Ca²⁺ Pools and Cell Growth: Arachidonic Acid Induces Recovery of Cells Growth-arrested by Ca²⁺ Pool Depletion*

(Received for publication, September 12, 1995, and in revised form, November 6, 1995)

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The intracellular Ca²⁺ pump blocker, thapsigargin, induces emptying of Ca²⁺ pools and entry of DDT-MF-2 smooth muscle cells into a quiescent G₀-like growth state. Although thapsigargin blocks pumps essentially irreversibly, high serum (20%) induces appearance of new pump protein, return of functional pools, and reentry of cells into the cell cycle (Waldron, R. T., Short, A. D., Meadows, J. J., Ghosh, T. K., and Gill, D. L. (1994) J. Biol. Chem. 269, 11927–11933). Through analysis of the effects of defined serum components and growth supplements, we reveal here that the factors in serum responsible for inducing recovery of Ca²⁺ pools and growth in thapsigargin-arrested DDT-MF-2 cells are exactly mimicked by the three essential fatty acids, arachidonic, linoleic, and α-linolenic acids. The EC₅₀ values for arachidonic and linoleic acids on growth induction of thapsigargin-arrested cells were the same, approximately 5 μM. Nonessential fatty acids, including myristic, palmitic, stearic, oleic, and arachidic acids, were without any effect. Although not proven to be the active component of serum, levels of arachidonic and linoleic acids in serum were sufficient to explain serum-induced growth recovery. Significantly, arachidonic or linoleic acids induced complete recovery of bradykinin-sensitive Ca²⁺ pools within 6 h of treatment of thapsigargin-arrested cells. Protein synthesis inhibitors (cycloheximide or puromycin) completely blocked the appearance of serum-induced or arachidonic acid-induced agonist-sensitive pools. The sensitivity and fatty acid specificity of Ca²⁺ pool recovery in thapsigargin-arrested cells were almost identical to that for growth recovery. No pool or growth recovery was observed with 5,8,11,14-eicosatetraynoic acid, the nonmetabolizable analogue of arachidonic acid, suggesting that conversion to eicosanoids underlies the pool and growth recovery induced by essential fatty acids. The results provide not only further information on the link between Ca²⁺ pools and cell growth but also evidence for a potentially important signaling pathway involved in inducing transition from a stationary to a proliferative growth state.

Cytoplasmic Ca²⁺ signals control diverse cellular functions

* This work was supported by National Institutes of Health Grant NS19304, National Science Foundation Grant MCB 9307746, and by a grant-in-aid from the Maryland Heart Association, Maryland Affiliate. 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.

‡ Supported by a postdoctoral fellowship from Formación del Personal Investigador en el Extranjero, Ministerio de Educación y Ciencia, Spain.

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The abbreviations used are: ER, endoplasmic reticulum; SERCA, sarcoplasmic/endooplasmic reticulum calcium ATPase; DMEM, Dulbecco's modified Eagle's medium; fura-2, 2-acetoxyxymethyl; BSA, bovine serum albumin; ETYA, 5,8,11,14-eicosatetraynoic acid.
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**EXPERIMENTAL PROCEDURES**

Cell Culture—DDT1MF-2 smooth muscle cells derived from hamster vas deferens were cultured in Dulbecco's modified Eagle's medium supplemented with 2.5% serum (Calf-Plus, Inovar, Gaithersburg, MD) as described previously (20). Calf-Plus is newborn calf serum supplemented with additional growth factors and is referred to as "serum" in this report. Fetal calf serum yields essentially identical results on growth and reversal of thapsigargin-induced growth arrest (13, 14).

