# Spatial and temporal aspects of cellular calcium signaling

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ABSTRACT **Cytosolic $Ca^{2+}$ signals are often organized in complex temporal and spatial patterns, even under conditions of sustained stimulation. In this review we discuss the mechanisms and physiological significance of this behavior in nonexcitable cells, in which the primary mechanism of $Ca^{2+}$ mobilization is through (1,4,5)$IP_3$-dependent $Ca^{2+}$ release from intracellular stores. Oscillations of cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_i$) are a common form of temporal organization; in the spatial domain, these $[Ca^{2+}]_i$ oscillations may take the form of $[Ca^{2+}]_i$ waves that propagate throughout the cell or they may be restricted to specific subcellular regions. These patterns of $Ca^{2+}$ signaling result from the limited range of cytoplasmic $Ca^{2+}$ diffusion and the feedback regulation of the pathways responsible for $Ca^{2+}$ mobilization. In addition, the spatial organization of $[Ca^{2+}]_i$ changes appears to depend on the strategic distribution of $Ca^{2+}$ stores within the cell. One type of $[Ca^{2+}]_i$ oscillation is baseline spiking, in which discrete $[Ca^{2+}]_i$ spikes occur with a frequency, but not amplitude, that is determined by agonist dose. Most current evidence favors a model in which baseline $[Ca^{2+}]_i$ spiking results from the complex interplay between $[Ca^{2+}]_i$ and (1,4,5)$IP_3$ in regulating the gating of (1,4,5)$IP_3$-sensitive intracellular $Ca^{2+}$ channels. Sinusoidal $[Ca^{2+}]_i$ oscillations represent a mechanistically distinct type of temporal organization, in which agonist dose regulates the amplitude but has no effect on oscillation frequency. Sinusoidal $[Ca^{2+}]_i$ oscillations can be explained by a negative feedback effect of protein kinase C on the generation of (1,4,5)$IP_3$ at the level of phospholipase C or its activating G-protein. The physiological significance of $[Ca^{2+}]_i$ oscillations and waves is becoming more established with the observation of this behavior in intact tissues and by the recognition of $Ca^{2+}$-dependent processes that are adapted to respond to frequency-modulated oscillatory $[Ca^{2+}]_i$ signals. In some cells, these $[Ca^{2+}]_i$ signals are targeted to control processes in limited cytoplasmic domains, and in other systems $[Ca^{2+}]_i$ waves can be propagated through gap junctions to coordinate the function of multicellular systems.—Thomas, A. P., Bird, G. S. J., Hajnóczky, G., Robb-Gaspers, L. D., Putney, J. W., Jr. Spatial and temporal aspects of cellular calcium signaling. *FASEB J.* 10, 1505–1517 (1996)**

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OSCILLATIONS IN $[Ca^{2+}]_i$, REFLECTING periodic fluctuations in membrane electrical activity, have been known for some time: the clearest example is the rhythmic cardiac action potentials that drive bursts of $Ca^{2+}$ release, entry and extrusion, and thereby maintain the pumping activity of the heart. The existence of $[Ca^{2+}]_i$ oscillations in nonexcitable cells was first suggested by Prince and Berridge (1), but this phenomenon was not widely appreciated until Woods et al. (2) directly demonstrated it in single, aequorin-injected hepatocytes. These investigators demonstrated that hormones acting through inositol lipid-specific phospholipase C (PLC)[2] caused discrete spikes or transients of $[Ca^{2+}]_i$ rising periodically from a baseline level of $[Ca^{2+}]_i$ similar to that in unstimulated cells. In the absence of an intuitive basis for this kinetic behavior in electrically nonexcitable cells, it was immediately clear that novel regulatory mechanisms must operate to produce these intriguing patterns. Subsequently, additional complexity was revealed when the spatial organization of $[Ca^{2+}]_i$ signaling was examined with digital imaging techniques; associated with $[Ca^{2+}]_i$ oscillations, $Ca^{2+}$ was seen to rise in cells in a nonuniform manner, giving rise to the appearance of $[Ca^{2+}]_i$ waves in the cytoplasm (3). In the years ensuing, much study has been devoted to basic control mechanisms that give rise to $[Ca^{2+}]_i$ oscillations, and this work has been the subject of earlier reviews (3–16). In most cases, $[Ca^{2+}]_i$ oscillations that occur independently of plasma membrane electrical activity are associated with stimulation of the inositol lipid-dependent signaling cascade and appear to rely, at least in part, on the release of intracellular $Ca^{2+}$ stores by (1,4,5)$IP_3$. In this review, we will attempt to summarize some of the most recent findings and ideas regarding

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[2]Abbreviations: PLC, phospholipase C; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release.

the types and mechanisms of these $[Ca^{2+}]_i$ oscillations, and discuss their significance in cellular signaling.

Before describing possible mechanisms of $[Ca^{2+}]_i$ oscillations, we must consider that $[Ca^{2+}]_i$ oscillations may be of different fundamental types, involving different mechanisms. Two major kinds of $[Ca^{2+}]_i$ oscillations are baseline transients or spikes and sinusoidal oscillations (5, 8). **Figure 1** illustrates these two oscillatory patterns. Baseline spikes are characterized by transient increases in $[Ca^{2+}]_i$ that rise rapidly from a baseline of $[Ca^{2+}]_i$, which is generally close to the resting level and can be maintained for relatively long periods between the individual $[Ca^{2+}]_i$ spikes. Sinusoidal oscillations more closely resemble true sine wave oscillations; they are generally of a higher frequency than baseline spikes >1/min, as opposed to frequencies <1/min for baseline spikes), and also generally appear as symmetrical oscillations superimposed on a sustained level of $[Ca^{2+}]_i$ usually above the prestimulus baseline level. Another notable difference between the two types of oscillations is that baseline spikes may sometimes continue throughout prolonged periods of stimulation, whereas sinusoidal oscillations tend to diminish with time, generally lasting for only a few minutes. However, the most significant and characteristic distinction between the two types of oscillations is the relationship of the oscillation amplitude and frequency to stimulus strength, or agonist concentration (16): for baseline spikes, increasing the agonist concentration increases the frequency of the spikes without affecting the amplitude or kinetics of the spikes, whereas for sinusoidal oscillations, increasing the agonist concentration increases the average $[Ca^{2+}]_i$ level without affecting the oscillation frequency. An additional, and likely significant, property of baseline spikes that differs from sinusoidal oscillations is that the latency before the first $[Ca^{2+}]_i$ spike is inversely related to the agonist concentration (17). The same mechanism underlying the varying frequency of spiking may also be responsible for the varying latency for the first spike, but this is not necessarily so. The persistent, constant amplitude baseline spikes require a positive feedback (sometimes called feed forward) mechanism to generate the spikes, with either a negative feedback or some capacity limitation (i.e., full depletion of an intracellular pool) to terminate each spike. On the other hand, negative feedback alone is sufficient to explain the behavior of the unstable, constant-frequency sinusoidal oscillations. Recent research and speculation has focused on the nature of the feed forward and termination mechanisms responsible for the baseline spikes and on the nature of the negative feedback mechanism responsible for the sinusoidal oscillations.

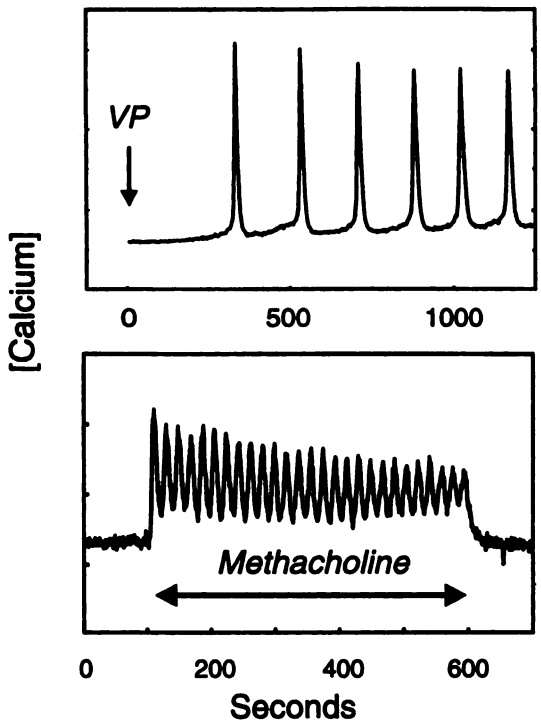


**Figure 1.** Baseline and sinusoidal $[Ca^{2+}]_i$ oscillations.

## BASELINE CALCIUM SPIKES AND ASSOCIATED CALCIUM WAVES

In many cells where $[Ca^{2+}]_i$ changes have been examined with sufficient temporal and spatial resolution, it has been found that oscillatory $[Ca^{2+}]_i$ spikes are also organized spatially at the subcellular level. This spatial organization often takes the form of $[Ca^{2+}]_i$ waves that spread through all or part of the cell during each $[Ca^{2+}]_i$ oscillation cycle. These $[Ca^{2+}]_i$ waves may serve an important function to ensure that the $[Ca^{2+}]_i$ signal is propagated at full strength to the appropriate subcellular targets (14); otherwise $Ca^{2+}$ would be restricted to act as a local message due to its limited range of diffusion within the cytosol (18). In fact, $[Ca^{2+}]_i$ waves may be an inevitable consequence of the mechanisms responsible for driving the oscillatory $[Ca^{2+}]_i$ spikes, such that the two processes can be considered the temporal and spatial manifestations of a single phenomenon. For this reason, the mechanisms responsible for both $[Ca^{2+}]_i$ oscillations and $[Ca^{2+}]_i$ waves will be discussed together.

