IP₃-Dependent Ca²⁺ Oscillations Switch into a Dual Oscillator Mechanism in the Presence of PLC-Linked Hormones

**HIGHLIGHTS**

- Ca²⁺ oscillations driven by IP₃R (class 1) and PLC (class 2) occur in the same cell.
- IP₃ uncaging elicits brief and often spatially localized class 1 Ca²⁺ oscillations.
- GPCRs elicit whole-cell Ca²⁺ oscillations and waves via a hybrid class 2 mechanism.
- Dual Ca²⁺ feedback on IP₃R and PLC ensures a robust response to hormonal stimulation.
IP₃-Dependent Ca²⁺ Oscillations Switch into a Dual Oscillator Mechanism in the Presence of PLC-Linked Hormones

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SUMMARY

Ca²⁺ oscillations that depend on inositol-1,4,5-trisphosphate (IP₃) have been ascribed to biphasic Ca²⁺ regulation of the IP₃ receptor (IP₃R) or feedback mechanisms controlling IP₃ levels in different cell types. IP₃ uncaging in hepatocytes elicits Ca²⁺ transients that are often localized at the subcellular level and increase in magnitude with stimulus strength. However, this does not reproduce the broad baseline-separated global Ca²⁺ oscillations elicited by vasopressin. Addition of hormone to cells activated by IP₃ uncaging initiates a qualitative transition from high-frequency spatially disorganized Ca²⁺ transients, to low-frequency, oscillatory Ca²⁺ waves that propagate throughout the cell. A mathematical model with dual coupled oscillators that integrates Ca²⁺-induced Ca²⁺ release at the IP₃R and mutual feedback mechanisms of cross-coupling between Ca²⁺ and IP₃ reproduces this behavior. Thus, multiple Ca²⁺ oscillation modes can coexist in the same cell, and hormonal stimulation can switch from the simpler to the more complex to yield robust signaling.

INTRODUCTION

Oscillations of intracellular Ca²⁺ represents a fundamental cellular signaling mechanism regulating a multitude of process such as fertilization, metabolism, and secretion (Rooney et al., 1989; Bootman et al., 1997b; Berridge et al., 2000; Berridge, 2016). In non-excitable cells, oscillations of cytosolic Ca²⁺ ([Ca²⁺]ₓ) are generated by activation of hormone receptors coupled to phospholipase C (PLC) that produce the second messenger inositol-1,4,5-trisphosphate (IP₃), which activates IP₃ receptor Ca²⁺ release channels (IP₃R) in the endoplasmic reticulum (ER). The Ca²⁺ signaling toolkit of channels, pumps, and regulatory proteins is well defined (Berridge et al., 2000; Berridge, 2017), but the mechanisms driving cyclical changes in [Ca²⁺]ₓ have yet to be resolved. In hepatocytes, several IP₃-dependent hormones regulate metabolism and a number of other functions via baseline-separated [Ca²⁺]ₓ oscillations. Hepatocytes exhibit frequency modulation, whereby the [Ca²⁺]ₓ oscillation frequency is determined by agonist concentration, but the individual Ca²⁺ spikes have constant amplitude and rate of rise, and propagate as intracellular [Ca²⁺]ₓ waves at a constant velocity, independent of agonist dose (Rooney et al., 1989; Thomas et al., 1991; Gaspers and Thomas, 2005; Bartlett et al., 2014). Moreover, these hormone-induced Ca²⁺ transients are an all-or-none phenomenon, whereby the Ca²⁺ waves propagate at full strength across the whole cell. In the intact liver, Ca²⁺ waves propagate from cell to cell through gap junctions, and hence across the entire liver lobules to regulate hepatic metabolism and glucose output (Gaspers et al., 2019). We propose that Ca²⁺-dependent activation of PLC provides a feedforward mechanism to elicit robust cell-wide Ca²⁺ increases throughout the cytoplasm, ensuring maximal activation of Ca²⁺-dependent processes. Alterations in Ca²⁺ homeostasis and signaling have been shown in diseases of the liver (Bartlett et al., 2017; Arruda and Hotamisligil, 2015), therefore elucidating the mechanisms that maintain Ca²⁺ oscillations is critical to understanding Ca²⁺ dysregulation in disease states such as fatty liver disease and diabetes.