**Growth Conditions and Measurement of Cell Proliferation—**

DDT1MF-2 smooth muscle cells were grown in 24-well dishes (1 \(\times 10^5\) cells/well). Thapsigargin-treated cells were prepared by adding 3-8 \(\mu\)M thapsigargin in DMEM with 2.5% serum for 3 h. Thapsigargin treatment was followed by three washes in thapsigargin-free DMEM with 2.5% serum following recovery under conditions specified. All recovery media contained DMEM with 2.5% serum together with additions as specified. For all cell proliferation experiments, the total time from the end of thapsigargin treatment until determination of cell number was 72 h. At the end of 72 h, cells were resuspended by pipette transferred to cuvettes, and counted spectrophotometrically. For each experiment, standard curves were obtained for cell number (by direct counting) and light scattering measured by absorbance at 600 nm. Absorbance values obtained at different dilutions of cells were compared with the linear portion of the standard curve and values for cell number obtained. All measurements for cell number were obtained in quadruplicate, and results presented are typical of at least three different experiments.

Measurement of Cytosolic Free Ca\(^{2+}\)—Cells were allowed to attach to poly-L-lysine-coated 25-mm glass coverslips in culture for at least 4 h prior to use. As described above, attached cells were treated with 3 \(\mu\)M thapsigargin for 3 h then, after washing in thapsigargin-free DMEM with 2.5% serum, transferred to recovery media as indicated in figures. Cells were incubated for 6 h (unless otherwise noted) under appropriate recovery conditions in DMEM with 2.5% serum, at the end of which Ca\(^{2+}\) levels were measured. Measurements of free cytosolic Ca\(^{2+}\) were similar to those described previously (21). Attached cells were transferred to Hepes-buffered Kreb's medium (107 mM NaCl, 6.6 mM KCl, 1.2 mM MgSO\(_4\), 1.2 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 11.5 mM glucose, 0.11% bovine serum albumin, 20 mM Hepes-KOH, pH 7.4) and loaded with fura-2/AM (2 \(\mu\)M) for 10 min at 20°C in the dark. Cells were then washed in the same medium and loaded dye allowed to deesterify for 15 min at 20°C in the dark. Under these conditions, approximately 95% of the dye was released into the cytosol and remaining by the signal remaining after saponin permeabilization (21). Coverslips were inserted into a Dvorak-Statler chamber (Nicholson Precision Instruments, Gaithersburg, MD) and cells viewed with a Nikon Diaphot microscope equipped with epifluorescence optics (Nikon 40X UV-Fluor objective). Additions were made by aspiration and addition of fresh bathing solution in an open chamber configuration. Excitation at 340 and 380 nm was obtained with a P1T1 D103 filter-based 75-watt xenon light source (PTI, S. Brunswick, NJ) and fluorescence emission at 505 nm (10-nm band-pass interference filter; Omega Optical, Brattleboro, VT) was measured with a D104 microscope photometer (PTI). Free intracellular Ca\(^{2+}\) concentrations were calculated from 340/380 fluorescence intensity ratios using the calculations of Grynkiewicz et al. (22) with a K\(_d\) of 135 nm. Maximum fluorescence ratio was determined in the presence of 40 \(\mu\)M ionomycin with 10 \(\mu\)M Ca\(^{2+}\) and minimum ratio in the presence of 40 \(\mu\)M ionomycin and 10 \(\mu\)M EGTA.

**RESULTS AND DISCUSSION**

In previous studies we have determined that the growth of DDT1MF-2 smooth muscle cells is profoundly altered by intracellular Ca\(^{2+}\) pump blockers, including thapsigargin, 2,5-di-tert-butylhydroquinone, and cyclopiazonic acid (2, 11, 12). In each case, Ca\(^{2+}\) pool emptying is correlated with entry of cells into a growth-arrested state. DNA synthesis is not inhibited per se, and cells appear to progress through S-phase before entry into a stable G\(_1\) like quiescent state (12). Cells remain stable in this state for 7 days, maintaining viability, normal morphology and mitochondrial function, and approximately 20% of the protein synthesis observed in normal dividing cells (11). The blocking action of thapsigargin on intracellular Ca\(^{2+}\) pumps is essentially irreversible (19, 23), and even brief (30 min) treatment of cells with thapsigargin followed by extensive washing and culture in thapsigargin-free medium for up to 7 days results in Ca\(^{2+}\) pools that remain empty (that is, unre sponsive to Ca\(^{2+}\) mobilizing agonists or Ca\(^{2+}\) pump blockers) and in cells that remain in a quiescent nondividing state (11, 12). However, we recently revealed that a brief treatment of thapsigargin-arrested cells with high serum (20% as opposed to the normal level of 2.5% used to grow DDT1MF-2 cells) in the absence of thapsigargin caused appearance of new functional Ca\(^{2+}\) pump protein (determined by measuring Ca\(^{2+}\) pump phosphorylated intermediate) in 1-3 h, reappearance of functional Ca\(^{2+}\) pools in 3-6 h, followed by reentry of cells into the cell cycle (13, 14).