### Properties of $[Ca^{2+}]_i$ spikes

The baseline $[Ca^{2+}]_i$ spiking type of oscillatory behavior has been observed in a wide variety of cells and in response to many different types of stimulus. Although there are considerable variations in the appearance of these $[Ca^{2+}]_i$ oscillations, the individual $[Ca^{2+}]_i$ spikes characteristically have a relatively rapid rising phase with a slower falling phase. The falling phase is the component of the $[Ca^{2+}]_i$ spikes that shows the greatest diversity. In fact, even within an individual cell, different agonists can give characteristically distinct shapes of $[Ca^{2+}]_i$ spikes that vary only in the decay phase (2, 17, 19, 20). Such variations in the shape of $[Ca^{2+}]_i$ spikes

with different agonists is a widespread observation in many cell types. However, there may be numerous distinct underlying causes for this diversity, including the subcellular localization of the $[Ca^{2+}]_i$ changes, the rate of (1,4,5)$IP_3$ formation, the type of signal transduction mechanism involved in the initial activation of PLC, and other receptor-specific differences in signal transduction such as receptor down-regulation and differential coupling to other second messenger pathways (14, 21, 22). Despite the diversity in the patterns of $[Ca^{2+}]_i$ spiking between cell types and with different agonists, a remarkable feature of $[Ca^{2+}]_i$ oscillations in many systems is that even quite complex patterns of $[Ca^{2+}]_i$ spiking appear to reflect intrinsic properties of the individual cells rather than being determined largely by stochastic behavior. Thus, when cells are stimulated repeatedly with the same type and dose of agonist, a reproducible cell-specific pattern of $[Ca^{2+}]_i$ spiking can be observed, which has been termed a $[Ca^{2+}]_i$ fingerprint (23).

An important property of oscillating $[Ca^{2+}]_i$ signals composed of baseline-separated spikes is that the frequency is dependent on agonist dose. As the agonist concentration is increased, the spiking frequency increases such that the integrated $[Ca^{2+}]_i$ elevation is a function of the strength of the stimulus (2, 5, 14, 16, 17, 19). Whereas this kind of frequency regulation is commonly observed with baseline $[Ca^{2+}]_i$ spiking, the relationship may not always hold for receptors that undergo desensitization. Receptor desensitization could result in a run down in oscillation frequency, which if it occurs rapidly enough may allow the appearance of only a single $[Ca^{2+}]_i$ spike in the continued presence of the agonist. In some systems, receptor desensitization may prevent multiple $[Ca^{2+}]_i$ spikes at high agonist doses, whereas stable baseline-separated $[Ca^{2+}]_i$ oscillations occur at very low doses. In other systems where receptor desensitization does not occur or is insufficient to overcome the stimulatory effects on PLC, the highest doses of agonist can give rise to a sustained elevation of $[Ca^{2+}]_i$ close to the maximal peak of the individual $[Ca^{2+}]_i$ spikes. However, the physiological levels of most systemic and local hormones are in the lower range of their dose response curves, where the predominant type of $[Ca^{2+}]_i$ signal is baseline-separated $[Ca^{2+}]_i$ spikes occurring at a constant frequency.

The dwell time between $[Ca^{2+}]_i$ spikes can range from a few seconds to tens of minutes, depending on the strength of the activating stimulus. By contrast, the duration and overall shape of individual $[Ca^{2+}]_i$ spikes tends to be constant throughout the oscillatory range of agonist doses. In most cases the amplitude of the $[Ca^{2+}]_i$ spikes is also independent of agonist dose, at least once a stable spiking frequency is established (2,17, 24). Thus, the only features of baseline-separated $[Ca^{2+}]_i$ spikes that are clearly regulated by the dose of the stimulating agonist are the initial latent period and the frequency of oscillation. This has given rise to the concept of frequency-modulated $[Ca^{2+}]_i$ signaling (2, 4, 5, 14, 19), wherein the biological targets of the $[Ca^{2+}]_i$ signal respond to the frequency of $[Ca^{2+}]_i$ spikes rather than to the amplitude of $[Ca^{2+}]_i$ change.

## Spatial aspects of $[Ca^{2+}]_i$ oscillations

It is important to consider the spatial properties of $Ca^{2+}$ as a cytosolic messenger before considering potential mechanisms for the generation of $[Ca^{2+}]_i$ spikes. As pointed out by Allbritton and Meyer (18), the high levels of relatively immobile cytoplasmic $Ca^{2+}$ buffers limit the range of $Ca^{2+}$ diffusion during a single $[Ca^{2+}]_i$ spike to within a few micrometers of its release site. Therefore, in the absence of a mechanism to propagate the $Ca^{2+}$ mobilization signal, $Ca^{2+}$ would be expected to act only as a local message within most cells. There are a number of potential mechanisms by which the $Ca^{2+}$ release signal can be spread through a cell. Two propagation mechanisms that rely primarily on intracellular $Ca^{2+}$ stores are *1)* diffusional distribution of the $Ca^{2+}$ mobilizing second messenger and *2)* regenerative $Ca^{2+}$ release. Because (1,4,5)$IP_3$ diffuses more rapidly and over a greater range than $Ca^{2+}$, it has been suggested that it can act as a global messenger in many cells (18). For example, it has been calculated that $Ca^{2+}$ and (1,4,5)$IP_3$ would have effective cytoplasmic diffusional ranges of 5 and 25 μm, respectively, during a 1 s release period under equivalent conditions (18). Of course, the level of (1,4,5)$IP_3$ (or other diffusive message) must oscillate in order to give rise to oscillations of $[Ca^{2+}]_i$ with this type of signal propagation.

The second type of $Ca^{2+}$ release propagation relies on regeneration of the $Ca^{2+}$ mobilizing signal by the released $Ca^{2+}$ itself. In its simplest form, this type of mechanism invokes activation of adjacent $Ca^{2+}$ release sites by the $Ca^{2+}$ mobilized from the preceding release sites. In principle, an intermediate $Ca^{2+}$-activated step could also be involved in the regenerative process, such as $Ca^{2+}$ activation of PLC to generate additional (1,4,5)$IP_3$. In contrast to diffusional mechanisms for spreading the $Ca^{2+}$ release signal, regenerative mechanisms can act over much longer distances, such as in some eggs and oocytes where $[Ca^{2+}]_i$ waves propagate over distances approaching 1 mm (25–28) and in multicellular systems where intercellular $[Ca^{2+}]_i$ waves propagate between gap junction-coupled cells over similar distances (29–32).

Regardless of the mechanism by which the $Ca^{2+}$ release signal is spread within a cell, the limited range of $Ca^{2+}$ diffusion necessitates the presence of $Ca^{2+}$ stores distributed strategically throughout the regions of the cell where $[Ca^{2+}]_i$ increases are to occur. A corollary of this is that the subcellular distribution of intracellular $Ca^{2+}$ stores can determine the spatial organization of the $[Ca^{2+}]_i$ signal. Approaches to investigate the subcellular organization of $Ca^{2+}$ stores include the use of specific antibodies (e.g., against the (1,4,5)$IP_3$ receptor) and spatially resolved functional measurements using $Ca^{2+}$-sensitive dyes or other methods to monitor cation

changes within the lumen of these stores (reviewed in ref 33). The former approach has the advantage that different isoforms of the release channel can be distinguished, but it is not possible to establish that all immunoreactive proteins are actually competent channels associated with functional intracellular $Ca^{2+}$ stores. Immunohistochemical techniques have revealed heterogeneity in the density and distribution of various components of the $Ca^{2+}$ stores that appear to underlie localized $[Ca^{2+}]_i$ responses in some cells (reviewed in refs 23, 33, 34). However, functional measurements have indicated that (1,4,5)$IP_3$-sensitive and/or ryanodine-sensitive $Ca^{2+}$ stores are present throughout the path of $[Ca^{2+}]_i$ wave propagation wherever both parameters have been examined. In hepatocytes, where oscillatory $[Ca^{2+}]_i$ waves propagate across the entire cell, the distribution of functional (1,4,5)$IP_3$-sensitive $Ca^{2+}$ stores has been imaged by monitoring (1,4,5)$IP_3$-induced changes in the fluorescence of chlortetracycline associated with the lumen of the stores and by monitoring (1,4,5)$IP_3$-activated $Mn^{2+}$ quenching of fura-2 compartmentalized within the stores (35). These studies yielded images of the (1,4,5)$IP_3$-sensitive stores that closely resembled the labeling pattern obtained for ER markers and had a similar distribution throughout the cytoplasm. These data are consistent with fractionation and immunohistochemical studies indicating that the (1,4,5)$IP_3$-sensitive $Ca^{2+}$ stores are primarily located in the ER (or SR) of most cells (3, 22, 33). Although the $Ca^{2+}$ release channels may not be distributed evenly within the ER, the location of the $Ca^{2+}$ stores in this organelle provides a means for $Ca^{2+}$ release and reuptake sites to be positioned appropriately throughout the cytosol.