Experimental and mathematical modeling studies have explored the [Ca²⁺]ₓ oscillatory phenomenon without reaching a consensus in terms of mechanism. Two distinct hypotheses have been proposed: class 1 oscillations, which arise solely due to the biphasic effects Ca²⁺ on IP₃ gating (Bezprozvanny et al., 1991; Thurley and Falcke, 2011; De Young and Keizer, 1992; Marchant and Parker, 2001) and rely only on a continuous elevation of IP₃, and class 2 oscillations, which rely on Ca²⁺ feedback regulation of IP₃ levels such that [Ca²⁺]ₓ oscillations are coupled to, and dependent on, IP₃ oscillations (Politi et al., 2006; Salazar et al., 2008;
Mathematical models based on biphasic regulation of the IP₃R by Ca²⁺, effectively IP₃-dependent CICR, yield Ca²⁺ oscillations with a high frequency and a narrow sensitivity to PLC activity and IP₃ concentration (Bezprozvanny et al., 1991; De Young and Keizer, 1992; Sneyd et al., 2017; Thurley and Falcke, 2011). This is borne out by the nature of the [Ca²⁺]ₗ signals observed in cell types where experimental evidence indicates that class 1 Ca²⁺ oscillations occur. Hepatocytes and many other secretory cells generate [Ca²⁺]ₗ oscillations with long interspike intervals (ISIs) and kinetics that are independent of agonist dose, which cannot be readily reproduced by class 1 oscillatory models. The long periods at basal [Ca²⁺]ₗ between Ca²⁺ transients at low hormone doses, together with a broad dynamic range of frequency modulation, suggests that these [Ca²⁺]ₗ oscillations are controlled by other feedback loops such as those that regulate IP₃ generation and metabolism (Meyer and Stryer, 1988; Politi et al., 2006; Gaspers et al., 2014; Kummer et al., 2000). In the hepatocyte modeling studies of Kummer et al. (Kummer et al., 2000), an autocatalytic activity of the G-protein drives dynamic PLC activation and hence IP₃ oscillations, with Ca²⁺ causing negative feedback on PLC. However, these activities have not been reported experimentally, and although their model reproduces baseline spiking, it also leads to chaotic behavior that is not usually observed in our studies. In general, models that rely on Ca²⁺-dependent IP₃ degradation or feedback inhibition of PLC activity do not fully reproduce the types of [Ca²⁺]ₗ oscillations generated by G-protein-coupled receptor (GPCR)-linked hormones in hepatocytes (Gaspers et al., 2014; Dupont et al., 2003). By contrast, class 2 mathematical models incorporating positive Ca²⁺ feedback on IP₃ formation can reproduce the [Ca²⁺]ₗ oscillatory patterns observed in these cells (Gaspers et al., 2014; Meyer and Stryer, 1988; Politi et al., 2006). In most models involving positive Ca²⁺ feedback on IP₃ formation, a component of Ca²⁺ activation of the IP₃R is also generally required to reproduce the observed waveform of baseline-separated [Ca²⁺]ₗ oscillations with a rapidly rising phase (Politi et al., 2006; Gaspers et al., 2014), making the relevant class 2 models a hybrid of class 2 and class 1 properties.

Our previous work has demonstrated that expression of a recombinant IP₃ buffer in hepatocytes slows or completely eliminates (at high expression levels) hormone-induced [Ca²⁺]ₗ oscillations and waves (Gaspers et al., 2014). These data, combined with mathematical modeling of the predicted effects of an IP₃ buffer, argue that a periodic rapid rise in IP₃ via Ca²⁺ feedforward activation of PLC is essential for generating [Ca²⁺]ₗ oscillations in hepatocytes. Previously, we showed that PLC inhibition does not affect [Ca²⁺]ₗ oscillations generated by IP₃ uncaging in hepatocytes, indicating that agonist-dependent activation of PLC is a prerequisite for the feedforward regulation seen with hormonal stimulation (Bartlett et al., 2015). Furthermore, we identified both positive and negative feedback regulation of Ca²⁺ oscillations by protein kinase C (PKC), at least part of which is due to inhibition of GPCR-dependent PLC activation. It is possible that this could underlie the unique and distinctively reproducible Ca²⁺ spike shapes elicited by activation of different GPCRs in hepatocytes (Rooney et al., 1989).

Subcellular Ca²⁺ blips and puffs are local events that can recruit other Ca²⁺ release sites to summate into global [Ca²⁺]ₗ oscillations and propagate as saltatory [Ca²⁺]ₗ waves (Bootman et al., 1997a; Bootman and Berridge, 1995). Intracellular uncaging of IP₃ has been employed as a tool to mimic hormone-induced Ca²⁺ responses and interrogate the basic parameters of localized and global Ca²⁺ release events (Marchant et al., 1999; Marchant and Parker, 2001; Smith et al., 2009; Lock et al., 2017). The local Ca²⁺ signals generated by uncaging IP₃ can provide information about the sensitivity of a given cell to IP₃ and the speed and extent of CICR via the resident Ca²⁺ release channels. Moreover, CICR at the level of IP₃R channels is sufficient to drive [Ca²⁺]ₗ oscillations and Ca²⁺ wave propagation. However, IP₃ uncaging does not recapitulate the [Ca²⁺]ₗ responses to physiological hormonal stimuli in hepatocytes.

The current consensus is that cells exhibit either class 1 or class 2 Ca²⁺ oscillations and that these occur unambiguously (Sneyd et al., 2006, 2017). However, the present data obtained in hepatocytes suggest that these mechanisms are not mutually exclusive. Photorelease of IP₃ alone elicits high-frequency [Ca²⁺]ₗ oscillations with an amplitude, width, and rate of rise that increase with stimulus strength. These responses can be observed in local Ca²⁺ puff sites or globally across the cell, and importantly, they are not perturbed by the expression of an intracellular IP₃ buffer. We conclude that these are CICR-dependent
[Ca\textsuperscript{2+}]c oscillations. However, there is a dramatic shift in the oscillatory pattern upon the addition of a hormone to cells responding to flash photolysis of caged IP\textsubscript{3}. The [Ca\textsuperscript{2+}]c oscillations in the presence of vasopressin (VP) become much broader, with a prolonged ISI, a very regular frequency, and manifesting as Ca\textsuperscript{2+} waves that consistently propagate through the entire cell. A mathematical model incorporating both CICR at the IP\textsubscript{3}R and hormone-dependent Ca\textsuperscript{2+} feedback on IP\textsubscript{3} generation faithfully reproduces both modes of [Ca\textsuperscript{2+}]c oscillations and the ability to transition between them. These data show that class 1 and class 2 [Ca\textsuperscript{2+}]c oscillations can coexist in the same cell and demonstrate that hormone-dependent [Ca\textsuperscript{2+}]c oscillations require dual feedback loops that dynamically control Ca\textsuperscript{2+} release and IP\textsubscript{3} levels during the oscillation cycle. Importantly, class 2 oscillations dominate over class 1 in the presence of hormone.