Although intriguing, the basis of action of high serum in inducing growth recovery was unknown. Therefore, we sought to determine the active component(s) within serum responsible for pool and growth recovery. Initial studies involved the fractionation and modification of serum by heat inactivation, charcoal-stripping, and dialysis. As shown in Fig. 1, as compared with the effect of 20% serum on growth recovery of thapsigargin-arrested cells, 20% serum heat-treated at either 56 or 78°C gave lower recovery. However, in both cases cells did recover...
Acid (fatty acid levels). In the absence of carrier BSA, growth recovery fraction of the fatty acid is bound, particularly at the higher concentration lower than 1 mM (Fig. 3). Significant growth recovery could be observed at linoleic acid concentrations as low as 100 nM, including 1% BSA, either alone or in combination, had no recovery-inducing activity. The EC_{50} of linoleic acid in the presence of 1% BSA was approximately 5 \mu M (Fig. 3). Significant growth recovery could be observed at linoleic acid concentrations lower than 1 \mu M and a maximal effect at approximately 100 \mu M. In the presence of BSA a substantial fraction of the fatty acid is bound, particularly at the higher fatty acid levels. In the absence of carrier BSA, growth recovery could be attained with less than 1 \mu M linoleic acid (not shown); however, without BSA, linoleic acid above 1 \mu M caused cell lysis and death as a result of membrane perturbation.

Key to determine was the specificity of fatty acid-mediated growth recovery of Ca^{2+} pool-depleted cells. As shown in Fig. 4, an important pattern of specificity was observed among fatty acids tested. Consistently, all the nonessential fatty acids tested between 14 and 20 carbons, including the saturated fatty acids, myristic, palmitic, stearic, and arachidic acids, and the unsaturated fatty acid, oleic acid, did not give any significant recovery of growth. In contrast, all three of the essential fatty acids, linoleic, \alpha-linolenic, and arachidonic acids, induced growth recovery of cells arrested by Ca^{2+} pool depletion (Fig. 4). Arachidonic acid was consistently more effective (that is, induced a greater rate of recovery) than linoleic acid, which itself was more effective than linoleic acid. The arachidonic acid dose-response curve for inducing growth recovery (Fig. 5) was similar to that for linoleic acid; the half-maximal effectiveness was between 3 and 5 \mu M, and significant recovery was usually seen with 100 \mu M arachidonic acid. The similar effectiveness of these closely related fatty acids is significant, indicating either a narrow structural requirement for their action or, as discussed below, that their effects on growth recovery are likely mediated by regulatory eicosanoids which can be specifically derived from each of the essential fatty acids (24).

An important question is whether essential fatty acids constitute the active component within serum-inducing growth recovery of pool-depleted cells. Whereas our results do not definitively prove that they are, evidence is consistent with this being the case. Heat inactivation, charcoal stripping, and dialysis are generally ineffective in removing fatty acids from serum, a large proportion of which is tightly bound to albumin. Analysis of the dose effectiveness of serum in inducing growth recovery in thapsigargin-arrested cells reveals a half-maximal effectiveness of approximately 7% serum (Fig. 6). Total nones-
terified fatty acid in the undiluted calf serum used was measured as approximately 530 μM; the essential fatty acids, linoleic, arachidonic, and linolenic acids, in combination, represent approximately 12% of total nonesterified fatty acid in serum and are in the ratio of approximately 90:10:1, respectively (25).