A $[Ca^{2+}]_i$ wave consists of a leading edge where $[Ca^{2+}]_i$ rises at a relatively constant rate, usually from close to the basal level, and advances through the cell in a progressive manner. In large cells such as *Xenopus* oocytes, the $[Ca^{2+}]_i$ wave is defined by a band of elevated $[Ca^{2+}]_i$, with a trailing edge where $[Ca^{2+}]_i$ is declining to the basal level. In many small cells, the $[Ca^{2+}]_i$ wave can completely fill the cell before the relaxation to basal begins to occur, giving rise to the appearance of a $[Ca^{2+}]_i$ tide (3, 9, 14). Repetitive $[Ca^{2+}]_i$ waves underlie the $[Ca^{2+}]_i$ oscillations in both of these types of system, although in the former case the $[Ca^{2+}]_i$ oscillations may only be apparent when $[Ca^{2+}]_i$ is monitored in a limited subcellular region. Oscillatory $[Ca^{2+}]_i$ waves can be induced both by activation of plasma membrane receptors linked to (1,4,5)$IP_3$ formation (25, 29, 36–38) and by direct introduction of nonmetabolizable (1,4,5)$IP_3$ analogs into the cytosol (27, 28, 39). $[Ca^{2+}]_i$ waves initiate at discrete subcellular sites, and then propagate with constant amplitude and velocity through the cell. Most (1,4,5)$IP_3$-dependent $[Ca^{2+}]_i$ waves propagate at velocities of 10—50 $\mu m \cdot s^{-1}$ (3, 10, 14, 27). Once a propagating $Ca^{2+}$ wave is initiated, the propagation velocity is generally found to be independent of the stimulus strength (agonist dose or intracellular level of (1,4,5)$IP_3$). Thus, the kinetics of $[Ca^{2+}]_i$ wave propagation are similar to the kinetics of individual $[Ca^{2+}]_i$ spikes in being a relatively constant parameter that is not regulated by the initiating stimulus. However, other factors can affect the velocity of $[Ca^{2+}]_i$ wave propagation, such as the $[Ca^{2+}]_i$ level in the interspike period, which can be elevated by enhanced influx of extracellular $Ca^{2+}$ (40). The rates of $[Ca^{2+}]_i$ wave propagation are relatively insensitive to temperature, with a $Q^{10}$ that is most consistent with a diffusion-limited process (27).

In many cases, successive $[Ca^{2+}]_i$ waves initiate from the same subcellular locus and then propagate along a consistent spatial path through the cell. Thus, in hepatocytes and pancreatic acinar cells, all $[Ca^{2+}]_i$ oscillations induced by stimulation with each of a range of different agonists can be shown to occur as $[Ca^{2+}]_i$ waves that begin in the same region adjacent to the plasma membrane, most often in the apical part of these cells (21, 34, 36–38, 41, 42). In pancreatic acinar cells, $[Ca^{2+}]_i$ waves do not always propagate throughout the cytoplasm, and at low levels of stimulation they can be limited to the initiation region in the apical part of the cell (21, 37, 42). By contrast, $[Ca^{2+}]_i$ waves in hepatocytes always propagate through the entire cell once they are initiated, even at very low agonist doses (34, 36, 41). Several of the components involved in (1,4,5)$IP_3$-mediated signal transduction can be organized heterogeneously within cells to yield spatially polarized $[Ca^{2+}]_i$ responses (reviewed in 3, 14, 22, 34). The polarization of $[Ca^{2+}]_i$ oscillations and waves in pancreatic acinar cells has been ascribed to a heterogeneous distribution of (1,4,5)$IP_3$ receptors, with either a higher density or more sensitive (1,4,5)$IP_3$ receptors being located in the initiation zone of the apical region (21, 34, 37, 38, 42). The relatively nonlocalized nature of (1,4,5)$IP_3$ as a second messenger may make it difficult to establish substantial (1,4,5)$IP_3$ gradients in most small cells (18). Therefore, the subcellular organization and isoform distribution of (1,4,5)$IP_3$ receptors is also likely to play an important role in generating polarized $[Ca^{2+}]_i$ signals in many other cell types.

In agonist-stimulated *Xenopus* oocytes, $[Ca^{2+}]_i$ waves may initiate at numerous discrete foci. These $[Ca^{2+}]_i$ waves initially spread as planar or radial waves from the initiation site, but the wave fronts annihilate when they collide. Spiral $[Ca^{2+}]_i$ waves can form where the symmetry of the wave front is disturbed in a refractory region caused by the prior propagation of a $[Ca^{2+}]_i$ wave, and once initiated, the spiral waves continue to propagate with a curvature that is determined by the properties of the regenerative $Ca^{2+}$ release and reuptake system (26, 27). Studies using *Xenopus* oocytes injected with (1,4,5)$IP_3$, nonmetabolizable (1,4,5)$IP_3$ analogs and caged (1,4,5)$IP_3$ have provided important insights into the process of $[Ca^{2+}]_i$ wave initiation (27, 28, 39, 43, 44). Both planar and spiral $[Ca^{2+}]_i$ waves can occur in the presence of (1,4,5)$IP_3$. However, at very low (1,4,5)$IP_3$ concentrations there are brief localized transients of $Ca^{2+}$ release (termed puffs) that fail to propagate as $[Ca^{2+}]_i$ waves (39). These $Ca^{2+}$ puffs occur at discrete sites spread

through a cytoplasmic band located 5–7 μm below the oocyte plasma membrane and they appear at fixed locations distributed at a density of about 1 per 30 $\mu m^2$. In some respects, $Ca^{2+}$ puffs have similar properties to the $[Ca^{2+}]_i$ oscillations to which they are believed to give rise. The frequency of $Ca^{2+}$ puffs at each locus increases with increasing (1,4,5)$IP_3$ concentration, whereas the amplitude is relatively unaffected. However, the decay phase of $Ca^{2+}$ puffs occurs more rapidly than for a full $[Ca^{2+}]_i$ wave and appears to depend primarily on diffusion of the released $Ca^{2+}$ away from the puff site. Individual puff sites demonstrate a refractory period of several seconds; after that, $Ca^{2+}$ release events occur in a stochastic manner, with no apparent interactions between sites. Based on the calculated $Ca^{2+}$ fluxes, Yao et al. (39) concluded that each site reflects the combined $Ca^{2+}$ release from several (1,4,5)$IP_3$-gated channels, presumably synchronized by the positive feedback responsible for the concerted $Ca^{2+}$ release observed in full $[Ca^{2+}]_i$ oscillations and $[Ca^{2+}]_i$ waves in the *Xenopus* oocyte. In addition to serving as sites of $[Ca^{2+}]_i$ wave initiation, the $Ca^{2+}$ puff sites have been suggested to act as $Ca^{2+}$ release units that propagate $[Ca^{2+}]_i$ waves when the (1,4,5)$IP_3$ level is sufficiently high to allow $Ca^{2+}$ diffusion from one site to trigger adjacent sites. As pointed out by Bootman and Berridge (45), evidence has been obtained for similar elemental $Ca^{2+}$ release units utilizing either (1,4,5)$IP_3$ or ryanodine receptors in a number of other cell types.

### Mechanisms responsible for baseline $[Ca^{2+}]_i$ spiking

Frequency-modulated $[Ca^{2+}]_i$ signaling resembles some electrically excitable systems, where the activating stimulus determines the frequency at which action potentials fire but the intrinsic properties of the individual action potentials are not determined by stimulus strength. In a similar manner, the individual cycles of $Ca^{2+}$ release and reuptake occurring during baseline-separated $[Ca^{2+}]_i$ oscillations appear to reflect intrinsic properties of the cell. The strength of the activating signal (agonist dose) determines the time taken for the system to reach a critical point, but once this point is reached a process of positive feedback results, which gives rise to a spike of $Ca^{2+}$ release with properties that are essentially independent of the initiating stimulus. Many models have been proposed to explain baseline-separated $[Ca^{2+}]_i$ oscillations, but in almost all of these the positive feedback is provided by $[Ca^{2+}]_i$.

Several lines of evidence indicate the importance of $[Ca^{2+}]_i$ in the generation of $[Ca^{2+}]_i$ oscillations. Under conditions of submaximal stimulation with agonists or (1,4,5)$IP_3$, direct introduction of $Ca^{2+}$ into the cytosol can initiate $[Ca^{2+}]_i$ spikes or propagating $[Ca^{2+}]_i$ waves (28, 46). Increases in cytoplasmic $Ca^{2+}$ buffering slow the frequency of agonist-induced $[Ca^{2+}]_i$ oscillations, whereas enhanced $Ca^{2+}$ influx from the extracellular medium leads to an increase in oscillation frequency (17, 40, 46, 47, 48). Stimulated $Ca^{2+}$ influx can also accelerate $[Ca^{2+}]_i$ wave velocity (40), and these effects on oscillation frequency and wave velocity may be mediated through a rise in the basal level of $[Ca^{2+}]_i$ (40, 47). Despite the important modulatory role of plasma membrane $Ca^{2+}$ influx, multiple $[Ca^{2+}]_i$ oscillations and waves can occur after chelation of extracellular $Ca^{2+}$ (17, 23, 48), although in some systems $[Ca^{2+}]_i$ spiking terminates under these conditions (49).

