6. T. Matsui *et al.*, *Science* **243**, 800 (1989); M. H. Kraus *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9193 (1989); G. D. Kruh *et al.*, *ibid.* **87**, 5802 (1990).
7. J. S. Rubin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 802 (1989).
8. P. W. Finch *et al.*, *Science* **245**, 752 (1989).
9. D. P. Bottaro *et al.*, *J. Biol. Chem.* **265**, 12767 (1990).
10. B. E. Weissman and S. A. Aaronson, *Cell* **32**, 599 (1983).
11. T. Miki, unpublished data.
12. J. L. Jainchill, S. A. Aaronson, G. J. Todaro, *J. Virol.* **4**, 549 (1969).
13. J. S. Rubin, unpublished data.
14. Genomic DNA from each transformant was cleaved by one of the infrequent cutters that can release the plasmids containing cDNA inserts. Digested DNA was ligated under diluted conditions and used to transform bacterial-competent cells. Plasmids were isolated from ampicillin- and kanamycin-resistant transformants and used to transfect NIH/3T3 cells to examine for focus formation. The *ect*1 plasmid was rescued by Xho I, while the *ect*2 and *ect*3 plasmids were rescued by Not I digestion.
15. M. Kozak, *Nucleic Acids Res.* **5**, 8125 (1987).
16. A. Safran *et al.*, *Oncogene* **5**, 635 (1990).
17. H. H. Reid, A. F. Wilks, O. Bernard, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1596 (1990); A. Mansukuhani, D. Moscatelli, D. Talarico, V. Levytska, C. Basilico, *ibid.*, p. 4378.
18. P. L. Lee, D. E. Johnson, L. S. Cousens, V. A. Fried, L. T. Williams, *Science* **245**, 57 (1989); E. B. Pasquale and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8722 (1989).
19. M. Ruta *et al.*, *Oncogene* **3**, 9 (1988); M. Ruta *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5449 (1989).
20. S. Kornbluth, K. E. Paulson, H. Hanafusa, *Mol. Cell. Biol.* **8**, 5541 (1988).
21. D. P. Bottaro, unpublished data.
22. C. Betsholtz, A. Johnsson, C.-H. Heldin, B. Westermark, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6440 (1986); T. P. Fleming *et al.*, *ibid.* **86**, 8063 (1989).
23. J. S. Rubin, unpublished data.
24. C. A. Dionne *et al.*, *EMBO J.* **9**, 2685 (1990).
25. E. B. Pasquale, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5812 (1990).
26. Y. Hattori *et al.*, *ibid.*, p. 5983.
27. D. E. Johnson, P. L. Lee, J. Lu, L. T. Williams, *Mol. Cell. Biol.* **10**, 4728 (1990).
28. DNA (10 μg) was digested by Sal I (Fig. 1A) or Eco RI (Fig. 1B), fractionated by agarose gel electrophoresis, and transferred to a nylon-supported nitrocellulose paper (Nitrocellulose-GTG, FMC Corp.). The blot in Fig. 1A was hybridized with the ^{32}P-labeled entire *ect*1 insert at 42°C and washed at 65°C in 0.1× saline sodium citrate (SSC), while the blot in Fig. 1B was hybridized with the ^{32}P-labeled 5′-*ect*1 probe (Fig. 2B) at 37°C and washed at 55°C in 0.1× SSC. Hybridization experiments were performed at the indicated temperature in a solution containing 50% formamide, 5× SSC, 2.5× Denhardt solution, 7 mM tris-HCl (pH 7.5), denatured calf thymus DNA (0.1 mg/ml), and tRNA (0.1 mg/ml). Location of DNA markers (BRL, Gaithersburg, MD) is indicated in kilobases.
29. Polyadenylated RNA preparations (5 μg each) were fractionated by formaldehyde gel, transferred to Nitrocellulose-GTG, and hybridized with the 5′-*ect*1 probe (Fig. 1C, lanes 1 and 2). After autoradiography, the filter was boiled to remove the probe and then hybridized with a β-actin probe (lanes 3 and 4). Location of markers (BRL, Gaithersburg, MD) is indicated in kilobases.
30. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
31. To control for the quality of the ^{125}I-labeled and unlabeled ligands, we tested each and all were mitogenic at picomolar concentrations on either BALB/MK (aFGF and KGF) or NIH/3T3 (aFGF and bFGF). As previously reported (*9*), the aFGF and bFGF preparations competed with similar efficiency for ^{125}I-labeled aFGF binding to NIH/3T3 cells.

