Reprint Series
4 January 1991, Volume 251, pp. 75–78

SCIENCE

# Generation of Calcium Oscillations in Fibroblasts by Positive Feedback Between Calcium and $IP_3$

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# Generation of Calcium Oscillations in Fibroblasts by Positive Feedback Between Calcium and $IP_3$

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**A wide variety of nonexcitable cells generate repetitive transient increases in cytosolic calcium ion concentration ($[Ca^{2+}]_i$) when stimulated with agonists that engage the phosphoinositide signalling pathway. Current theories regarding the mechanisms of oscillation disagree on whether $Ca^{2+}$ inhibits or stimulates its own release from internal stores and whether inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DG) also undergo oscillations linked to the $Ca^{2+}$ spikes. In this study, $Ca^{2+}$ was found to stimulate its own release in REF52 fibroblasts primed by mitogens plus depolarization. However, unlike $Ca^{2+}$ release in muscle and nerve cells, this amplification was insensitive to caffeine or ryanodine and required hormone receptor occupancy and functional $IP_3$ receptors. Oscillations in $[Ca^{2+}]_i$ were accompanied by oscillations in $IP_3$ concentration but did not require functional protein kinase C. Therefore, the dominant feedback mechanism in this cell type appears to be $Ca^{2+}$ stimulation of phospholipase C once this enzyme has been activated by hormone receptors.**

MANY NONEXCITABLE CELLS EXhibit periodic increases (spikes) in the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) when stimulated with hormones or growth factors (*1*). The biochemical mechanism and physiological significance of these $[Ca^{2+}]_i$ oscillations are still highly controversial. At least four classes of generating mechanisms have been proposed (Table 1). These can be distinguished by whether inositol 1,4,5-trisphosphate concentrations oscillate as well as $[Ca^{2+}]_i$ and whether cytosolic $Ca^{2+}$ stimulates or inhibits further release of $Ca^{2+}$ from intracellular stores. The first model was formulated on the basis of the observation that in some cell types, elevated $[Ca^{2+}]_i$ inhibits the ability of $IP_3$ to release additional $Ca^{2+}$ from internal stores (*2*). If this negative feedback has a sufficient time delay, it could explain $Ca^{2+}$ oscillations that occur without $IP_3$ oscillations. A second model that postulates steady $IP_3$ elevation proposes that $IP_3$ merely transfers $Ca^{2+}$ from the $IP_3$-sensitive internal stores to a separate $IP_3$-insensitive pool from which it is repetitively dumped by $Ca^{2+}$-induced $Ca^{2+}$ release (*3*). This model is probably the most popular at present. Other models postulate that $IP_3$ concentrations do oscillate. For example, initial receptor stimulation of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) hydrolysis might be self-limiting. This negative feedback would be mediated by diacylglycerol (DG) production and $IP_3$-mediated release of $Ca^{2+}$, which together would activate protein kinase C to phosphorylate the receptor or G protein and inhibit them, thus shutting off $PIP_2$ hydrolysis (model 3). Only when phosphatases had reversed the phosphorylation would another coordinated burst of $IP_3$, DG, and $Ca^{2+}$ release be generated (*4*). A fourth model (*5*) proposes that phospholipase C can be stimulated not only by agonist but by cytosolic $Ca^{2+}$. Therefore an initial weak activation would self-amplify because $Ca^{2+}$ released by $IP_3$ would further increase $IP_3$ production. This positive feedback would fail when the $Ca^{2+}$ store was mostly depleted; only after a period of refilling could the burst of $IP_3$ and $Ca^{2+}$ be repeated. This hypothesis, like model 2, predicts that an increase in $[Ca^{2+}]_i$ can release of stored $Ca^{2+}$, but in model 4 the positive feedback is mediated by $IP_3$, whereas in model 2 it is an inherent property of an $IP_3$-independent $Ca^{2+}$ pool.