The opening of intracellular $Ca^{2+}$ channels can be examined in intact cells by monitoring retrograde flux of $Mn^{2+}$ from the cytosol to quench fura-2 compartmentalized within the $Ca^{2+}$ stores (50, 51). This approach has the advantage that changes in the permeability of (1,4,5)$IP_3$-activated intracellular channels (measured as the rate of $Mn^{2+}$ quench) can be monitored in situ in a manner that does not depend on $Ca^{2+}$ fluxes. These studies have shown that vasopressin causes a dose-dependent increase in channel activity in hepatocytes pretreated with thapsigargin to drain the intracellular $Ca^{2+}$ stores and eliminate feedback effects due to released $Ca^{2+}$, as expected from the dependence of (1,4,5)$IP_3$ formation on vasopressin dose (**Fig. 2*A***). In the absence of thapsigargin, the $Mn^{2+}$ quench of compartmentalized fura-2 occurs in a stepwise manner at low vasopressin doses that give $[Ca^{2+}]_i$ oscillations, but occurs in a continuous manner at higher doses that cause sustained $[Ca^{2+}]_i$ increases (Figs. 2*B*, *C*). The maximal rate of $Mn^{2+}$ quench, which corresponds to the $Ca^{2+}$ release phase, is the same under both conditions (50). These data suggest that feedback effects of the $Ca^{2+}$ released during each $[Ca^{2+}]_i$ spike cause channel activation to an extent (presumably maximal) similar to that obtained with a saturating dose of agonist.

Mechanisms for the generation of baseline $[Ca^{2+}]_i$ spiking can be classified into two groups, defined by whether or not the $[Ca^{2+}]_i$ oscillations are secondary to oscillations of (1,4,5)$IP_3$ levels. The receptor-controlled model originally proposed by Cobbold and co-workers (2, 52) postulated that $[Ca^{2+}]_i$ oscillations reflect an oscillatory formation of (1,4,5)$IP_3$ resulting from negative feedback on (1,4,5)$IP_3$ formation by PKC, similar to that described below for sinusoidal $[Ca^{2+}]_i$ oscillations. In their model, the rapid rising phase of $[Ca^{2+}]_i$ release occurs when receptor-activated G-proteins accumulate to a threshold level leading to rapid stimulation of PLC; then the diacylglycerol generated in parallel with (1,4,5)$IP_3$ stimulates PKC to phosphorylate and inactivate a component of the receptor-activated PLC system. However, the effects of activators and inhibitors of PKC in hepatocytes are not entirely consistent with this model (14, 16, 41), and it does not readily account for $[Ca^{2+}]_i$ waves or the role of $[Ca^{2+}]_i$ in establishing the positive feedback underlying the rapid rising phase of $[Ca^{2+}]_i$. As a result, Cobbold et al. (19) modified their receptor-controlled model to incorporate a role for positive feedback by $[Ca^{2+}]_i$ on PLC during the rising phase of the $[Ca^{2+}]_i$ spikes and at the wave front of propagating $[Ca^{2+}]_i$ waves. This type of cross-coupling between $[Ca^{2+}]_i$ levels and (1,4,5)$IP_3$ for-

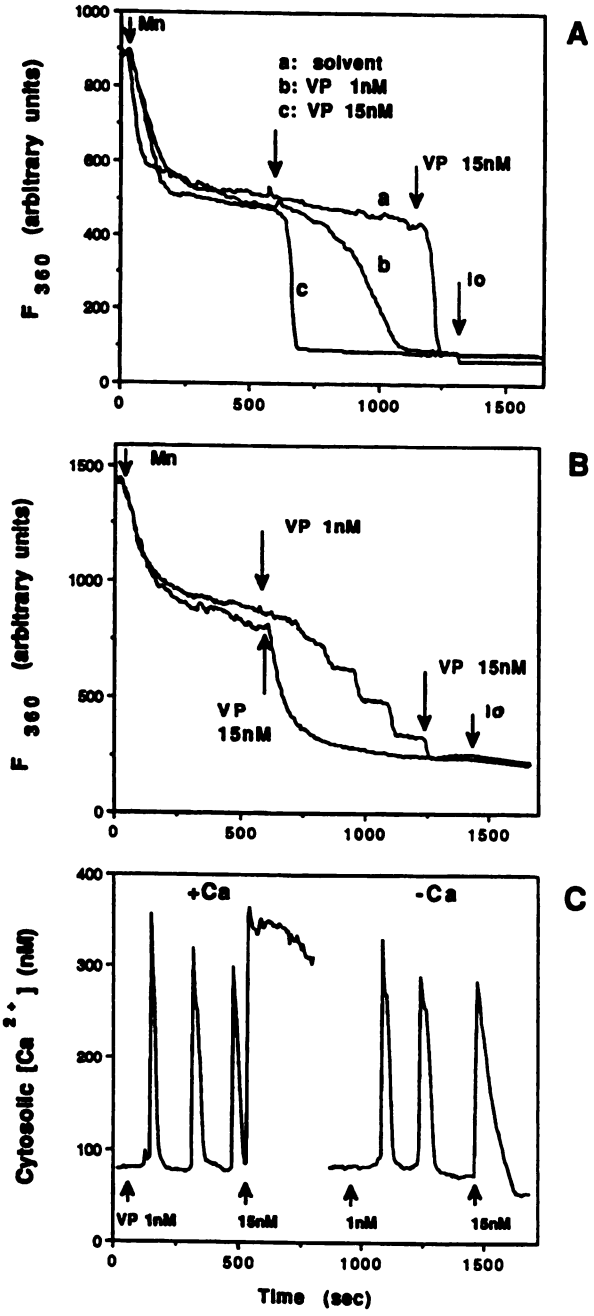


**Figure 2.** Vasopressin-induced $Mn^{2+}$ quench of compartmentalized fura-2 in intact hepatocytes. Isolated hepatocytes were loaded with fura-2/AM under conditions that gave significant compartmentalization of the dye, primarily in the ER (35, 50). The cells were incubated in the absence of extracellular $Ca^{2+}$, except for the left trace of panel *C*. *A)* The cells were preincubated with 2 μM thapsigargin. Panels *A* and *B* show the $Mn^{2+}$ quench of fura-2 measured at the $Ca^{2+}$-insensitive wavelength of 360 nm, and panel *C* shows $[Ca^{2+}]_i$ signals in parallel incubations calculated from the ratio of 340/380 nm fluorescence. Additions were: Mn, 100 μM $MnCl_2$; VP, 1 or 15 nM vasopressin; Io, 5 μM ionomycin. In panels *A* and *B*, the initial rapid quench after $Mn^{2+}$ addition reflects plasma membrane influx and the complete quench of cytosolic fura-2. The subsequent quench responses to vasopressin are due to stimulated entry of $Mn^{2+}$ into the intracellular stores. There is a significant lag period in both the $[Ca^{2+}]_i$ and $Mn^{2+}$ quench responses at 1 nM vasopressin, and the quench steps in panel *B* are believed to reflect the periodic opening of $(1,4,5)IP_3$-sensitive channels during the rising phase of $[Ca^{2+}]_i$ oscillations. Data adapted from ref 50.

mation underlies the other major model where oscillations of $(1,4,5)IP_3$ are considered to drive $[Ca^{2+}]_i$ oscillations and waves, as proposed by Meyer and Stryer (8). In their model, the rapid phase of $[Ca^{2+}]_i$ increase results from feedback activation of PLC by $[Ca^{2+}]_i$ together with a cooperative activation of intracellular $Ca^{2+}$ release by $(1,4,5)IP_3$ and $[Ca^{2+}]_i$. The relaxation phase of the $[Ca^{2+}]_i$ spikes is postulated to result from accumulation of the released $Ca^{2+}$ into a distinct $(1,4,5)IP_3$-insensitive intracellular store and/or inactivation of the $(1,4,5)IP_3$ receptors, which allows PLC activity and $(1,4,5)IP_3$ levels to decline as $[Ca^{2+}]_i$ falls. In the cross-coupling model, $[Ca^{2+}]_i$ wave propagation results primarily from the pulsatile generation of $(1,4,5)IP_3$, which can diffuse over distances in the order of 25 μm, as discussed above. Longer distance propagation of $[Ca^{2+}]_i$ waves may result from amplification of $(1,4,5)IP_3$ generation through $Ca^{2+}$ stimulation of additional PLC molecules located in "amplification units" along the path of the $[Ca^{2+}]_i$ wave (18).

Berridge (4, 5) first proposed a two-pool model in which $[Ca^{2+}]_i$ oscillations can occur in the presence of a constant level of $(1,4,5)IP_3$. In this model, $Ca^{2+}$ is released slowly from $(1,4,5)IP_3$-sensitive intracellular stores at a rate determined by the steady-state concentration of $(1,4,5)IP_3$, until the released $Ca^{2+}$ reaches a threshold where it triggers a rapid $Ca^{2+}$ release from a distinct $(1,4,5)IP_3$-insensitive store through a process of $Ca^{2+}$-induced $Ca^{2+}$ release (CICR). Relatively long interspike periods can result if the CICR store must first be topped up with $Ca^{2+}$ derived from the $(1,4,5)IP_3$-sensitive $Ca^{2+}$ pool before the precipitous $Ca^{2+}$ release phase. This process is analogous to CICR mediated by the ryanodine receptor $Ca^{2+}$ channels of muscle. In some systems where $(1,4,5)IP_3$ acts as the $Ca^{2+}$-mobilizing second messenger, the $[Ca^{2+}]_i$ spikes are sensitive to modulation by ryanodine and caffeine (38, 53, 54). However, these agents are not consistently observed to affect agonist-induced $[Ca^{2+}]_i$ oscillations in nonexcitable cells, and it appears that many of their effects may be mediated by alterations in the formation or action of $(1,4,5)IP_3$ (14). Furthermore, according to the CICR model it should be possible to induce spikes of $Ca^{2+}$ release and generate $[Ca^{2+}]_i$ waves by direct introduction of $Ca^{2+}$ into the cells in the absence of $(1,4,5)IP_3$. With a few exceptions (42, 53, 55), $Ca^{2+}$ microinjection or photorelease of caged $Ca^{2+}$ does not mimic the $[Ca^{2+}]_i$ oscillations and waves induced by $(1,4,5)IP_3$ and agonist stimulation (27, 28). In addition, $[Ca^{2+}]_i$ oscillations and the propagation of $[Ca^{2+}]_i$ waves are prevented by $(1,4,5)IP_3$ receptor blockade with heparin (21, 28, 56) or $(1,4,5)IP_3$ receptor antibody (55).