Therefore, 7% serum contains a combined essential fatty acid concentration of approximately 5 μM, agreeing with the half-maximal effectiveness of linoleic or arachidonic acids given in Figs. 3 and 5. The somewhat broad concentration dependence of arachidonic and linolenic acids likely reflects dissociation from albumin which has a number of different binding sites for fatty acids over the sub- and low micromolar range (26). Albumin both protects cells from the detergent effects of fatty acids and provides a means of delivering fatty acids to cells; the actual free concentrations of fatty acids present in experiments are obviously considerably lower than the total added.

Although extremely unlikely based on the remarkable affinity and slow dissociation rate of thapsigargin from SERCA pump protein (19, 23), a trivial explanation for the actions of essential fatty acids or serum on recovery of thapsigargin-treated cells was possible removal or stripping of thapsigargin from cells. To determine any such effect, experiments were conducted to measure the effectiveness of thapsigargin on cells in the presence of fatty acids and serum. One such experiment is shown in Fig. 7 where the concentration dependence of thapsigargin in preventing growth of cells under standard conditions (2.5% serum) is compared with its effects either in the presence of 20% serum or 100 μM linoleic acid (together with 1% BSA and 2.5% serum). It is clear that the effectiveness of thapsigargin is virtually identical under each condition, indicating that these agents do not bind, sequester, or otherwise prevent the inhibitory action of thapsigargin. The concentration dependence of thapsigargin on Ca²⁺ pump blockade and emptying of Ca²⁺ pools in intact cells is similar to that for growth inhibition (12, 13), and similarly, serum, BSA, linoleic acid, or arachidonic acid had no measurable effects on the ability of thapsigargin to empty pools (data not shown).

In other control experiments, we examined the effects of essential and nonessential fatty acids on growth of normal cells, that is, cells not treated with pump blockers. Linoleic acid added to DDT_MF-2 cells at up to 500 μM (with 1% BSA) under otherwise standard culture conditions had no significant effect on cell proliferation. Arachidonic acid actually had a significant growth inhibitory effect when added above 10 μM; at 100 μM the rate of cell growth was reduced by approximately 40%. Other nonessential fatty acids had no effect on normal cell growth. The effects of linoleic and arachidonic acids on normal cell growth concur well with those described by others on the effects of essential fatty acids on smooth muscle proliferation (27). These results indicate that reversal of growth arrest and induction of entry of quiescent Ca²⁺ pool-depleted cells into the cell cycle is a specific action of essential fatty acids which is distinct from any general effects on cell proliferation. Indeed, taking into account its inhibitory action on cell growth rate, the action of arachidonic acid on recovery of quiescent cells is some of the above experiments is actually underestimated. In further control experiments, neither arachidonic nor linoleic acids had any measurable effect upon the size or function of Ca²⁺ pools in normal cells.

From the above experiments, it is clear that arachidonic acid and the other essential fatty acids can mimic the action of high serum in promoting growth recovery of cells that have entered a stable quiescent state following Ca²⁺ pool depletion. Such recovery of growth was the end result measured after 3 days. Previously we demonstrated that one of the initial events fol-
Arachidonic Acid and Ca\textsuperscript{2+} Pools