We have used the fibroblast cell line REF52 as a model system to study the mechanisms that generate $[Ca^{2+}]_i$ oscillations. This cell line was chosen because it gives unusually consistent oscillations (*6*): when appropriately stimulated by combined depolarization and treatment with mitogens or hormones such as vasopressin, essentially all the cells generate repetitive spikes in $[Ca^{2+}]_i$ (Fig. 1A). The amplitude and frequency of the spikes vary somewhat from cell to cell but in any one cell are remarkably consistent for hours. One set of experiments was directed at the question of whether $Ca^{2+}$, delivered by wounding or photolysis of a light-sensitive chelator, inhibits or stimulates further release from internal stores. A second series of experiments was to synchronize the $[Ca^{2+}]_i$ spikes in a population to see whether $IP_3$ fluctuates in parallel with $[Ca^{2+}]_i$. In a third group of experiments,

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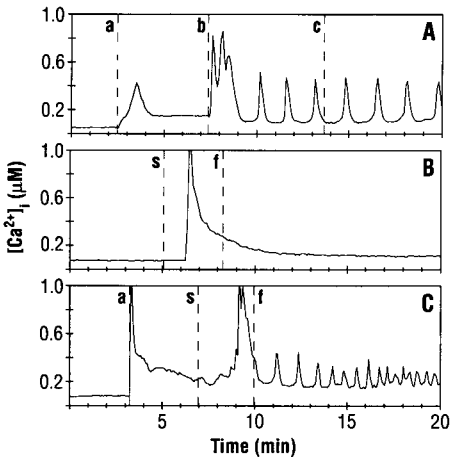


**Fig. 1.** $[Ca^{2+}]_i$ oscillations in REF52 fibroblasts and the effects of $Ca^{2+}$ overload and of pharmacological interference with $Ca^{2+}$-induced $Ca^{2+}$ release. (**A**) $[Ca^{2+}]_i$ in individual REF52 fibroblasts oscillated with rhythmical accuracy after stimulation with gramicidin (500 nM) (a) and vasopressin (50 nM) (b). Oscillations continued unperturbed by addition of ryanodine (10 μM) (c) (*14*). (**B** and **C**) Momentary wounding (s, start; f, finish) by mock microinjection in the absence (B) or presence (C) of a hormone (vasopressin, 50 nM) (a) linked to inositol phospholipid metabolism. $[Ca^{2+}]_i$ was measured in individual cells with fura-2 imaging as described (*6, 15*); unless otherwise indicated, the extracellular medium was Hanks balanced salt solution that contained 1.3 mM $Ca^{2+}$.

protein kinase C activity was removed to see whether it has an essential role in generating oscillations.

A characteristic of $Ca^{2+}$-induced $Ca^{2+}$ release in muscle and some neurons (*7*) is its sensitivity to caffeine or ryanodine; however, these agents, applied at low or high concentrations (up to 10 mM and 10 μM, respectively), had no effect on cytosolic $Ca^{2+}$ either in resting or already oscillating REF52 cells (Fig. 1A). Also, a massive $Ca^{2+}$ transient could be induced by deliberate mechanical wounding with a micropipette, but this $Ca^{2+}$ overload never induced oscillations by itself (Fig. 1B), although $[Ca^{2+}]_i$ traversed all levels, from basal to supraphysiological concentrations in both directions. However, if the cells were pretreated with the mitogen vasopressin, wounding did initiate oscillations (Fig. 1C). Thus if there is $Ca^{2+}$-induced $Ca^{2+}$ release, it is not a conventional caffeine- or ryanodine-sensitive process, and it requires not only $Ca^{2+}$ overload but receptor activation as well.