The two pool mechanism for the generation of $[Ca^{2+}]_i$ oscillations was modified to a one-pool model (11, 15, 27, 28, 57) when it became apparent that the $(1,4,5)IP_3$ receptor $Ca^{2+}$ channel is itself sensitive to activation by $Ca^{2+}$ in the presence of subthreshold levels of $(1,4,5)IP_3$ (42, 58–60). These studies demonstrated that the $Ca^{2+}$ release function of the $(1,4,5)IP_3$ receptor has a bell-shaped dependence on $[Ca^{2+}]_i$, with activation in the

lower range of $[Ca^{2+}]_i$ and inhibition at the higher $[Ca^{2+}]_i$ levels attained during the peak of baseline-separated $[Ca^{2+}]_i$ spikes. These properties allow the $(1,4,5)IP_3$ receptor to function as a self-limiting CICR channel in the presence of $(1,4,5)IP_3$. Thus, the one pool model for the generation of $[Ca^{2+}]_i$ oscillations can be referred to as $IP_3$-dependent CICR. In this model, a slow rate of $Ca^{2+}$ release occurs in the presence of a constant low level of $(1,4,5)IP_3$, until the local $[Ca^{2+}]_i$ rises to a point at which $(1,4,5)IP_3$ receptor sensitization begins to occur. At this point, the positive feedback effect of the released $Ca^{2+}$ on the $(1,4,5)IP_3$ receptor causes a rapid acceleration of $Ca^{2+}$ mobilization, giving rise to the fast rising phase of the $[Ca^{2+}]_i$ spike. In addition, local diffusion of the released $Ca^{2+}$ can lead to sensitization of other nearby $(1,4,5)IP_3$ receptors and initiate the same autocatalytic process of $Ca^{2+}$ release at these sites. In this way, the distribution and $Ca^{2+}$ sensitivity of the $(1,4,5)IP_3$ receptors gives rise to an excitable medium through which $[Ca^{2+}]_i$ waves propagate by the $IP_3$-dependent CICR mechanism (11, 15, 27, 28, 57). At $(1,4,5)IP_3$ levels above a critical value, the positive feedback effects of $Ca^{2+}$ will be sufficient to ensure that all available $(1,4,5)IP_3$ receptor $Ca^{2+}$ channels are activated. This can explain why the rate of $[Ca^{2+}]_i$ rise and the extent of intracellular channel activation are the same throughout the range of agonist and $(1,4,5)IP_3$ concentrations that give rise to both oscillatory and sustained $[Ca^{2+}]_i$ increases.

It remains to be determined whether the $Ca^{2+}$ release phase of each $[Ca^{2+}]_i$ spike results in complete emptying of the available $Ca^{2+}$ stores. Photolysis of caged $(1,4,5)IP_3$ at the peak of an agonist-induced $[Ca^{2+}]_i$ spike does not result in further $Ca^{2+}$ release in hepatocytes (51). However, this could reflect inactivation of the $(1,4,5)IP_3$ receptor rather than exhaustion of the $Ca^{2+}$ stores, because measurements of $Ca^{2+}$ store content with Mag-fura-2 in these cells suggest that only part of the thapsigargin-sensitive $Ca^{2+}$ pool is mobilized during each $[Ca^{2+}]_i$ oscillation (61). Regardless of whether $Ca^{2+}$ release terminates due to store depletion, it is clear that the $Ca^{2+}$ release channels must become inactivated to allow refilling of the stores before the next $[Ca^{2+}]_i$ spike. In the $IP_3$-dependent CICR model, the declining phase of the $[Ca^{2+}]_i$ spikes has been ascribed to the negative feedback effects of the released $Ca^{2+}$ on the $(1,4,5)IP_3$ receptor (44, 58–60). However, recent work by Hajnoczky and Thomas (62; unpublished results) has demonstrated that the $Ca^{2+}$-sensitized form of the $(1,4,5)IP_3$ receptor also undergoes a form of ligand-induced inactivation in the sustained presence of $(1,4,5)IP_3$, which may also contribute to the termination of the $[Ca^{2+}]_i$ spikes and provide a refractory period for $Ca^{2+}$ store refilling.

The $IP_3$-dependent CICR model for the generation of $[Ca^{2+}]_i$ oscillations has become widely accepted, in part because of the wealth of data supporting this model in *Xenopus* oocytes. However, in many respects the $IP_3$-dependent CICR model is functionally equivalent to the cross-coupling model. Both utilize a single $(1,4,5)IP_3$-sensitive $Ca^{2+}$ pool, with release sites distributed strategically along the path of $[Ca^{2+}]_i$ wave propagation, and in both cases the positive feedback is provided by $[Ca^{2+}]_i$. As a result, it is difficult to distinguish between the two models in many cellular systems. One prediction of the $IP_3$-dependent CICR model is that an elevation of cytosolic $(1,4,5)IP_3$ levels should be sufficient to induce $[Ca^{2+}]_i$ oscillations and waves independent of PLC activation. Consistent with this, microinjection of *Xenopus* oocytes with nonmetabolizable $(1,4,5)IP_3$ analogs has been shown to initiate oscillatory $[Ca^{2+}]_i$ waves (27, 28, 39). However, it could be argued that the injected $(1,4,5)IP_3$ serves only to release sufficient $Ca^{2+}$ in order to allow feedback activation of PLC and that the $[Ca^{2+}]_i$ oscillations actually result from a cross-coupling mechanism in these experiments. Strong evidence against this possibility has come from experiments in which caged $(1,4,5)IP_3$ was photolysed in a band through the cytoplasm of *Xenopus* oocytes (27). This procedure induced regenerative $[Ca^{2+}]_i$ waves that did not propagate out of the region where $(1,4,5)IP_3$ had been released, indicating that a preexisting elevation of $(1,4,5)IP_3$ is a necessary component of the excitable medium through which $[Ca^{2+}]_i$ waves propagate in these cells. This would not be expected if $[Ca^{2+}]_i$ waves could propagate by $Ca^{2+}$-stimulated regenerative $(1,4,5)IP_3$ formation. Further evidence against the cross-coupling model comes from the finding that injection of $Ca^{2+}$ does not induce $[Ca^{2+}]_i$ waves in unstimulated *Xenopus* oocytes, although $[Ca^{2+}]_i$ waves can be induced by $Ca^{2+}$ injection into oocytes previously loaded to a subthreshold level with a nonmetabolizable $(1,4,5)IP_3$ analog (27, 28). Because $Ca^{2+}$ injections do not trigger $[Ca^{2+}]_i$ waves in most cells, some proponents of cross-coupling models have suggested that the $Ca^{2+}$ stimulation of PLC is only effective in the presence of receptor activation (18, 19). However, this requirement is not consistent with the induction of $[Ca^{2+}]_i$ waves by microinjected $(1,4,5)IP_3$ in the absence of receptor stimulation.

Although the $IP_3$-dependent CICR mechanism is now fairly well established for the *Xenopus* oocyte system, the data obtained for small mammalian cells have not been so clear. Direct introduction of $(1,4,5)IP_3$ analogs causes $[Ca^{2+}]_i$ oscillations in some cells, as first shown by Petersen and co-workers (37, 63). However, in some cell types, injection or photorelease of $(1,4,5)IP_3$ analogs does not result in oscillatory $[Ca^{2+}]_i$ signals or even the rapid all-or-nothing $Ca^{2+}$ release characteristic of the rising phase of baseline $[Ca^{2+}]_i$ spikes (51, 64, 65). In hepatocytes, where frequency-modulated $[Ca^{2+}]_i$ oscillations have a constant amplitude over a wide range of agonist doses, the amplitudes of $[Ca^{2+}]_i$ spikes induced by photolysis of caged $(1,4,5)IP_3$ were graded with the amount of $(1,4,5)IP_3$ liberated (51). These observations suggest that the spatial or temporal pattern of $(1,4,5)IP_3$ presentation may be important. Alternatively, these findings could be interpreted to indicate that an elevation of $(1,4,5)IP_3$ is not sufficient to induce regenerative $Ca^{2+}$ release in

these cells, suggesting that there may be a role for a receptor-activated step in the propagation mechanism. In the study by Chiavaroli et al. (51), the latent period to the first $[Ca^{2+}]_i$ spike after hormone addition appeared to reflect the time taken for (1,4,5)$IP_3$ to accumulate to a threshold value, consistent with the suggestion that the kinetics of (1,4,5)$IP_3$ generation may be important. However, pretreatment of the cells with thapsigargin to eliminate feedback effects of $[Ca^{2+}]_i$ slowed the accumulation of (1,4,5)$IP_3$, suggesting a component of $Ca^{2+}$ feedback activation of PLC during the latent period, although thapsigargin had no effect on the steady-state (1,4,5)$IP_3$ levels associated with the interspike period (51; G. S. J. Bird and J. W. Putney, unpublished results). Using a protocol in which $Ca^{2+}$ was added back to agonist-stimulated fibroblasts, Harootunian et al. (56) also obtained evidence for a role of $Ca^{2+}$ activation of (1,4,5)$IP_3$ generation in the initiation of $[Ca^{2+}]_i$ spikes. Despite these observations implicating $Ca^{2+}$ feedback stimulation of PLC, the $Ca^{2+}$ dependence of the enzyme is questionable (see refs 14, 16), and it remains to be determined whether there is sufficient $Ca^{2+}$ sensitivity of receptor-stimulated PLC in vivo in the range required to elicit the rapid rising phase of $[Ca^{2+}]_i$ during $[Ca^{2+}]_i$ oscillations. Moreover, $[Ca^{2+}]_i$ oscillations and waves can be induced without receptor activation and in the absence of any measurable elevation of (1,4,5)$IP_3$ by thiol-reactive agents that have been shown to enhance the sensitivity of the (1,4,5)$IP_3$ receptor to (1,4,5)$IP_3$. Thus, treatment of cells with thimerosal or with *tert*-butyl hydroperoxide to oxidize intracellular glutathione apparently sensitizes the (1,4,5)$IP_3$ receptor to the point where basal levels of (1,4,5)$IP_3$ are sufficient to cause baseline $[Ca^{2+}]_i$ spiking and regenerative $[Ca^{2+}]_i$ waves with properties similar to those induced by agonists (21, 66, 67).