RESULTS
Graded [Ca\textsuperscript{2+}]c Responses to Flash Photolysis of Caged IP\textsubscript{3}

The genetically encoded Ca\textsuperscript{2+} indicators GCaMP3 and RGECO-1 were used to detect [Ca\textsuperscript{2+}]c responses elicited by IP\textsubscript{3} uncaging and activation of the PLC-coupled VP receptor (V1R). Expression of molecular Ca\textsuperscript{2+} indicators offers a number of advantages over chemical indicators such as fluo4. The apparent affinities for Ca\textsuperscript{2+} of GCaMP3 and RGECO\textsubscript{1} (405 and 449 nM, respectively; Akerboom et al., 2013) are comparable with that of fluo4 (335 nM), but they have a higher quantum yield and are typically expressed at a lower effective concentration. They are also less diffusible and so are less likely to perturb local Ca\textsuperscript{2+} dynamics. It is also an advantage that they have an exclusive cytosolic location (unlike the chemical indicators, which also load into subcellular compartments). Empirically, we find that these genetically encoded Ca\textsuperscript{2+} indicators are well suited to the resolution of small and local [Ca\textsuperscript{2+}]c signals. In the present study, we analyzed the local, global, and propagating properties of [Ca\textsuperscript{2+}]c oscillations elicited by global IP\textsubscript{3} uncaging and VP treatment, with a view to dissecting the mechanisms driving baseline-separated [Ca\textsuperscript{2+}]c oscillations.

Hormonal regulation of metabolism in hepatocytes has been extensively characterized by our group, and we have shown that [Ca\textsuperscript{2+}]c oscillation frequency, rather than amplitude, encodes signal strength (Bartlett et al., 2014; Gaspers and Thomas, 2005; Rooney et al., 1989; Thomas and Robb-Gaspers, 1996). Different GPCRs give rise to [Ca\textsuperscript{2+}]c oscillations with distinct Ca\textsuperscript{2+} spike shapes, reflected primarily in the rate of decay of each Ca\textsuperscript{2+} spike, and this too is independent of agonist dose. By contrast, photorelease of caged IP\textsubscript{3} in GCaMP3-expressing hepatocytes induced [Ca\textsuperscript{2+}]c oscillations whose amplitude and duration increased in a dose-dependent manner (Figure 1A; see also Transparent Methods). Analysis of global (whole-cell) [Ca\textsuperscript{2+}]c responses showed that as the number of UV pulses was increased, the [Ca\textsuperscript{2+}]c peak height, peak width (measured as full width at half maximum; FWHM), rate of rise, and frequency increased, and there was a concomitant decrease in ISI (Figures 1B–1E). For each of these experiments, 5 nM VP was also added. VP-induced [Ca\textsuperscript{2+}]c oscillations had amplitudes and rates of Ca\textsuperscript{2+} rise that were similar to the maximum response to IP\textsubscript{3} uncaging, whereas the [Ca\textsuperscript{2+}]c peak widths and ISI were much longer than was observed with IP\textsubscript{3} uncaging.

We have previously demonstrated that hormone-induced Ca\textsuperscript{2+} oscillations are inhibited by the PLC inhibitor U73122, whereas oscillations induced by photorelease of caged IP\textsubscript{3} are insensitive to PLC inhibition (Bartlett et al., 2015). Thus, without GPCR activation the mechanisms required to drive regenerative IP\textsubscript{3} increases are lacking. Furthermore, in contrast to the sustained hormone-induced [Ca\textsuperscript{2+}]c oscillations, the responses elicited by caged IP\textsubscript{3} run down, as shown by the decrease in Ca\textsuperscript{2+} transient amplitude (Figure 1A). Taken together, these data demonstrate that an increase in IP\textsubscript{3} alone does not recapitulate the effect of GPCR activation.

Continuous Uncaging of IP\textsubscript{3}

Caged IP\textsubscript{3} can also be photolyzed slowly using a xenon light source and UV illumination over a prolonged period (as opposed to the discrete UV laser flashes reported in the previous section) to more closely mimic the continuous generation of IP\textsubscript{3} during hormonal stimulation. In the majority of cells (72% ± 6%, mean ± SEM, 45 cells from 3 independent experiments), continuous IP\textsubscript{3} uncaging resulted in a slow monophasic rise in [Ca\textsuperscript{2+}]c throughout the cell, but [Ca\textsuperscript{2+}]c oscillations were also observed in ~25% cells (Figures S1A and S1B). Similar to the observations with flash photolysis of caged IP\textsubscript{3}, these [Ca\textsuperscript{2+}]c oscillations had a higher frequency and shorter spike duration than hormone-induced oscillations, whereas the rate of [Ca\textsuperscript{2+}]c rise was the same (Figures S1C and S1D). We have previously shown that expression of the ligand-binding domain of rat IP\textsubscript{3}R type 1 (LBD) acts as an intracellular IP\textsubscript{3} buffer, which slows hormone-induced [Ca\textsuperscript{2+}]c oscillations and waves in primary hepatocytes (Gaspers et al., 2014). In that study we
showed that LBD expression did not affect the rate of rise or amplitude of the slow monophasic \([\text{Ca}^{2+}]_c\) response to continuous IP3 uncaging. As shown in Figure 2, LBD expression also had no effect on the peak width, ISI, or rate of rise of the oscillatory \([\text{Ca}^{2+}]_c\) responses generated by slow uncaging of IP3. Thus, in the absence of hormone, when IP3 reaches the threshold for CICR-mediated \([\text{Ca}^{2+}]_c\) oscillations the kinetic properties of these oscillations is unaffected by the presence of an IP3 buffer, as predicted for a class 1 model.