A much more controllable method of generating sudden increases in $[Ca^{2+}]_i$ is with photolabile $Ca^{2+}$ chelators that release $Ca^{2+}$ upon illumination (*8*). The chelator nitr-7 was chosen because its high affinity for $Ca^{2+}$ before photolysis minimized steady-state buffering of $[Ca^{2+}]_i$. In otherwise unstimulated cells, flash photolysis of nitr-7 caused a sudden increase in $[Ca^{2+}]_i$, which then decreased in a monotonic decay (Fig. 2A). In nitr-7-loaded cells that were stimulated to oscillate, a similar flash delivered in the resting period between endogenous $[Ca^{2+}]_i$ spikes produced not only the immediate increase in $[Ca^{2+}]_i$ due to the trigger pulse of $Ca^{2+}$, but also an additional, delayed increase in $[Ca^{2+}]_i$ (Fig. 2B). This secondary increase, which peaked about 10 s after the trigger, provided direct evidence of positive feedback through $[Ca^{2+}]_i$ in oscillating cells. Because the flash-induced $[Ca^{2+}]_i$ response occurred well before the next endogenous spike would have been generated, the secondary peak was not due to the normal timing of the oscillations. To determine whether this positive feedback was mediated by conventional $Ca^{2+}$-induced $Ca^{2+}$ release or enhanced $IP_3$ production, we microinjected cells with heparin, the most specific blocker currently available for the intracellular $IP_3$ receptor (*9*). Heparin blocked the delayed amplification of the trigger $Ca^{2+}$ delivered by nitr-7 photolysis (Fig. 2C). This result argues that the positive feedback occurs via $IP_3$ production. As expected, the same dose of heparin inhibited release of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores elicited either by vasopressin or photolysis of intracellular caged $IP_3$ (*6*) and prevented oscillations due to mitogens plus depolarization, but did not inhibit $Ca^{2+}$ influx through voltage-gated channels.

We next looked for direct evidence of $IP_3$ oscillations that might accompany $[Ca^{2+}]_i$ oscillations. Because $IP_3$ measurements cannot yet be made on single cells, it was necessary to find a way to synchronize the $[Ca^{2+}]_i$ oscillations of a population of cells without changing hormone concentrations. Oscillations halted on removal of extracellular $Ca^{2+}$, even though the concentrations of hormone and depolarizing agents were unchanged (Fig. 3A). After restoration of extracellular $Ca^{2+}$, the $[Ca^{2+}]_i$ oscillations resumed. The first spike in $[Ca^{2+}]_i$ occurred 20 to 30 s after the readdition of $Ca^{2+}$ (Fig. 3A), roughly synchronized within the population of cells, whose average therefore showed a peak at that time (Fig. 3B). This synchrony was then rapidly lost because the individual cells each had somewhat different periods of oscillation (Fig. 3A), so that the population mean $[Ca^{2+}]_i$ (Fig. 3B) returned to an intermediate plateau. Population measurements of $IP_3$ in parallel experiments showed a closely similar spike and plateau (Fig. 3C), as would be expected if $IP_3$ does oscillate in synchrony with $[Ca^{2+}]_i$. Control experiments verified that the burst of $IP_3$ production depended on intracellular

**Table 1.** Mechanisms proposed in the literature (*1*) to explain how $[Ca^{2+}]_i$ oscillations may be generated in electrically nonexcitable cells. Abbreviations are as follows: HR, hormone-receptor complex or other activated receptor on the plasma membrane; G, GTP-binding coupling protein; PLC, phospholipase C; DG, diacylglycerol; $IP_3$, *myo*-inositol-1,4,5-trisphosphate; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release; PKC, protein kinase C. Stimulatory linkages are shown as solid arrows, inhibitory linkages as dashed arrows. The dashed pathways labeled exhaustion indicate that positive feedback of $Ca^{2+}$ on its own release is soon limited by exhaustion of those stores. The matrix at the right shows the experimental tests applied here, where + and −, respectively, indicate agreement and disagreement with each theoretical mechanism and a blank indicates that the theory makes no strong prediction.

| Model | $[IP_3]$ oscillates | $Ca^{2+}$ feedback | Mechanism | $Ca^{2+}$ feedback positive | Needs hormone, blocked by heparin | $[IP_3]$ oscillates | PKC downregulation permissive |
|---|---|---|---|---|---|---|---|
| 1 | No | Negative | HR → G → PLC → $IP_3$ → $Ca^{2+}$ release; DG | − | | − | |
| 2 | No | Positive | HR → G → PLC → $IP_3$ → $Ca^{2+}$ release; DG; CICR from separate pool; exhaustion | + | − | − | |
| 3 | Yes | Negative | HR → G → PLC → $IP_3$ → $Ca^{2+}$ release; DG; PKC | − | | + | − |
| 4 | Yes | Positive | HR → G → PLC → $IP_3$ → $Ca^{2+}$ release; exhaustion; DG | + | + | + | |