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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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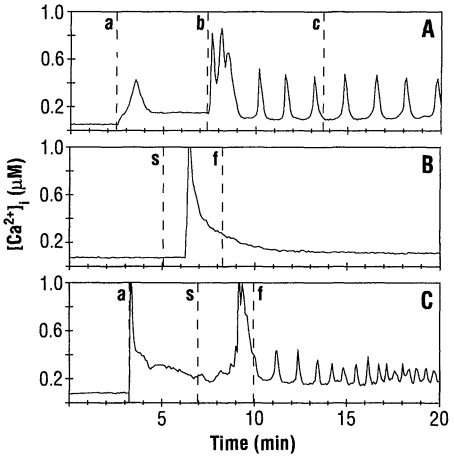

Fig. 1. $[Ca^{2+}]_i$ oscillations in REF52 fibroblasts and the effects of Ca^{2+} overload and of pharmacological interference with Ca^{2+}-induced Ca^{2+} release. (**A**) $[Ca^{2+}]_i$ in individual REF52 fibroblasts oscillated with rhythmical accuracy after stimulation with gramicidin (500 nM) (a) 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; PLC → DG	−		−	
2	No	Positive	HR → G → PLC → IP_3 → Ca^{2+} release; PLC → DG; Ca^{2+} release → CICR from separate pool; CICR from separate pool → exhaustion	+	−	−	
3	Yes	Negative	HR → G → PLC → IP_3 → Ca^{2+} release; PLC → DG; DG → PKC; PKC → HR			+	−
4	Yes	Positive	HR → G → PLC → IP_3 → Ca^{2+} release; Ca^{2+} release → exhaustion; PLC → DG; DG → Ca^{2+} release	+	+	+	

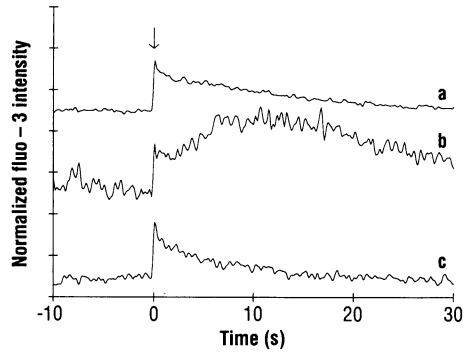

Fig. 2. Delayed release by Ca^{2+} of further Ca^{2+}, requirement for hormone, and inhibition by heparin. (a) After a sudden release of Ca^{2+} by flash photolysis of nitr-7 (arrow) in otherwise unstimulated cells, $[Ca^{2+}]_i$ decayed monotonically. The rising phase due to nitr-7 kinetics is complete 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.

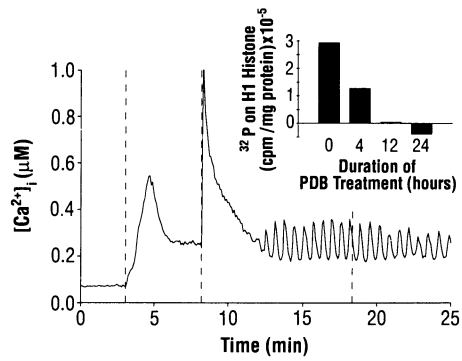

Fig. 4. Persistence of $[Ca^{2+}]_i$ oscillations after downregulation of protein kinase C. In downregulated cells, addition of fresh PDB (1 μM) had no effect on the oscillations, whereas in cells treated with PDB for the first time, as little as 5 nM was sufficient to suppress oscillations (*6*). a, 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.

