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Imaging and manipulation of cytosolic ions and messengers during cell activation

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Summary

Optical methods have recently become available for continuously imaging the free concentrations of important ions and second messengers such as calcium, sodium and hydrogen inside living cells. These ion levels are found to undergo remarkable changes upon stimulation of quiescent cells with growth factors known to stimulate phosphoinositide breakdown. In serum-starved REF-52 fibroblasts, growth factors such as serum, vasopressin, or PDGF (platelet-derived growth factor) cause intracellular $[Na^+]$ to increase from about 4 mM to 8 mM. If mitogen treatment is combined with pharmacological depolarization of the membrane potential, repetitive $[Ca^{2+}]_i$ spikes result in these rat fibroblasts. The mechanism of this oscillation has been investigated by light-flash release of intracellular messengers such as inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), Ca^{2+}, and diacylglycerol, as well as more traditional biochemical techniques. The key feedback pathway appears to be Ca^{2+}-stimulation of phospholipase C production of Ins(1,4,5)P_3.

Introduction

Recent technical advances now enable cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$), Na^+ concentrations ($[Na^+]_i$), and pH to be continuously imaged inside individual living cells with micrometer spatial resolution and subsecond time resolution (for recent reviews see Tsien, 1988, 1989). This methodology relies on the molecular engineering of indicator dyes whose fluorescence is strong and highly sensitive to those ions (Grynkiewicz *et al.* 1985; Minta and Tsien, 1989; Rink *et al.* 1982). Binding of these ions shifts the fluorescence spectrum of the corresponding indicator. The ratio of excitation or emission amplitudes at two wavelengths measures the free ion concentration while canceling out intensity variations due to non-uniform cell thickness or dye content. A fluorescence microscope equipped to acquire images at two wavelengths and ratio them can thus produce dynamic images of intracellular messenger levels.

Results and discussion

Currently our most fully developed use of this methodology is the analysis of the response of the REF-52 line of rat fibroblasts to mitogenic stimulation. Serum-starved cells typically show $[Na^+]_i$ of only about 4 mM. Within a few minutes after addition of serum vasopressin, or PDGF, $[Na^+]_i$ doubles to about 8 mM

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(Harootunian *et al.* 1989*a*). This surprisingly large increase cannot be blocked by cytoplasmic alkalinization, a finding that suggests that the Na^+ influx is primarily due to pathways other than Na^+/H^+ exchange. When similar mitogenic stimulation using serum, vasopressin, bradykinin, thrombin, bombesin or ATP is combined with membrane potential depolarization by gramicidin, high $[K^+]$, or sodium pump blockage, repetitive spikes of high $[Ca^{2+}]_i$ result every few tens of seconds to minutes (Harootunian *et al.* 1988). Though these oscillations require static depolarization and Ca^{2+} influx, they do not involve cyclic fluctuations of membrane potential as in excitable cells, but rather rhythmic dumping of intracellular stores. Similar oscillations can be elicited without depolarization if GTPγS (guanosine-5′-*O*-(3-thiotriphosphate) or AlF_4^- are administered to activate G proteins (Harootunian *et al.* 1989*b*). Many other cases of $[Ca^{2+}]_i$ oscillations have been reported in other cell types (for reviews see Berridge and Galione, 1988; Berridge and Irvine, 1989; Rink and Hallam, 1989; Rink and Jacob, 1989), but the amenability of REF-52 cells to microinjection and the reproducibility and precise rhythm of their $[Ca^{2+}]_i$ spikes make them an unusually favorable system for analyzing their biochemical mechanism. An essential adjunct to single-cell imaging is the ability suddenly to release Ca^{2+}, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), and diacylglycerol (DG) by photolysis of caged precursors. Ca^{2+} is released from the light-sensitive chelator nitr-7, whose high pre-photolysis affinity for Ca^{2+} makes it better for these experiments than the commercially available nitr-5 (Adams *et al.* 1988). Ins(1,4,5)P_3 is generated by photocleavage of its 1-(2-nitrophenyl)ethyl ester (Walker *et al.* 1987); *sn*-1,2-dioctanoylglycerol is released by irradiation of its 2-nitro-4,5-dimethoxybenzyl ether.