**Subcellular Organization of Ca\(^{2+}\) Signals Elicited by Global Flash Photolysis of Caged IP3**

The preceding data describe only the integrated whole-cell \([\text{Ca}^{2+}]_c\) events elicited by IP3 uncaging, but IP3 uncaging can also cause localized subcellular \(\text{Ca}^{2+}\) release. Local \(\text{Ca}^{2+}\) release responses termed \(\text{Ca}^{2+}\) puffs have been observed in Xenopus oocytes after photorelease of caged IP3 (Marchant and Parker, 2001), and similar localized \(\text{Ca}^{2+}\) release events have also been reported in mammalian cell lines (Smith et al., 2009; Thomas et al., 2000; Tovey et al., 2001). Local \(\text{Ca}^{2+}\) release events have not previously been reported in hepatocytes in response to photorelease of caged IP3 (Bartlett et al., 2015; Gaspers et al., 2014) or following hormone stimulation (Rooney et al., 1990; Thomas et al., 1996). In the present study, the use of a genetically encoded \(\text{Ca}^{2+}\)
indicator that has less effect on Ca\textsuperscript{2+} diffusion and buffering than small molecule chemical indicator dyes has revealed spatial heterogeneity in the [Ca\textsuperscript{2+}]c responses to submaximal levels of photoreleased IP\textsubscript{3}. As shown in Figure 3, low levels of IP\textsubscript{3}, particularly in the threshold range elicited by 1 or 2 UV pulses, caused localized [Ca\textsuperscript{2+}]c transients at discreet subcellular locations in ~40% cells (23 of 59 cells, 6 independent experiments). In the other cells analyzed, where the initial IP\textsubscript{3} uncaging presumably exceeded this threshold, oscillatory propagating Ca\textsuperscript{2+} waves or a single global [Ca\textsuperscript{2+}]c response were observed (summarized in Figure 3E). Importantly, the data of Figure 3E show a progression from predominantly local events, to propagating oscillations, to global [Ca\textsuperscript{2+}]c elevations as the UV flash density was increased.

The localized Ca\textsuperscript{2+} release events observed with IP\textsubscript{3} uncaging in hepatocytes (Figure 3A) were 3.3 ± 0.3 μm in diameter (mean ± SEM, n = 23 cells from 6 independent experiments), which is similar to the Ca\textsuperscript{2+} puffs reported in other cell types, including Xenopus oocytes (Marchant and Parker, 2001; Tovey et al., 2001; Thomas et al., 2000). We therefore refer to the localized hepatocyte Ca\textsuperscript{2+} transients in the present study as Ca\textsuperscript{2+} puffs, although they differ somewhat from most previous reports in having a longer duration. Another difference from previous reports of Ca\textsuperscript{2+} puffs elicited by IP\textsubscript{3} is that they typically occurred at a single repeating location at the cell periphery in the primary cultured hepatocyte (87% of cells). Significantly, these “eager” Ca\textsuperscript{2+} release sites corresponded to the site of Ca\textsuperscript{2+} wave initiation by hormones in the same cell. This polarization may reflect the reported subcellular distribution of IP\textsubscript{3} receptors in hepatocytes, with the more sensitive type 2 IP\textsubscript{3}R predominantly in the apical region where hormone-induced Ca\textsuperscript{2+} waves originate and type 1 IP\textsubscript{3}R uniformly distributed across the cell (Hernandez et al., 2007; Nagata et al., 2007). In cells with a diffuse distribution of IP\textsubscript{3}Rs, Ca\textsuperscript{2+} puffs occur in multiple locations, although there are still discrete eager sites (Smith et al., 2009; Tovey et al., 2001; Marchant and Parker, 2001). Thus, the functional polarization of the hepatocyte allows for a stable Ca\textsuperscript{2+} release site that presumably engages multiple IP\textsubscript{3}R clusters to form a discrete integrated Ca\textsuperscript{2+} puff site.
Figure 3. Spatial Properties of \([\text{Ca}^{2+}]_c\), Responses Elicited by Flash Photolysis of caged IP3

Isolated hepatocytes were transfected with GCaMP3, cultured overnight, and then loaded with caged IP3 (2 μM, 1 h). (A) Single video frames showing focal \([\text{Ca}^{2+}]_c\) release events. Scale bar, 10 μM. (B and C) Representative traces of changes in \([\text{Ca}^{2+}]_c\); at the local puff site (red) and distal pole (blue) of two individual hepatocytes during IP3 uncaging. (D) Interpuff interval (IPI, red) and interspike interval (ISI, blue) during the 3 UV pulse response from (C). (E) Ordinal plot of the \([\text{Ca}^{2+}]_c\) response pattern for cells with increasing UV pulse density (bursts of 1–4 flashes, as indicated). Response patterns of increasing strength are classified as follows: no response, local \([\text{Ca}^{2+}]_c\) transients at a discrete puff site, partial propagation where \([\text{Ca}^{2+}]_c\) waves only propagated across the cell intermittently, propagation where \([\text{Ca}^{2+}]_c\) waves consistently propagated across the cell, and global for whole-cell \([\text{Ca}^{2+}]_c\) responses that were not spatially resolved (summary of 59 cells from 6 independent experiments).