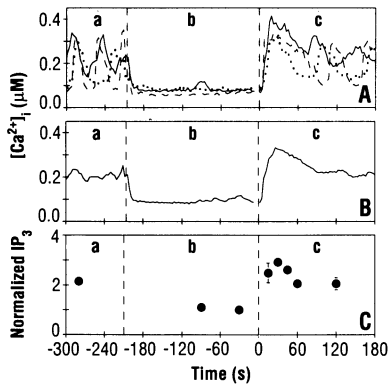

Fig. 3. Synchronization of $[Ca^{2+}]_i$ oscillations and detection of an accompanying pulse of IP_3. (**A**) $[Ca^{2+}]_i$ versus time traces for three representative individual cells. (**B**) A computer-calculated average for 15 cells in the field of view. Oscillations were synchronized for one cycle 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.

REFERENCES AND NOTES

1. M. J. Berridge, *J. Biol. Chem.* **265**, 9583 (1990); R. Jacob, *Biochim. Biophys. Acta* **1052**, 427 (1990); T. J. Rink and J. E. Merritt, *Curr. Opin. Cell Biol.* **2**, 198 (1990); R. W. Tsien and R. Y. Tsien, *Annu. Rev. Cell Biol.* **6**, 715 (1990).
2. R. Payne, T. M. Flores, A. Fein, *Neuron* **4**, 547 (1990); I. Parker and I. Ivorra, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 260 (1990).
3. A. Goldbeter, G. Dupont, M. J. Berridge, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1461 (1990); M. J. Berridge and R. F. Irvine, *Nature* **341**, 197 (1989); T. A. Rooney, E. J. Sass, A. P. Thomas, *J. Biol. Chem.* **264**, 17131 (1989); M. Wakui, B. V. L. Potter, O. H. Petersen, *Nature* **339**, 317 (1989).
4. N. M. Woods, K. S. R. Cuthbertson, P. H. Cobbold, *Cell Calcium* **8**, 79 (1987); M. J. Berridge, P. H. Cobbold, K. S. R. Cuthbertson, *Philos. Trans.*

R. Soc. London Ser. B **320**, 325 (1988).
5. T. Meyer and L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5051 (1988).
6. A. T. Harootunian, J. P. Y. Kao, R. Y. Tsien, *Cold Spring Harbor Symp. Quant. Biol.* **53**, 945 (1988); *J. Cell Biol.* **109**, 99a (1989).
7. M. Endo, M. Tanaka, Y. Ogawa, *Nature* **228**, 34 (1970); A. Fabiato, *Am. J. Physiol.* **245**, C1 (1983); M. Valdeolmillos, S. C. O'Neill, G. L. Smith, D. A. Eisner, *Pflügers Arch.* **413**, 676 (1989); D. Lipscombe *et al.*, *Neuron* **1**, 335 (1988); K. Kuba, *J. Physiol.* **298**, 547 (1980); A. Malgaroli, R. Fesce, J. Meldolesi, *J. Biol. Chem.* **265**, 3005 (1990).
8. S. R. Adams, J. P. Y. Kao, G. Grynkiewicz, A. Minta, R. Y. Tsien, *J. Am. Chem. Soc.* **110**, 3212 (1988); J. H. Kaplan, *Annu. Rev. Physiol.* **52**, 897 (1990).
9. S. Kobayashi, T. Kitazawa, A. V. Somlyo, A. P. Somlyo, *J. Biol. Chem.* **264**, 17997 (1989); P. Palade, C. Dettbarn, B. Alderson, P. Volpe, *Mol. Pharmacol.* **36**, 673 (1989); T. K. Ghosh, P. S. Eis, J. M. Mullaney, C. L. Ebert, D. L. Gill, *J. Biol. Chem.* **263**, 11075 (1988).
10. A. Rodriguez-Pena and E. Rozengurt, *Biochem. Biophys. Res. Commun.* **120**, 1053 (1984).
11. A. Harootunian, unpublished data.
12. Y. A. Hannun and R. M. Bell, *Science* **243**, 500 (1989); U. T. Rüegg and G. M. Burgess, *Trends Pharmacol. Sci.* **10**, 218 (1989). Though the pharmacological specificity of these inhibitors and protein kinase C downregulation must be viewed with caution, the lack of effect of such disparate means of enzyme inhibition on $[Ca^{2+}]_i$ oscillations is considered to be meaningful.
13. D. A. Eberhard and R. W. Holz, *Trends Neurosci.* **11**, 517 (1988); D. Renard, J. Poggioli, B. Berthon, M. Claret, *Biochem. J.* **243**, 319 (1987); S. G. Rhee, P. G. Suh, S. H. Ryu, S. Y. Lee, *Science* **244**, 546 (1989).
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).
16. J. P. Y. Kao, A. T. Harootunian, R. Y. Tsien, *J. Biol. Chem.* **264**, 8179 (1989).
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.
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Glycosylphosphatidylinositol: A Candidate System for Interleukin-2 Signal Transduction