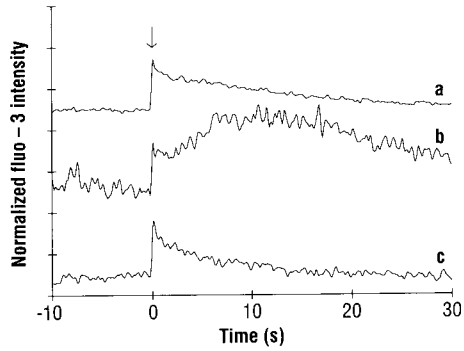


**Fig. 2.** Delayed release by $Ca^{2+}$ of further $Ca^{2+}$, requirement for hormone, and inhibition by heparin. (a) After a sudden release of $Ca^{2+}$ by flash photolysis of nitr-7 (arrow) in otherwise unstimulated cells, $[Ca^{2+}]_i$ decayed monotonically. The rising phase due to nitr-7 kinetics is complete in milliseconds (*8*) and is not resolved at the time scale shown. (b) Cells treated with vasopressin (50 nM) and gramicidin (500 nM) responded to the nitr-7 $Ca^{2+}$ pulse by further elevation of $[Ca^{2+}]_i$ peaking ~10 s after the flash. (c) Microinjection of heparin prevented nitr-7 photolysis from triggering a delayed $Ca^{2+}$ increase. In the top two traces cells were loaded with permeant esters (5 μM fluo-3/AM, 2 μM nitr-7/AM) in a manner identical to the fura-2 experiments. The cell in the bottom trace was injected at ~1% of its volume with a solution of fluo-3 (10 mM), nitr-7 (5 mM), heparin (100 mg/ml) (Sigma #H5640), and 25 mM $K^+$-Hepes, pH 7.3, then exposed to vasopressin and gramicidin, as in trace b. The photolytic flash from a xenon flashlamp (Chadwick-Helmuth, Monrovia, California) was delivered through the epi-illumination port of a Zeiss IM35 microscope, while $[Ca^{2+}]_i$ was monitored with fluo-3 and a photomultiplier (*16*). Increasing fluorescence from fluo-3 indicates increasing $[Ca^{2+}]_i$; the three traces have been arbitrarily offset vertically for clarity.

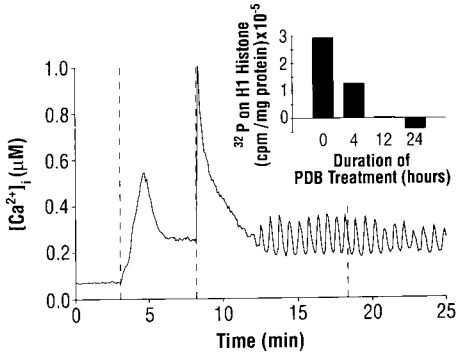


**Fig. 4.** Persistence of $[Ca^{2+}]_i$ oscillations after downregulation of protein kinase C. In downregulated cells, addition of fresh PDB (1 μM) had no effect on the oscillations, whereas in cells treated with PDB for the first time, as little as 5 nM was sufficient to suppress oscillations (*6*). a, 500 nM gramicidin; b, 50 nM vasopressin. $[Ca^{2+}]_i$ was measured with fura-2 as in Fig. 1. (**Inset**) Verification of the time course and completeness of the downregulation; protein kinase C activity was determined by a standard histone phosphorylation assay (*19*).

$[Ca^{2+}]_i$ rather than directly on extracellular $Ca^{2+}$. Thus when cells were preloaded with the $Ca^{2+}$ chelator BAPTA, then subjected to the same protocol of $Ca^{2+}$ deprivation and restoration, the additional buffering of $[Ca^{2+}]_i$ suppressed the peaks in both $[Ca^{2+}]_i$ and $IP_3$. A complementary experiment was to measure the effect of ionomycin (2.5 μM) on $IP_3$ concentrations. This ionophore clamped $[Ca^{2+}]_i$ in all the cells (whether previously oscillating or not) to a steady concentration of about 0.5 μM, similar to or slightly higher than the peak of individual cells' $[Ca^{2+}]_i$ spikes. Ionomycin alone had a negligible effect on $IP_3$ concentration (88 ± 16% of the value of untreated controls). Asynchronously oscillating cells had somewhat higher average $IP_3$ concentrations (178 ± 19% of control), but further addition of ionomycin to these cells gave much higher amounts (445 ± 47%). These results explain those of Figs. 1 and 2 by showing that elevated $[Ca^{2+}]_i$ by itself is neither necessary nor sufficient to increase to increase $IP_3$, yet it synergizes with receptor occupancy to stimulate maximal $IP_3$ production.