To characterize the local and global \([\text{Ca}^{2+}]_c\) events in each cell, \([\text{Ca}^{2+}]_c\) in the region of interest at the \([\text{Ca}^{2+}]_c\) puff site and at the distal pole of the cell were analyzed. Figures 3B and 3C show example \([\text{Ca}^{2+}]_c\) traces from two cells with the puff site and distal pole superimposed. In these studies the \([\text{Ca}^{2+}]_c\) signals at the distal region reflect the propagation of \([\text{Ca}^{2+}]_c\) waves from the puff site (Rooney et al., 1990; Gaspers et al., 2019; Gaspers and Thomas, 2003). We have determined the frequency of oscillations at each site from the interval between \([\text{Ca}^{2+}]_c\) transients; by convention the term inter-puff interval (IPI) is used for events at the \([\text{Ca}^{2+}]_c\) puff site and inter-spike interval (ISI) for events that propagate across the whole cell. Submaximal levels of IP3 uncaging frequently generated localized \([\text{Ca}^{2+}]_c\) oscillations at the puff site that did not propagate across the whole cell, as shown in Figure 3B, 1 UV flash, and Figure 3C, 2 UV flashes. As the stimulus strength was increased (number of UV flashes), \([\text{Ca}^{2+}]_c\) waves were observed to propagate across the cell, radiating from the puff site. However, the effect of IP3 uncaging on \([\text{Ca}^{2+}]_c\) responses at the distal pole of the cell did not always mirror the \([\text{Ca}^{2+}]_c\) oscillations at the puff site. In some cases only the first \([\text{Ca}^{2+}]_c\) transient at the puff site propagated across the cell as a single \([\text{Ca}^{2+}]_c\) wave (Figure 3B). When multiple \([\text{Ca}^{2+}]_c\) transients at the puff site did propagate as \([\text{Ca}^{2+}]_c\) waves they often declined in amplitude. Importantly, not all \([\text{Ca}^{2+}]_c\) transients at the puff site were transmitted across the hepatocyte to the distal pole (Figure 3C). This failure to propagate manifests in the difference in IPI and ISI plotted in Figure 3D for the 3 UV uncaging response in Figure 3C (see also Figure 5E). The continuum of increasingly effective propagation with increasing UV flash density is summarized in Figure 3E.

In contrast to IP3 uncaging, stimulation with a PLC-linked hormone such as VP leads to \([\text{Ca}^{2+}]_c\) oscillations that consistently propagate across the entire cell throughout the active dose range, with the characteristic long-duration \([\text{Ca}^{2+}]_c\) spikes described earlier (Figures 1 and 52; Gaspers and Thomas, 2005; Bartlett et al., 2014; Rooney et al., 1990). Moreover, low concentrations of hormone cause baseline-separated broad \([\text{Ca}^{2+}]_c\) oscillations with long 1- to 5-min ISIs. We attempted to mimic this behavior by eliciting periodic IP3 uncaging with a similar frequency using single UV flash events (Figure S3). However, this did not reproduce the typical whole-cell baseline-separated \([\text{Ca}^{2+}]_c\) oscillations observed with hormone. Instead multiple local \([\text{Ca}^{2+}]_c\) transients were observed with the initial UV flashes. Although these did propagate to the distal pole as more IP3 was released into the hepatocyte, the \([\text{Ca}^{2+}]_c\) spikes elicited by periodic uncaging never took on the broad profile of those observed with VP.

Effect of IP3 Uncaging during Hormone-Induced \([\text{Ca}^{2+}]_c\) Oscillations

Photolysis of caged IP3 during a train of hormone-induced \([\text{Ca}^{2+}]_c\) oscillations has been explored as an approach to determine whether receptor-mediated \([\text{Ca}^{2+}]_c\) oscillations are dependent on oscillating or steady-state IP3 levels (Sneyd et al., 2006, 2017). Mathematical modeling predicts that class 1 oscillating cells show an increase in frequency following a pulse of IP3, and this has been demonstrated experimentally (Sneyd et al., 2006). For class 2 oscillating cells it has been predicted that an increase in [IP3] will result in a delay before \([\text{Ca}^{2+}]_c\) oscillations recommence, because the IP3 level needs to fall before a subsequent \([\text{Ca}^{2+}]_c\) spike can be initiated (Sneyd et al., 2006).

In hepatocytes, photolysis of caged IP3 during a train of VP-induced \([\text{Ca}^{2+}]_c\) oscillations either slightly reduced the \([\text{Ca}^{2+}]_c\) oscillation frequency (Figure 4A, 22/32 cells) or in some cases led to a temporary arrest of oscillations, as predicted from class 2 models (Figure 4B, 7/32 cells). In cells where the global hormone-induced \([\text{Ca}^{2+}]_c\) oscillations were arrested, small local \([\text{Ca}^{2+}]_c\) puffs were often observed during the interruption in the whole-cell \([\text{Ca}^{2+}]_c\) oscillations. The decrease in oscillation frequency observed in the majority of cells is shown in Figure 4C, with mean pre- and post-photolysis values of 0.76 ± 0.07 min⁻¹ and 0.59 ± 0.09 min⁻¹, respectively (mean ± SEM for 32 hepatocytes from 4 independent experiments; 5 min pre- and post-UV; p < 0.001 by paired Student’s t test). When the same UV pulse protocol was applied to VP-stimulated hepatocytes not loaded with caged IP3, the pre- and post-flash values were
0.84 ± 0.07 min⁻¹ and 0.77 ± 0.09 min⁻¹, respectively (15 cells from 2 independent experiments; p = 0.15). The decrease in frequency following IP₃ uncaging was associated with an increase in Ca²⁺ transient width from 17.1 ± 1.1 s to 19.8 ± 1.6 s FWHM (Figure 4D; mean ± SEM for 26 hepatocytes from 4 independent experiments; p < 0.01 by paired Student’s t test). As shown in Figure 4E, stronger IP₃ uncaging (3 UV laser pulses) during a train of VP-induced [Ca²⁺]c oscillations caused a more pronounced prolongation of the [Ca²⁺]c spikes, but did not increase the oscillation frequency as would be expected for a class 1 mechanism. Presumably under the conditions of Figure 4E the uncaged IP₃ extends the time required for IP₃ levels to decay and hence delays Ca²⁺ refilling of the ER. Taken together, the data of Figure 4 are consistent with the hypothesis that hormones elicit predominantly class 2 [Ca²⁺]c oscillations that depend on fluctuations of IP₃ in hepatocytes, because an increase in [IP₃] did not translate to an increase in oscillation frequency.