Following high serum treatment of pool-depleted cells was induction of new functional SERCA pump activity and inositol 1,4,5-trisphosphate-releasable Ca\textsuperscript{2+} pools (13, 14). Obviously it was important to assess whether a similar early expression of Ca\textsuperscript{2+} pools resulted from treatment with essential fatty acids or whether their effect on growth recovery was manifested after a different series of events. As shown in Fig. 8, there is clearly a rapid fatty acid-mediated induction of new Ca\textsuperscript{2+} pools. Cells after treatment with thapsigargin have no measurable inositol 1,4,5-trisphosphate-sensitive ino\textsuperscript{2+} pools, and they remain without these pools for many days in a quiescent but otherwise viable state (12, 13). After 6-h treatment of thapsigargin-arrested cells with 20% serum, the cells had regained fully operational pools as judged by a maximal Ca\textsuperscript{2+} response to 10\,\mu M bradykinin (Fig. 8A). 6 h after treatment of similarly arrested cells with 100\,\mu M arachidonic acid and 1% BSA, an identical bradykinin-releasable pool was observed (Fig. 8B). This provides further evidence that the effects of high serum and arachidonic acid are equivalent and therefore that the action of high serum could be attributed to essential fatty acids contained within it. Although our recent studies revealed that high serum treatment induces the appearance of new pump protein (14), we had not previously determined whether protein synthesis was required for return of this activity. The results in Fig. 8 reveal that this is the case and that regardless of whether recovery is induced by serum or arachidonic acid, the appearance of bradykinin-sensitive Ca\textsuperscript{2+} pools was almost identical to that for growth induction.

Thus, as shown in Fig. 9, 6 h of treatment of thapsigargin-arrested cells with 100\,\mu M arachidonic or linoleic acids caused complete induction of functional Ca\textsuperscript{2+} pools; 100\,\mu M linoleic acid consistently induced appearance of Ca\textsuperscript{2+} pools that were less than maximal in this time period. The nonessential fatty...

**Fig. 8.** Arachidonic acid and 20% serum both induce recovery of agonist-sensitive Ca\textsuperscript{2+} pools in thapsigargin-arrested DDT\textsuperscript{-} MF-2 cells via a process dependent on protein synthesis. Cells were thapsigargin-treated under standard conditions, then exposed for 6 h to either 2.5% serum (control), 20% serum, 20% serum with 16\,\mu M puromycin, or 20% serum with 35\,\mu M cycloheximide (CHX) (A) or 2.5% serum with 1% BSA (control), 100\,\mu M arachidonic acid with 1% BSA (AA), 100\,\mu M arachidonic acid with 1% BSA and 16\,\mu M puromycin, or 100\,\mu M arachidonic acid with 1% BSA and 35\,\mu M cycloheximide (CHX) (B). Puromycin and cycloheximide were both added 30 min prior to addition of high serum or arachidonic acid. Under each condition Ca\textsuperscript{2+} release was measured in response to 10\,\mu M bradykinin (BK) added at the arrow. Conditions were otherwise as stated under “Experimental Procedures.” Results are typical of three similar experiments.

**Fig. 9.** Fatty acid specificity of agonist-sensitive Ca\textsuperscript{2+} pool recovery in thapsigargin-arrested DDT, MF-2 cells. Thapsigargin-treated cells were exposed for 6 h to either 100\,\mu M arachidonic acid (AA), 100\,\mu M linoleic acid (LA), 100\,\mu M \(\alpha\)-linolenic acid (L\textsubscript{N}A), 100\,\mu M palmitic acid (PA), or 100\,\mu M stearic acid (SA), in each case added with 1% BSA in the presence of the standard growth medium containing 2.5% serum. Ca\textsuperscript{2+} release was measured in response to 10\,\mu M bradykinin (BK) added at the arrow. Results are typical of three similar experiments, and conditions were otherwise as stated under “Experimental Procedures.”

