21. Premack, B. A., McDonald, T. V., and Gardner, P. (1994) J. Immunol. 152, 5226–5240
22. Guerrero, A., Fay, F. S., and Singer, J. J. (1994) J. Gen. Physiol. 104, 375–394
23. Guerrero, A., Singer, J. J., and Fay, F. S. (1994) J. Gen. Physiol. 104, 395–422
24. Enda, M. (1985) Curr. Top. Membr. Transp. 25, 181–229