Figure 4. Effect of IP₃ Uncaging on [Ca²⁺]c Responses Elicited by Vasopressin

(A–D) Isolated hepatocytes were transfected with GCaMP3, cultured overnight and then loaded with caged IP₃ (2 μM; 1 h). Hepatocytes were stimulated with vasopressin (VP) and then IP₃ uncaging was achieved by a brief (1-s) pulse of 340-nm light from the microscope fluorescence illuminator Xeon lamp. Representative traces showing (A) a slight slowing of Ca²⁺ oscillation frequency or (B) the temporary arrest of oscillations. Summary data showing a decrease in oscillation frequency (C) and an increase in peak width (D) after the IP₃ uncaging event (data are mean ± SEM from ≥26 individual hepatocytes from 4 independent experiments, **p < 0.01, ***p < 0.001 paired Student’s t test).

(E) Representative trace of hepatocyte stimulated with VP and then IP₃ uncaging with 3 UV flashes from UV laser.
Figure 5. Fast \([\text{Ca}^{2+}]_c\) Oscillations Elicited by IP$_3$ Uncaging Transition to Broad Baseline-Separate \([\text{Ca}^{2+}]_c\) Oscillations in the Presence of Hormone

Isolated hepatocytes were transfected with GCaMP3, cultured overnight, and then loaded with caged IP$_3$ (2 µM; 1 h). Rapid trains of 1, 2, 3, or 4 UV pulses were applied as indicated (arrows) followed by the addition of VP (5 nM).

(A and B) Representative traces showing cytosolic \([\text{Ca}^{2+}]_c\) oscillations at puff site (red) and distal pole (blue) of two cells in response to IP$_3$ uncaging and VP (see also Videos S1 and S2).

(C) Mathematical model combining class 1 and class 2 oscillation mechanisms predicts the observed behavior in this paradigm (red line \([\text{Ca}^{2+}]_c\), green line \([\text{IP}_3]\)).

(D) IPI and ISI of cell shown in (B) in response to UV pulses and VP (transition period between UV and VP responses not shown).

(E–G) Comparison of ISI (E), spike width (FWHM) (F), and rate of rise (G) at the puff site and distal pole of hepatocytes.
**Addition of hormone during IP$_3$-induced [Ca$^{2+}$]$_c$ oscillations causes a qualitative shift in Ca$^{2+}$ oscillation properties**

Our previous work (Bartlett et al., 2015) and data presented in the current study, demonstrate that release of IP$_3$ alone is incapable of reproducing oscillatory [Ca$^{2+}$]$_c$ transients with the same properties as those observed with hormone stimulation. To further test the hypothesis that baseline-separated Ca$^{2+}$ oscillations depend on cross-coupling of IP$_3$ and Ca$^{2+}$, we reversed the paradigm of Figure 4 and examined the effect of adding VP during a train of oscillations induced by IP$_3$ uncaging. Flash photolysis events were incrementally increased, and then VP was added while cells were still responding to the uncaged IP$_3$. The [Ca$^{2+}$]$_c$ signals in puff sites and at the distal pole of the cell were then compared between uncaging and hormone-induced responses. Strikingly, the application of VP resulted in a dramatic shift in oscillatory behavior from high-frequency, small-width oscillations, only some of which propagated across the whole cell (as summarized in Figure 3E), to lower-frequency broad [Ca$^{2+}$]$_c$ oscillations that always engaged the entire cell (Figures 5A and 5B; see also Videos S1 and S2). The IPI and ISI for the cell shown in Figure 5B are plotted in Figure 5D. These data show reliable and complete propagation of the hormone-induced [Ca$^{2+}$]$_c$, oscillations throughout the cell, whereas the higher-frequency [Ca$^{2+}$]$_c$, transients with IP$_3$ uncaging were irregular and inconsistent in engaging the distal pole of the cell. Videos S1 and S2 show this cell (in center) and other cells that exhibit similar behavior. Video S1 shows the changes in [Ca$^{2+}$]$_c$ in response to UV flash events and then subsequent VP addition; increases in GCaMP3 fluorescence intensity reflect increased Ca$^{2+}$. Note that the UV flashes are global across the whole file, but appear as white dots on the images due to the position of the spinning disk at the moment of the flash. Video S2 is a differential image series of the same data; the red intensity is proportional to the rate of GCaMP3 fluorescence increase overlaid on the gray scale image. This video is included to better resolve the spatially localized [Ca$^{2+}$]$_c$, changes, as well as the wave front during Ca$^{2+}$ wave propagation. It should be noted that only the rising phase (not sustained) of each Ca$^{2+}$ transient is observed in the red differential image (Video S2), so this does not show the full duration of Ca$^{2+}$ transients (seen in Video S1).

This switch in [Ca$^{2+}$]$_c$ oscillation properties between IP$_3$ uncaging and activation of a PLC-linked GPCR such as VP can be recapitulated in a mathematical model that combines class 1 and class 2 oscillation mechanisms (see Transparent Methods). Specifically, the simulation presented in Figure 5C incorporates positive and negative feedback at the IP$_{3R}$ using parameters similar to those described previously (Sneyd et al., 2006), which is sufficient to generate [Ca$^{2+}$]$_c$, oscillations in the presence of incremental sustained elevations of IP$_3$ (green line). A stepped increase in [IP$_3$] increases the oscillation frequency with no requirement for dynamic changes of IP$_3$. Introduction of VP, which activates PLC and allows positive feedback of Ca$^{2+}$ on IP$_3$ formation, yields much broader [Ca$^{2+}$]$_c$, oscillations with sustained periods of baseline [Ca$^{2+}$]$_c$, similar to those observed in Figures 5A and 5B. The model even recapitulates the secondary Ca$^{2+}$ peaks often seen during the falling phase of [Ca$^{2+}$]$_c$ in the experimental data in response to VP (this is also seen in cells that have not experienced prior IP$_3$ uncaging events, Figure S2). Importantly, the qualitatively different oscillation patterns shown in Figure 5C are obtained with a single model that allows both intrinsic IP$_3$R regulation by Ca$^{2+}$ and Ca$^{2+}$ regulation of IP$_3$ levels in the presence of hormone.