Taken together, the data described above are most consistent with $IP_3$-dependent CICR as the predominant mechanism underlying the baseline $[Ca^{2+}]_i$ oscillations and $[Ca^{2+}]_i$ waves observed in the majority of cells. However, an outstanding question related to the mechanisms of frequency-modulated baseline spiking is exactly how the period between spikes is set by the strength of the activating signal (agonist dose). The refractory period for $IP_3$-dependent CICR is relatively short and appears to be determined primarily by the activity of the $Ca^{2+}$ pumps responsible for refilling the intracellular $Ca^{2+}$ stores (26, 44, 57, 68). Measurements of $[Ca^{2+}]_i$ indicate that this occurs relatively rapidly compared to oscillation frequency, especially at low levels of agonist stimulation where consistent interspike periods lasting for 10–30 min are observed in some cells. Although the (1,4,5)$IP_3$ level regulates the frequency of the spontaneous $Ca^{2+}$ puffs in *Xenopus* oocytes, this occurs in a stochastic manner (39), suggesting that other factors may contribute to the frequency modulation of baseline $[Ca^{2+}]_i$ spikes. One possibility is that $Ca^{2+}$ release and uptake sites are segregated, such that $Ca^{2+}$ must redistribute within luminally linked $Ca^{2+}$ stores before it is readily available for the next $Ca^{2+}$ release cycle (35, 50, 69). The extent to which this needs to occur before there is sufficient $Ca^{2+}$ release to trigger the next $[Ca^{2+}]_i$ spike could be a function of (1,4,5)$IP_3$ concentration. In addition, a significant proportion of the $Ca^{2+}$ released from intracellular stores during each spike is lost to the extracellular medium (70). As a result, the rate of $Ca^{2+}$ influx across the plasma membrane can play an important role in setting the periodicity of $[Ca^{2+}]_i$ oscillations. As pointed out in the modeling studies by De Young and Cozier (71), interactions between $IP_3$-dependent CICR and $Ca^{2+}$ feedback activation of PLC could extend the interspike period and provide an additional point of control linked to receptor activation. In addition, regulation at the level of the (1,4,5)$IP_3$ receptor itself is likely to be important. The negative feedback effects of $[Ca^{2+}]_i$ on the (1,4,5)$IP_3$ receptor reverse relatively rapidly (44, 58, 72), but the ligand-induced inactivation by (1,4,5)$IP_3$ reverses more slowly when $[Ca^{2+}]_i$ returns to the basal level in the continuous presence of (1,4,5)$IP_3$ (62). The (1,4,5)$IP_3$ receptor is also a target for several protein kinases and phosphatases, which could modulate its (1,4,5)$IP_3$ sensitivity in a manner coordinated with the $[Ca^{2+}]_i$ oscillations.

## SINUSOIDAL OSCILLATIONS

Although there are considerably fewer reports in the literature of sinusoidal $[Ca^{2+}]_i$ oscillations than of baseline spikes, strictly speaking the sinusoidal oscillations may be the only form of discontinuous $[Ca^{2+}]_i$ signaling for which the term oscillations is appropriate. Sinusoidal oscillations in $[Ca^{2+}]_i$ are roughly symmetrical fluctuations usually superimposed on a raised basal level of $[Ca^{2+}]_i$. The most significant characteristic of this type of oscillation is their constant frequency at different agonist concentrations (6, 73–76). As pointed out by Gray (73), these sinusoidal oscillations are considerably simpler than the baseline spike type of $[Ca^{2+}]_i$ oscillations, and can most simply be explained by a single negative feedback on the $[Ca^{2+}]_i$ signaling mechanism. For one system, the lacrimal acinar cell activated through its muscarinic cholinergic receptor, it appears that the negative feedback responsible for the sinusoidal oscillations is due to protein kinase C (76). In contrast to the results with baseline spiking in hepatocytes discussed above, sinusoidal oscillations in lacrimal acinar cells are completely blocked by pharmacological activation, inhibition, or down-regulation of protein kinase C. Complete inhibition occurs regardless of the level of agonist activation or the level of $[Ca^{2+}]_i$, indicating that the inhibition does not simply result from alterations in the degree of PLC activation. The negative inhibition appears to occur at the level of either the PLC or the G-protein linking receptor activation to PLC, because: *1)* pharmacological activation of protein kinase C by phorbol esters inhibits the production of (1,4,5)$IP_3$ in response to muscarinic agonists (76)

or the $[Ca^{2+}]_i$ signal in response to GTPγS (**Fig. 3**), and *2)* bypassing (1,4,5)$IP_3$ production by intracellular application of stable analogs of (1,4,5)$IP_3$ always gives sustained, nonoscillating $[Ca^{2+}]_i$ signals (76). Thus, in the lacrimal acinar cell, the negative feedback mechanism for sinusoidal oscillations is similar to the mechanism proposed for baseline spiking in hepatocytes by Woods et al. (52). Activation of PLC increases diacylglycerol, which in turn activates protein kinase C, which feeds back and inhibits PLC; this leads to a diminution in diacylglycerol production, diminished protein kinase C activity, and relief of the inhibition of PLC. Continuous cycling of this feedback loop generates oscillations in PLC activity that, in the absence of some feed forward input, gradually damp down to a sustained level under tonic control by the opposing forces of receptor activation and protein kinase C inhibition. In this particular scheme, $[Ca^{2+}]_i$ is simply a passive follower of the oscillating (1,4,5)$IP_3$ production and plays no obvious active role in generating or modulating the oscillations. Thus, this particular type of oscillation might more appropriately be called "diacylglycerol oscillations" or "PLC/protein kinase C oscillations" (16). Note that Werner et al. (77) reported fluctuations in the levels of diacylglycerol in thrombin-activated platelets that were interpreted as resulting from oscillating diacylglycerol production.

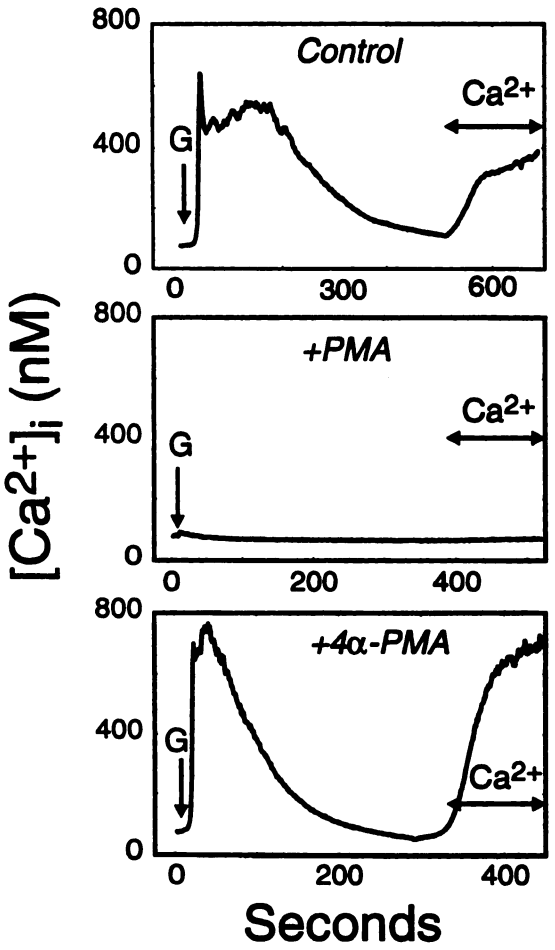


**Figure 3.** Negative feedback by protein kinase C occurs downstream of receptor activation. $[Ca^{2+}]_i$ was monitored in single mouse lacrimal cells by fura-2 fluorescence. The cells were activated by microinjection of the G-protein activator GTPγS (at G: pipette concentration was 10 mM), which results in a release of intracellular $Ca^{2+}$ (seen immediately after injection in a $Ca^{2+}$ deficient medium), followed by an entry of $Ca^{2+}$ across the plasma membrane (seen on reintroduction of 1.8 mM $Ca^{2+}$ to the bathing medium). Top: control. Middle: The cell was treated with 1.6 μM phorbol myristate acetate (PMA), which blocks the $[Ca^{2+}]_i$ signal due to GTPγS injection. Bottom: Pretreatment with 1.6 μM of the inactive isomer of PMA, 4α-PMA, did not block the $[Ca^{2+}]_i$ signal.