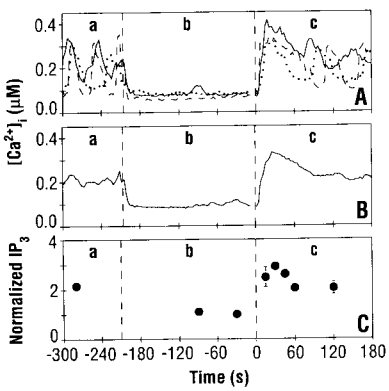


**Fig. 3.** Synchronization of $[Ca^{2+}]_i$ oscillations and detection of an accompanying pulse of $IP_3$. (**A**) $[Ca^{2+}]_i$ versus time traces for three representative individual cells. (**B**) A computer-calculated average for 15 cells in the field of view. Oscillations were synchronized for one cycle in the continuous presence of vasopressin (50 nM) and gramicidin (1 μM) by a protocol of extracellular $Ca^{2+}$ withdrawal (in Dulbecco's buffered saline that contained the vasopressin, gramicidin, EGTA (2 mM), glucose (5.6 mM) but no added $Ca^{2+}$, followed by $Ca^{2+}$ restoration (0.9 mM). $[Ca^{2+}]_i$ was measured with fura-2 imaging as in Fig. 1. (**C**) $IP_3$ assays at selected time points in parallel experiments. The $IP_3$ was measured by a competitive binding assay (*17*). Qualitatively similar results were obtained by preloading the cells with [$^3$H]inositol and measuring labeled $IP_3$ by HPLC (*18*). a, 0.9 mM $Ca^{2+}$; b, 2 mM EGTA; c, 0.9 mM $Ca^{2+}$.

Although the above results suggest a mechanism for $[Ca^{2+}]_i$ oscillations that does not involve protein kinase C, it was still desirable to test explicitly whether this enzyme has an essential function in the generation of oscillations. In REF52 cells, as in many other cell types, acute stimulation of protein kinase C by phorbol esters slows or inhibits $[Ca^{2+}]_i$ oscillations (*6*), but such observations do not show that kinase is necessary for oscillations. A better test is to eliminate the enzyme by downregulation, which was accomplished in confluent REF52 cultures by overnight treatment with 4β-phorbol 12,13-dibutyrate (PDB) (2 μM). After this treatment, protein kinase C activity was measured by a conventional assay (*10*) and found to be completely absent. However, these cells could still be induced to oscillate by the usual combination of vasopressin and depolarization (Fig. 4). Moreover, the oscillations were no longer sensitive to treatment with a high dose of additional phorbol ester, as one would expect if protein kinase C had been completely downregulated. In analogous experiments (*11*), sphinganine (20 to 60 μM), staurosporine (100 nM), and H-7 (10 to 20 μM), which are blockers of protein kinase C (*12*), did not affect $[Ca^{2+}]_i$ oscillations. These results show that protein kinase C is not essential for the maintenance or timing of the $[Ca^{2+}]_i$ oscillations.

These results, and others previously reported (*6*), are most consistent with model 4 (Table 1), in which the major feedback loop is $Ca^{2+}$ stimulation of phospholipase C to generate $IP_3$, which releases more $Ca^{2+}$ if the internal stores are sufficiently full (*5*). It is currently controversial (*13*) whether phospholipase C is significantly stimulated by $[Ca^{2+}]_i$ increases, and if so, whether the stimulation is in parallel to or synergistic with receptor–G protein activation. Although the present results with intact REF52 fibroblasts show synergistic stimulation, the plethora of G proteins and isozymes of phospholipase C suggest that the interaction with $[Ca^{2+}]_i$ is likely to vary from tissue to tissue. Likewise the mechanisms of $[Ca^{2+}]_i$ oscillations are probably variable, but the types of experimental tests introduced here should be helpful in determining these mechanisms in other tissues.