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The mechanism of interleukin-2 (IL-2) signal transduction was analyzed by use of an inducible B lymphoma. Like normal antigen-activated B lymphocytes, the lymphoma cells respond to IL-2 by proliferating and differentiating into antibody-secreting cells; both responses are blocked by a second interleukin, IL-4. Analyses of the signaling pathway showed that IL-2 stimulated the rapid hydrolysis of an inositol-containing glycolipid to yield two possible second messengers, a myristylated diacylglycerol and an inositol phosphate-glycan. The myristylated diacylglycerol response exhibited the same IL-2 dose dependence as the growth and differentiative responses, and the generation of both hydrolysis products was inhibited by IL-4. These correlations implicate the glycosyl-phosphatidylinositol system in the intracellular relay of the IL-2 signal.

THE PATHWAYS BY WHICH LYMPHOkine signals are relayed in B and T lymphocytes have yet to be determined. Analyses of IL-2– and IL-4–induced responses indicate that the classical second messenger systems are not involved (*1–3*). Thus, in cells where the lymphokine signals can be distinguished from those initiated at the antigen receptor, the binding of IL-2 and IL-4 to their respective high-affinity receptors does not stimulate calcium mobilization or increase production of phosphatidyl inositol (PI) metabolites. The lymphokine responses cannot be mimicked by ionomycin and/or phorbol ester treatment and are not affected by inhibitors of cyclic adenosine monophosphate (cAMP)– or cyclic guanosine monophosphate (cGMP)–dependent kinases. Finally, none of the lymphokine receptors has a cytoplasmic domain with the characteristic structure of a tyrosine kinase (*4*). In view of these findings, it seems likely that lymphokine signals are transduced by unidentified second messengers and/or by kinases that are associated directly with the lymphokine receptors in the membrane.

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A novel second messenger system has been implicated in insulin (*5*) and nerve growth factor (NGF) signaling (*6*). The precursor is believed to be one of a structurally related set of glycosyl-phosphatidylinositol (Gly-PI) molecules (*7*). Although the structures of the different Gly-PI forms have not been precisely defined, these molecules are known to contain a distinctive hydrophobic domain, usually 1,2-dimyristoylacylglycerol, and a PI that is glycosidically linked to a glycan moiety through glucosamine. When insulin or NGF binds to its receptor, a hormone-sensitive Gly-PI is hydrolyzed, probably through the activation of a specific phospholipase C. The result is the rapid appearance of myristylated diacylglycerol (myr-DAG) in the membrane and the release of inositol phosphate–glycan (IP-glycan) into the cytoplasm. A second messenger function for myr-DAG has yet to be established, but IP-glycan has been shown to mediate some of the effects of insulin on lipid and carbohydrate metabolism (*8*).

To explore the possibility that lymphokine signaling operates through this pathway, we used an IL-2 inducible B cell line. The BCL_1 lymphoma was derived from a cell that had received the early signals in a primary immune response (*9*). Like their normal counterparts (*10*), BCL_1 cells express functional IL-2 receptors and are capable of responding to the lymphokine (*11, 12*). Under standard culture conditions