## OTHER TYPES OF $[Ca^{2+}]_i$ OSCILLATIONS

At least two additional kinds of oscillations have been reported in nonexcitable cells with properties clearly distinguishing them from the baseline spikes and sinusoidal oscillations already discussed. The first is a rapid spiking type of $[Ca^{2+}]_i$ oscillation that generally is either too rapid or too compartmentalized to be detected by fluorescent indicators. These have been detected by monitoring the activity of $Ca^{2+}$-sensitive $K^+$ or $Cl^-$ channels in exocrine gland cells (20, 53, 63). These rapid spikes might be precursors for larger spikes and $[Ca^{2+}]_i$ waves (20). In pancreatic acinar cells, oscillations of this kind can be induced with low concentrations of either acetylcholine or cholecystokinin, even though at higher concentrations these two agents give sinusoidal oscillations or baseline spiking, respectively (53). Rapid spikes can also be induced by perfusion of patch pipettes with GTPγS, analogs of (1,4,5)$IP_3$, and even elevated $[Ca^{2+}]$ (53). Thus, these rapid spikes appear to result from a CICR mechanism; this could involve a novel intracellular release mechanism or novel kinetic behavior of the (1,4,5)$IP_3$ receptor channel.

A second, rather interesting, form of $[Ca^{2+}]_i$ oscillation was observed in a majority of thapsigargin-activated parotid acinar cell, as reported by Foskett et al. (78, 79). These oscillations occurred with frequencies on the order of several minutes, similar to those reported for baseline spikes. Except for mitochondria, exocrine glands appear to be without thapsigargin-insensitive intracellular $Ca^{2+}$ pools (80). Carbachol, which substantially increases (1,4,5)$IP_3$ in these cells, did not affect thapsigargin-induced oscillations, indicating that the (1,4,5)$IP_3$-sensitive $Ca^{2+}$ pool remains empty throughout the cycles (78). Unlike the case for baseline spikes or sinusoidal oscillations, removal of extracellular $Ca^{2+}$ caused abrupt and immediate cessation of thapsigargin-induced oscillations (78). Furthermore, other manipulations that affected the rate of $Ca^{2+}$ influx, including modulation of the degree of stimulation of capacitative $Ca^{2+}$ entry, were found to modify the frequency with which thapsigargin-induced $[Ca^{2+}]_i$ oscillations were observed (79). Thus, it seems likely that these oscillations reflect fluctuations in either $Ca^{2+}$ entry or $Ca^{2+}$ extrusion at the plasma membrane. When $Ca^{2+}$ was restored to $Ca^{2+}$-depleted, thapsigargin-treated parotid cells, $[Ca^{2+}]_i$ always rose rapidly but then fell by about 50%, even in those cells that did not oscil-

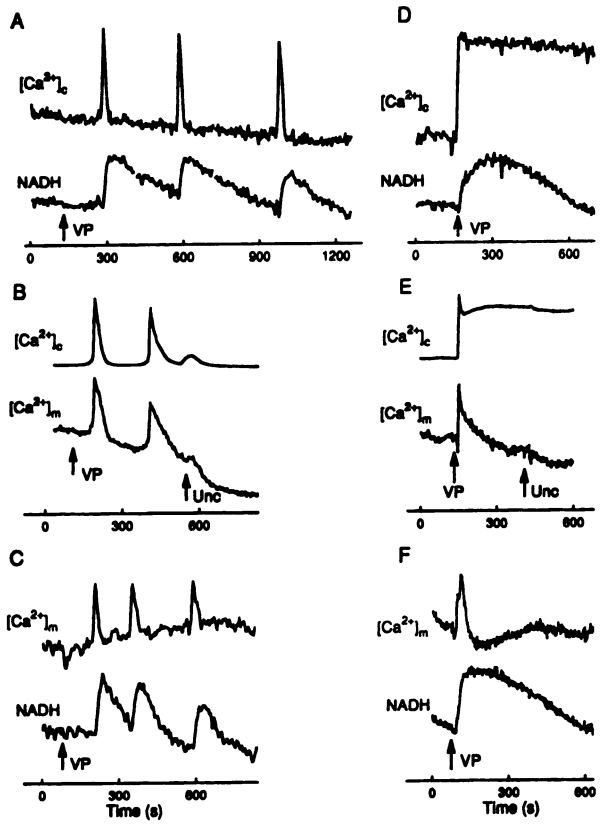


**Figure 4.** Simultaneous measurement of $[Ca^{2+}]_i$, $[Ca^{2+}]_m$ and pyridine nucleotide oscillations in vasopressin-treated hepatocytes. Isolated hepatocytes were loaded with fura-2 and/or rhod-2 to monitor cytosolic $Ca^{2+}$ ($[Ca^{2+}]_c$) and mitochondrial $Ca^{2+}$ ($[Ca^{2+}]_m$), respectively. Measurements of $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, and pyridine nucleotide fluorescence were carried out as described previously (88). The left panels show responses to a low dose of vasopressin (VP) that gave oscillations of $[Ca^{2+}]_i$; the right panels show responses at a saturating vasopressin dose that gave sustained $[Ca^{2+}]_i$ increases. In each experiment, two parameters were measured simultaneously: $[Ca^{2+}]_c$ and NADH (*A*, *D*); $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ (*B*, *E*); and $[Ca^{2+}]_m$ and NADH (*C*, *F*).

late (78, 79). A similar $[Ca^{2+}]_i$ overshoot, followed by a compensatory mechanism, was observed when inorganic blockers of $Ca^{2+}$ influx were washed out (79). This suggests a negative feedback of $[Ca^{2+}]_i$ on the plasma membrane $Ca^{2+}$ influx pathway during the $Ca^{2+}$ entry phase, which these authors now propose as the primary mechanism driving thapsigargin-induced $[Ca^{2+}]_i$ oscillations (79). The recent report of a slow feedback inhibition of capacitative $Ca^{2+}$ entry by $[Ca^{2+}]_i$ could provide a molecular mechanism for this phenomenon (81).

## PHYSIOLOGICAL SIGNIFICANCE OF CALCIUM OSCILLATIONS AND WAVES

The most commonly observed form of $[Ca^{2+}]_i$ oscillations is the baseline spike. Various authors have considered the theoretical advantages and implications of spikes of $[Ca^{2+}]_i$ whose frequency, but not amplitude, depends on the concentration of agonist (4, 19, 8, 14, 16). Meyer et al. (82) point out that the unusual kinetic behavior of the calmodulin/CaM-kinase interaction, which they termed "calmodulin trapping," would allow this response system to detect and respond to calcium spike frequency. It has also been suggested that the significance of calcium spiking behavior is that it permits information to be encoded and detected over a much broader range of signaling levels than with sustained, tonic $[Ca^{2+}]_i$ increases. This may be especially important in the very low range of hormone concentrations that are most often encountered in vivo. Extremely low concentrations of certain PLC-linked hormones, when applied over prolonged periods, can induce subtle biological responses, such as changes in gene expression (83, 84). If $[Ca^{2+}]_i$ signals occurred in a graded and sustained manner, it is difficult to imagine how hormone concentrations well below the $EC_{50}$ for $[Ca^{2+}]_i$ signaling could induce average, sustained increases in $[Ca^{2+}]_i$ that would be biologically detectable against background $[Ca^{2+}]_i$ levels. But with $[Ca^{2+}]_i$ oscillations whose frequency depends on agonist dose, extremely low concentrations of agonist can induce sparsely dispersed spikes of $[Ca^{2+}]_i$, which are readily distinguishable from the small elevations of $[Ca^{2+}]_i$ that might result from nonspecific $Ca^{2+}$ leak pathways or stochastic variations in the mechanisms responsible for maintaining basal $Ca^{2+}$ homeostasis. Low-frequency $[Ca^{2+}]_i$ spikes can be converted into a more continuous activation of the $Ca^{2+}$-stimulated response if the inactivation mechanism that reverses the $Ca^{2+}$ effect is relatively slow. Moreover, if the inactivation mechanism is sufficiently slow that it is not complete during the interspike period, even low-frequency $[Ca^{2+}]_i$ oscillations can be integrated into a strong biological signal over a prolonged period of time, for example, by incremental $Ca^{2+}$-dependent phosphorylation of regulatory proteins on sites that are resistant to dephosphorylation (16). In addition to integration of $[Ca^{2+}]_i$ spikes at the cellular level, oscillatory $[Ca^{2+}]_i$ signals can also be integrated at the tissue level. Thus, oscillations of $[Ca^{2+}]_i$ that result in asynchronous pulsatile responses in individual cells or groups of cells will be integrated into a smooth and continuous response in the total output of the tissue.