Experiments were designed to differentiate between several oscillation mechanisms already proposed in the literature: (1) Cobbold and collaborators (Woods *et al.* 1987; Berridge *et al.* 1988) have suggested that phosphorylations due to protein kinase C are the dominant negative feedback mechanism controlling the interspike interval. (2) Berridge and collaborators (Berridge *et al.* 1988; Berridge and Irvine, 1989; Goldbeter *et al.* 1990) have proposed that Ins(1,4,5)P_3 releases Ca^{2+} from one intracellular pool, causing Ca^{2+} overload and Ca^{2+}-dependent Ca^{2+} release (CICR) from an Ins(1,4,5)P_3-insensitive pool. (3) Payne *et al.* (1988) and Parker and Ivorra, (1990) have noted that high $[Ca^{2+}]_i$ may inhibit the ability of Ins(1,4,5)P_3 to release more Ca^{2+}; this negative feedback could generate oscillations if some kinetic delays were introduced. (4) Meyer and Stryer (1988) have suggested that positive cooperativity of Ins(1,4,5)P_3 in releasing Ca^{2+} from internal stores, together with Ca^{2+} stimulation of phospholipase C to generate more Ins(1,4,5)P_3 (Eberhard and Holz, 1988), would be a potent feedback loop capable of generating oscillations.

Our current data suggest that the Meyer and Stryer (1988) model, with some modifications, is the most likely to describe the $[Ca^{2+}]_i$ oscillations in REF-52 cells. This conclusion is based on the following key observations: (1) although phorbol esters do slow or inhibit oscillations, *cyclical* activation/deactivation of

protein kinase C does not seem necessary for the rhythm generation, since flash photolytic generation of diacylglycerol slows the rhythm for several successive cycles, not just the first after the flash. Also, protein kinase C can be thoroughly down-regulated by12–24 h exposure to phorbol esters, after which oscillations can be produced as normal, though of course they can no longer be inhibited by reapplication of phorbol esters. (2) In the Berridge model, Ins(1,4,5)P_3 levels are elevated in a steady non-oscillatory manner upon agonist stimulation. Sudden delivery of additional Ins(1,4,5)P_3 should either have no effect, or, if it releases some Ca^{2+}, should advance the next cycle of CICR. In fact, photorelease of Ins(1,4,5)P_3 elevates $[Ca^{2+}]_i$ at all phases of the oscillation cycle and causes phase-resetting (and sometimes considerable delay) of the subsequent endogenous oscillations. Also, REF-52 cells are quite insensitive to the traditional pharmacological tests for CICR such as caffeine and ryanodine. Cells can be subjected to deliberate moderate to massive $[Ca^{2+}]_i$ elevation, for example by wounding with a micropipet, but oscillations never result from such Ca^{2+} overload alone, and still require addition of the mitogenic hormone, presumably to stimulate PIP_2 breakdown. (3) Photorelease of Ca^{2+} in oscillating cells causes not only an immediate but also a secondary rise in $[Ca^{2+}]_i$ about 10 s later; the second phase is blocked by heparin, which is known to block Ins(1,4,5)P_3 receptors (e.g. see Kobayashi *et al.* 1989). Also, in some microinjected cells, the endogenous oscillations can be seen to proceed as waves which are initiated at localized sites of high Ca^{2+}, due to imperfect resealing from the microinjection. Thus in REF-52 cells, Ca^{2+} exerts mainly *positive* feedback on its own release, and this is probably Ins(1,4,5)P_3 mediated. (4) GTPγS causes $[Ca^{2+}]_i$ oscillations that proceed at lower $[Ca^{2+}]_i$ levels and that are relatively independent of extracellular Ca^{2+}, an effect explicable by the reported ability of GTPγS to shift the Ca^{2+}-activation curve of phospholipase C to lower Ca^{2+} concentrations (e.g. see Taylor and Exton, 1987). (5) We are attempting to synchronize the $[Ca^{2+}]_i$ oscillations sufficiently so that direct assay of Ins(1,4,5)P_3, which necessarily must be done on large populations of cells, can show whether its levels oscillate as well.

Future investigations of intracellular signaling, especially to determine the functional significance of complex single-cell responses such as oscillations and spatial gradients, would be aided if further optical methods could be developed. For example, a light-sensitive Ca^{2+} chelator whose affinity could be reversibly cycled from high to low and back by different wavelengths of illumination (unlike current designs, which use irreversible photochemistry: Adams *et al.* 1988, 1989) would be useful for generating artificial oscillations and spatial gradients. Methods for measuring protein phosphorylation and reporter gene transcription at the single cell level would also be highly desirable to complement the imaging of second messenger signals.

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