At subcellular resolution, the broad hormone-dependent [Ca$^{2+}$]$_c$, oscillations in hepatocytes typically propagated starting at the same puff site that was activated during IP$_3$ uncaging. Following addition of VP, a single broad [Ca$^{2+}$]$_c$, response with complicated oscillatory behavior was often observed during the transition to baseline-separated [Ca$^{2+}$]$_c$, oscillations (Figures 5A and 5B). [Ca$^{2+}$]$_c$, transients generated by uncaging IP$_3$ had a higher frequency at the puff site (Figure 5E), a narrower spike width (Figure 5F), and a faster rate of [Ca$^{2+}$]$_c$ rise (Figure 5G) compared with the distal pole of the cell. Hormone-induced Ca$^{2+}$ oscillations had a much greater width (FWHM, Figure 5F), with greater ISI (Figures 5D and 5E), compared with responses generated solely by uncaging IP$_3$. Unlike the responses generated by IP$_3$ uncaging, no differences in spike width, ISI, or rate of rise between the puff site and distal regions of the cell were observed with hormone-dependent [Ca$^{2+}$]$_c$, oscillations. These data show a clear switch in [Ca$^{2+}$]$_c$, oscillation properties between IP$_3$ uncaging and stimulation via a PLC-linked GPCR in hepatocytes. Our modeling studies reveal that the ability of hormones to generate all-or-none oscillatory propagating Ca$^{2+}$ waves can be ascribed to the Ca$^{2+}$-dependent stimulation of PLC, which only occurs with GPCR activation. This positive feedback of Ca$^{2+}$ on PLC ensures that cellular IP$_3$ levels consistently reach a level that is sufficient to engage the whole...
cell during each Ca\(^{2+}\) oscillation in the presence of hormone. By contrast, in the absence of hormone there is no stimulation of PLC activity when IP\(_3\) is uncaged, resulting in local responses at the "eager" Ca\(^{2+}\) release sites and fast narrow [Ca\(^{2+}\)]\(_c\) transients that do not reliably propagate throughout the cell.

**Discussion**

Ca\(^{2+}\) oscillation and waves generated by the PLC-linked GPCRs are a major class of Ca\(^{2+}\) signals that regulate a host of intracellular processes. In hepatocytes, hormone-induced [Ca\(^{2+}\)]\(_c\) oscillations regulate cellular metabolism, bile secretion, gene expression, and glucose homeostasis (Amaya and Nathanson, 2013; Bartlett et al., 2014). The hepatocyte was one of the first cell types to be shown to signal through Ca\(^{2+}\) oscillations, and these cells remain one of the best examples of frequency-modulated signaling, with a broad frequency response range and long-period baseline-separated [Ca\(^{2+}\)]\(_c\) transients (Bartlett et al., 2014; Hajnoczky et al., 1995; Rooney et al., 1989; Woods et al., 1986, 1987). The mechanisms that give rise to [Ca\(^{2+}\)]\(_c\) oscillations have been the topic of much study and discussion, which has been complicated by the existence of two distinct paradigms that have been evidenced in different cellular systems: class 1 oscillations that are essentially intrinsic to the IP\(_3\)R and rely on positive and negative feedback effects of Ca\(^{2+}\) on the Ca\(^{2+}\) release channel (Sneyd et al., 2006, 2017; Thurley and Falcke, 2011) and class 2 oscillations in which Ca\(^{2+}\) feedback acts at the level of IP\(_3\) formation and/or breakdown (Dupont and Erneux, 1997; Gaspers et al., 2014; Haroutunian et al., 1991; Politi et al., 2006; Sneyd et al., 2006). It has generally been considered that the main determinants dictating the utilization of these mechanisms lie in the cellular complement of Ca\(^{2+}\) toolkit components, and that the higher-frequency-intrinsic IP\(_3\)R oscillator will tend to dominate in the presence of sufficiently elevated IP\(_3\).

In the present study, we demonstrate the coexistence of both types of oscillatory Ca\(^{2+}\) signaling in primary rat hepatocytes and show that in contrast to the IP\(_3\)R-level class 1 oscillation mechanism dominating, class 2 mechanisms become important when an agonist is added to activate the GPCR-dependent PLC and elicit IP\(_3\) formation. Moreover, Ca\(^{2+}\) oscillations elicited by uncaging IP\(_3\) and those generated by the hormone VP are qualitatively different and generated by distinct mechanisms, but, nevertheless, share components of the same Ca\(^{2+}\) toolkit. Both mechanisms can be recapitulated in a single mathematical model without changing any model parameters, but simply allowing for an agonist-dependent formation of IP\(_3\) in a Ca\(^{2+}\)-regulated manner. The simplicity of this divergent behavior in a convergent model makes it clear that the same elements of the Ca\(^{2+}\) signaling toolkit give rise to the full range of Ca\(^{2+}\) signaling in hepatocytes. Thus, at least in the hepatocyte, the class 1 and class 2 oscillation mechanisms are not mutually exclusive, but integrate to give rise to the complex patterns of [Ca\(^{2+}\)]\(_c\) signals seen in these cells. In a hybrid class 2 model, Ca\(^{2+}\) and IP\(_3\) oscillations are co-dependent, and in effect cross-coupled through mutual feedback regulation. Positive Ca\(^{2+}\) feedback on PLC ensures that sufficient IP\(_3\) is produced for a global cell-wide [Ca\(^{2+}\)]\(_c\) response and drives Ca\(^{2+}\) release, whereas positive Ca\(^{2+}\) feedback on the IP\(_3\)R ensures the rapid rising phase of the Ca\(^{2+}\) transients. In the context of the "Ca\(^{2+}\) toolkit" described by Berridge and others (Berridge et al., 2000; Bootman and Berridge, 1995; Bootman et al., 1997b), the key determinant of this type of Ca\(^{2+}\) oscillation is the involvement of G\(_{a\_}\)-linked GPCRs that engage the Ca\(^{2+}\)-sensitive PLC\(_B\) enzymes. By contrast, photorelease of caged IP\(_3\) initiates solely class 1 Ca\(^{2+}\) oscillations, and whereas this is a non-physiological stimulus, other physiological stimuli that act through IP\(_3\) but do not activate a Ca\(^{2+}\)-sensitive PLC could still elicit [Ca\(^{2+}\)]\(_c\) oscillations through predominantly class 1 mechanisms in hepatocytes, for example, growth factors that signal through PLC\(_Y\) (Baffy et al., 1992) and agonists that enhance IP\(_3\)R activity through, for example, cAMP-dependent phosphorylation (Joseph and Ryan, 1993; Bartlett et al., 2014).