Direct measurements of the functional consequences of $[Ca^{2+}]_i$ oscillations have been difficult to obtain because of the limitations imposed by the need to measure the $Ca^{2+}$-mediated responses at the level of single cells, preferably in a time-resolved manner. In one system, the rat gonadotrope, it has been possible to obtain real-time measurements of secretion at the single cell level by use of membrane capacitance. Using this approach together with fluorometric $[Ca^{2+}]_i$ measurements, oscillations in secretory rate commensurate with oscillations in $[Ca^{2+}]_i$ were reported (85). Other studies have utilized electrophysiological approaches to monitor the activation of $Ca^{2+}$-dependent plasma membrane ion currents in parallel with $[Ca^{2+}]_i$ oscillations (21, 53, 86). In addition, the fluorescence of pyridine and flavin nucleotides has been utilized to monitor oscillations in metabolic responses as-

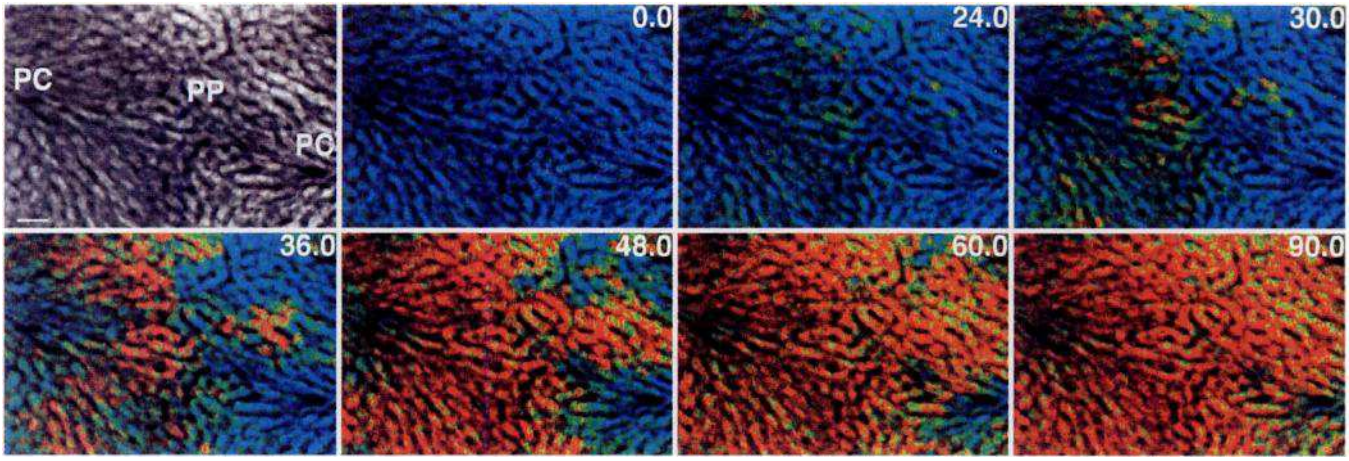


**Figure 5.** Intercellular propagation of a $[Ca^{2+}]_i$ wave in the intact perfused rat liver. Confocal images of fluo3 fluorescence during infusion of a perfused rat liver with 150 pM vasopressin were obtained as described previously (32). The initial image shows the basal fluorescence on a gray scale. The periportal (PP) and pericentral (PC) zones were identified by following the distribution of infused fluoresceine-labeled albumin. The scale bar represents 50 μm. The remaining images show the fluorescence changes as a ratio to the initial intensity at each pixel presented on a pseudocolor scale, with the spectral range from blue to red representing a ratio scale range of 0.95 to 1.50.

sociated with oscillating $[Ca^{2+}]_i$ signals (87, 88). In the study by Hajnoczky et al. (88), vasopressin-induced $[Ca^{2+}]_i$ oscillations in single hepatocytes were accompanied by redox oscillations that were mediated by mitochondrial $Ca^{2+}$-sensitive dehydrogenases (**Fig. 4*A***). As shown in Fig. 4*B*, *C*, this activation of mitochondrial energy production could be accounted for by the rapid and efficient transmission of each $[Ca^{2+}]_i$ spike into a spike of mitochondrial $Ca^{2+}$ ($[Ca^{2+}]_m$) increase. However, the spikes of increased NADH and reduced flavoprotein (not shown) decayed more slowly than either $[Ca^{2+}]_i$ or $[Ca^{2+}]_m$. As a result, $[Ca^{2+}]_i$ oscillations were integrated at the level of the metabolic response; at $[Ca^{2+}]_i$ oscillation frequencies above 0.5 $min^{-1}$, the mitochondrial redox responses were effectively sustained close to the peak response (88). By contrast, sustained $[Ca^{2+}]_i$ increases induced by maximal vasopressin doses were associated with only a single transient increase of NADH (Figs. 4*D*, *F*). This can be explained by the close coupling of (1,4,5)$IP_3$-induced $Ca^{2+}$ release to mitochondrial $Ca^{2+}$ uptake (89), which allows an increase in $[Ca^{2+}]_m$ to occur only during the initial $Ca^{2+}$ release phase of the sustained $[Ca^{2+}]_i$ signal (Fig. 4*E*). Thus, these data demonstrate that a $Ca^{2+}$ response system, in this case mitochondrial energy metabolism, can be tuned to the oscillatory range of $[Ca^{2+}]_i$ signaling and actually tune out sustained $[Ca^{2+}]_i$ signals (88).

In addition to the temporal consequences of $[Ca^{2+}]_i$ oscillations, the spatial organization of these signals have important physiological implications. In some cells, the rapid $[Ca^{2+}]_i$ spikes that occur at low agonist concentration are not reflected in global changes in $[Ca^{2+}]_i$. These may reflect physiologically important and highly localized bursts of $Ca^{2+}$ release, whose major role is to regulate functions close to the plasma membrane. Localized $[Ca^{2+}]_i$ changes of this type are most clearly documented in exocrine cells, where they may provide $[Ca^{2+}]_i$ signals that are especially efficient either in regulating exocytosis of digestive enzymes or in providing pulsatile changes in monovalent ion fluxes that regulate fluid movements (21, 37, 42, 86). $[Ca^{2+}]_i$ gradients and localized changes in $[Ca^{2+}]_i$ have also been associated with chemotactic responses of motile cells (90). On the other hand, regenerative $[Ca^{2+}]_i$ waves that propagate through the cytoplasm of cells may be important to ensure that $Ca^{2+}$-activated targets distal to the initial sites of $Ca^{2+}$ release are exposed to a full-strength $[Ca^{2+}]_i$ signal. For example, the wave of cortical granule fusion associated with fertilization of medaka eggs is driven by a wave of intracellular $Ca^{2+}$ release that propagates around the entire egg from the site of sperm fusion (25). Because cytoplasmic $Ca^{2+}$ diffusion is limited to a few micrometers during a $[Ca^{2+}]_i$ spike, regenerative $[Ca^{2+}]_i$ waves are also likely to be important in propagating $Ca^{2+}$ signals within small mammalian cells. In addition to cytoplasmic targets, $[Ca^{2+}]_i$ waves can be propagated into intracellular organelles such as mitochondria (88, 89) and nuclei (91). Thus, intracellular $[Ca^{2+}]_i$ waves may be thought of as an extension of plasma membrane signal transduction designed to transmit the $[Ca^{2+}]_i$ signal within the cell. The $[Ca^{2+}]_i$ wave propagation mechanism provides the necessary amplification and maintains the potentially important information encoded in the frequency of the $Ca^{2+}$ spikes.

In some cases where cells are coupled through gap junctions, the propagation of $[Ca^{2+}]_i$ waves is not limited to the cell in which the $[Ca^{2+}]_i$ signal is initiated (reviewed in ref 31). Although intercellular signaling can be mediated by electrical excitability, $[Ca^{2+}]_i$ waves generated by regenerative release of $Ca^{2+}$ from intracellular stores also propagate from cell to cell, often without loss of amplitude. The mechanism of intercellular $[Ca^{2+}]_i$ wave propagation is believed to be an extension of the in-

tracellular propagation mechanism, with either (1,4,5)$IP_3$ or $Ca^{2+}$ passing through the gap juntions to convey the signal into adjacent cells. The propagation of $[Ca^{2+}]_i$ waves between cells can serve to coordinate the responses of coupled cells. For example, mechanical stimulation of ciliated airway epithelial cells increases ciliary beat frequency in the target cell, and this response is then propagated in an (1,4,5)$IP_3$-dependent manner to neighboring cells (30). Propagation of $[Ca^{2+}]_i$ oscillations through gap junctions between cells of pancreatic acini has also been reported and may serve to coordinate the secretory responses of these cell clusters (92). In addition to coordinating local functions, intercellular $[Ca^{2+}]_i$ waves that propagate between astrocytes have also been proposed to act as a long-range signaling system or even form the basis of an "astroctye network" that processes information in a manner analogous to neural networks (29, 31, 93, 94). All of the studies described above have been carried out using either cells in monolayer culture or small segments of explanted tissue. Robb-Gaspers and Thomas (32) recently demonstrated that $[Ca^{2+}]_i$ waves also propagate between hepatocytes in the intact perfused liver, where hormone delivery occurs via the normal circulatory pathway and the 3-dimensional interactions between cells are fully maintained. Infusion of low doses of vasopressin into the portal circulation causes oscillations of $[Ca^{2+}]_i$, each of which initiates in specific hepatocytes of the periportal zone and then propagates through gap junctions from cell to cell along the hepatic plates into the pericentral zones (**Fig 5**). These intercellular $[Ca^{2+}]_i$ waves are not secondary to the flow of perfusate, because they still originate from the periportal zone when the direction of perfusion is reversed to allow vasopressin infusion from the hepatic vein (32). These intercellular $[Ca^{2+}]_i$ waves effectively coordinate the response of the entire lobule, which is the functional unit of the liver. The oscillatory $[Ca^{2+}]_i$ waves in the intact liver show the same properties of constant amplitude and frequency modulation by agonist dose as observed in isolated cell preparations. Thus, the extensive intercellular connections in the intact tissue do not preclude the expression of cellular oscillation mechanisms, but instead appear to provide an extension of the cytoplasmic excitable medium through which the functions of large numbers of cells can be regulated in a coordinated manner. FJ

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