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14. Ryanodine at 10 μM immediately halted spontaneous $[Ca^{2+}]_i$ oscillations in neonatal heart cells (A. T. Harootunian, unpublished data). Oscillations in REF52 fibroblasts occurred in the presence of 1 to 10 μM ryanodine and 1 to 10 mM caffeine. Caffeine at 25 mM lowered $[Ca^{2+}]_i$ and halted oscillations, a result that was not unexpected, because caffeine at such high doses been reported to block voltage-dependent $Ca^{2+}$ channels and phosphodiesterase [D. Lipscombe *et al.*, in (7)].
15. The $[Ca^{2+}]_i$ was measured with a Zeiss IM35 microscope and imaging system described previously [R. Y. Tsien and A. T. Harootunian, *Cell Calcium* **11**, 93 (1990)]. Cells were loaded with fura-2 by incubating the cells for 1 hour in a Hepes-buffered Dulbecco's modified Eagle's medium containing fura-2/AM (100 nM). Experiments were performed at 30°C in Hanks balanced salt solution (oscillations occur between 25° and 37°C).
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17. The $IP_3$ mass assays were performed with an $IP_3$ binding protein kit (Amersham) [M. Seishima, Y. Yada, S. Nagao, S. Mori, Y. Nozawa, *Biochem. Biophys. Res. Commun.* **156**, 1077 (1988); N. M. Dean and M. A. Beaven, *Anal. Biochem.* **183**, 199 (1989); M. C. Michel *et al.*, *J. Biol. Chem.* **264**, 4986 (1989).] Experiments were done on confluent monolayers of cells on 25-mm round cover slips in Dulbecco's phosphate-buffered saline with glucose (5.6 mM) at 30°C. Reactions were halted by rapid transfer of cover slips into ice-cold trifluoracetic acid (1 M). Samples were centrifuged to remove proteins, lyophilized to remove acid, and finally resuspended to a volume of 100 μl and neutralized. $IP_3$ was quantified from a standard curve of known amounts of $IP_3$ (0.25 to 25 pmol) competitively releasing $[^3H]IP_3$ from the binding protein. The $IP_3$ concentration was normalized to the amount of total cellular protein. Experiments were run in duplicate on separate cover slips of cells; error bars smaller than the symbol size were omitted. $IP_3$ maxima were observed 30 s after $Ca^{2+}$ readdition in five of five similar experiments.
18. A pulse of $IP_3$ on readdition of extracellular $Ca^{2+}$ to oscillating cells was detected by high-performance liquid chromatography (HPLC) of $[^3H]IP_3$ from cells previously labeled with [$^3$H]inositol. The time course of the $IP_3$ pulse was similar to that seen by the $IP_3$ mass assay and peaked 30 s after $Ca^{2+}$ was added back. The $IP_3$ concentration at that point was 1.8 times more than the preceding period in EGTA. The $[^3H]IP_3$ concentrations were determined with techniques described by S. K. Ambler, B. Thompson, P. A. Solski, J. H. Brown, and P. Taylor [*Mol. Pharmacol.* **32**, 376 (1987)].
19. The protein kinase C activity assay involved labeling of H1 histone with radioactive phosphate in a mixed micelle system as described [D. Mochly-Rosen and D. E. Koshland, Jr., *J. Biol. Chem.* **262**, 2291 (1987); U. Kikkawa, Y. Takai, R. Minakuchi, S. Inohara, Y. Nishizuka, *ibid.* **257**, 13341 (1982)]. Labeling of histone H1 was defined as the difference between activity with and without $Ca^{2+}$, phosphatidylserine, and diolein.
20. We thank S. Adams for providing the nitr-7 and nitr-7/AM, B. Platz and P. Taylor for help with the HPLC separation of $[^3H]IP_3$, and A. Flint for help with the protein kinase C assays. Supported by NIH grants GM 31004 and NS 27177 and the Howard Hughes Medical Institute.

27 August 1990; accepted 26 October 1990