**Fig. 10.** Concentration dependence of arachidonic acid-induced recovery of agonist-sensitive Ca\textsuperscript{2+} pools in thapsigargin-arrested DDT, MF-2 cells. Thapsigargin-treated cells were exposed to arachidonic acid (AA) at the concentrations shown with 1% BSA for 1 h. Cells were washed and recovery continued for a further 18 h in standard growth medium containing 2.5% serum. Control cells received 1% BSA without arachidonic acid. Ca\textsuperscript{2+} release was measured in response to 10\,\mu M bradykinin (BK) added at the arrow. Conditions were otherwise as stated under “Experimental Procedures.” Results are typical of three similar experiments.
acids, stearic and palmitic acid, did not result in any measurable bradykinin-sensitive Ca\(^{2+}\) pools. The sensitivity of arachidonic acid-mediated pool recovery was also similar to that for arachidonic acid-induced growth recovery. As shown in Fig. 10, the response to 100 \(\mu M\) arachidonic acid was maximal, lower concentrations giving a smaller response. Although the maximum peak height appeared to be approximately correlated with arachidonic acid level, at concentrations below 1 \(\mu M\) arachidonic acid the rate of onset of the bradykinin-activated Ca\(^{2+}\) signal was attenuated (data not shown). Thus, cells treated with 100 \(nM\) arachidonic acid showed a consistent recovery of pools, but the rate at which emptying occurred in response to bradykinin was slower. It is possible that functional Ca\(^{2+}\) pools may be less extensively formed at this lower level of stimulation by arachidonic acid, resulting in not only a smaller amount of releasable Ca\(^{2+}\) but also a slower onset of Ca\(^{2+}\) release. An important question is whether arachidonic acid acts directly to stimulate Ca\(^{2+}\) pool regeneration and growth recovery or whether it becomes metabolized to an active derivative. From the results shown in Fig. 11, it is unlikely that arachidonic acid itself is the activating species. Thus, the structural analogue, eicosatetraynoic acid (ETYA), at 100 \(\mu M\) did not induce any recovery of pools. This analogue is unable to undergo metabolism to active products via the cyclooxygenase, cyclooxygenase, or monoxygenase pathways (28). ETYA has been shown to mimic the actions of arachidonic acid in cases where metabolism of the fatty acid is not required (28–30). Based on this, it appears likely that arachidonic acid metabolites are required in order to induce cell recovery. ETYA also did not induce any growth of thapsigargin-arrested cells; and like arachidonic acid, ETYA induced some inhibition of the rate of growth of normal cells, reducing the rate by almost 50% at 100 \(\mu M\).

The results presented here provide compelling evidence that essential fatty acids can induce recovery of cells that have been growth arrested by Ca\(^{2+}\) pool depletion. This recovery involves both the return of Ca\(^{2+}\) pumping pools and the transition of cells from a Go\(_1\)-like growth state back into the cell cycle. The action of essential fatty acids is very similar to that of addition of high serum to cells (13, 14). At present, we have not proven whether essential fatty acids are the active recovery-inducing component within serum or whether serum induces the formation of arachidonic acid or other fatty acids by, for example, stimulation of receptor-linked phospholipase A\(_2\) activity. The latter appears unlikely since preliminary experiments have shown no effect of phospholipase A\(_2\) inhibitors on serum-induced recovery. Moreover, the actual levels of arachidonic and linoleic acids present within serum appear sufficient to explain the actions of serum. The other important question is whether arachidonic acid requires conversion to eicosanoids in order to have its growth inducing effects. Obviously, the essential fatty acids, including arachidonic, linoleic, and linolenic acids, are each major substrates for conversion to prostaglandins, prosta-cyclins, thromboxanes, leukotrienes, and other eicosanoids (24). In view of the noneffectiveness of ETYA, it is likely that conversion is required. It is also clear that ETYA is an effective blocker of the entry of arachidonic acid and other essential fatty acids into each of the pathways through which eicosanoids are formed, including the cyclooxygenase, lipoxygenase, and monoxygenase pathways (24, 28). Recent experiments indicate that ETYA also blocks arachidonic acid-induced pool and growth recovery. However, further dissection of the effective metabolites is required before information on exactly which eicosanoid products are effective in mediating recovery of cells from growth arrest induced by Ca\(^{2+}\) pool depletion.

Acknowledgments—We thank Thyuy Nguyen for expert technical assistance and Drs. Allison D. Short and Tarun K. Ghosh for helpful advice and discussions.

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\(^2\) M. N. Graber, A. Alfonso, and D. L. Gill, unpublished results.