An important characteristic of the [Ca\(^{2+}\)]\(_c\) oscillations elicited by PLC\(_B\)-linked GPCRs is their robustness, both in the temporal domain and in their spatial organization. In the presence of hormones such as VP, Ca\(^{2+}\) transients occur with a regular period and relatively little variation of ISI, whereas the Ca\(^{2+}\) transients occurring in response to IP\(_3\) uncaging are much more stochastic. This implies a larger contribution from deterministic components under the conditions of hormone stimulation (Thurley et al., 2014), which likely reside in the metabolic steps of IP\(_3\) formation and breakdown. Presumably it is the linked cascade of metabolic steps and Ca\(^{2+}\) feedback on these that gives rise to the other notable difference between [Ca\(^{2+}\)]\(_c\) oscillations induced by IP\(_3\) uncaging and hormone treatment, which is the much greater duration of the Ca\(^{2+}\) transients elicited by hormone. These broader [Ca\(^{2+}\)]\(_c\) transient kinetics are known to be independent of agonist dose, but do show characteristically distinct shapes with different agonists (Bartlett et al., 2014; Green et al., 1993; Rooney et al., 1989).
A potential explanation for the agonist-specific \([\text{Ca}^{2+}]_c\) spike shapes is differential modulation of the generation and/or metabolism of IP3 by distinct GPCRs. An additional component of the G3-linked PLC pathway is the generation of diacylglycerol and activation of PKC, concurrent with the production of IP3. Many components of the Ca\(^{2+}\) signaling toolkit are substrates for PKC isoforms, and the activity of these kinases could contribute to oscillatory IP3 production (Nash et al., 2001; Woodring and Garrison, 1997; Bartlett et al., 2015). GPCRs can be phosphorylated by PKC (Nash et al., 2001), which decreases their coupling to G proteins. Consistent with this, we and others have shown that PKC inhibition enhances hormone-induced Ca\(^{2+}\) signaling, whereas acute PKC activation with phorbol ester decreases Ca\(^{2+}\) oscillation frequency (Bartlett et al., 2015; Sanchez-Bueno et al., 1990). PKC phosphorylation may also modify the activity of IP3Rs (Matter et al., 1993; Arguin et al., 2007), and thus has the potential to affect the onset or duration of class 1 oscillations. Indeed, we have shown that acute phorbol ester treatment increases the frequency of Ca\(^{2+}\) oscillations induced by uncaging IP3 (Bartlett et al., 2015). However, it is important to note that a PKC feedback mechanism to regulate Ca\(^{2+}\) or IP3 metabolism was not included in the modeling data presented here. Thus, although PKC activity may account for agonist-dependent differences in Ca\(^{2+}\) transient dynamics, the only factor required for the switch between class 1 and class 2 oscillations in our model is allowing the Ca\(^{2+}\) feedforward activation of PLC (Ca\(^{2+}\) and IP3 cross-coupling).

In addition to the longer duration of the Ca\(^{2+}\) transients in the presence of hormones, it is also noteworthy that hormone-induced \([\text{Ca}^{2+}]_c\) oscillations sustain much longer periods of baseline \([\text{Ca}^{2+}]_c\), between Ca\(^{2+}\) transients when compared with IP3 uncaging. During these periods there is no overt stochastic Ca\(^{2+}\) spiking like that seen with IP3 uncaging. Indeed, hormone-mediated Ca\(^{2+}\) signaling not only yields more robust low-frequency oscillations but also appears to suppress stochastic behavior between Ca\(^{2+}\) transients. Nevertheless, both IP3 uncaging and agonist stimulation are associated with dose-dependent increases in \([\text{Ca}^{2+}]_c\) oscillation frequency (present work and Bartlett et al., 2014; Bartlett et al., 2015; Gaspers et al., 2014).

The greater robustness of Ca\(^{2+}\) signaling with hormones when compared with direct photorelease of IP3 is also apparent at the spatial level. The \([\text{Ca}^{2+}]_c\) oscillations elicited by global uncaging of IP3 are characterized by local events, which may be sustained in only a small subcellular region, or propagate to other parts of the cell. Even within the same cell, and during a single uncaging event, there can be a mixture of local Ca\(^{2+}\) transients, partial propagation, and global Ca\(^{2+}\) waves. When they do occur, these global Ca\(^{2+}\) signals resulting from IP3 uncaging vary in their kinetic properties in different parts of the cell, tending to decrease in rates of Ca\(^{2+}\) rise and increase in duration from the initial puff site to the distal pole of the cell. By contrast, the \([\text{Ca}^{2+}]_c\) oscillations induced by hormone treatment always propagate throughout the cell as a full Ca\(^{2+}\) wave, and do so with kinetic properties that are sustained across the cell. Significantly, the hormone-induced \([\text{Ca}^{2+}]_c\) oscillations and waves typically originate from the same “eager” Ca\(^{2+}\) puff sites that are seen with IP3 uncaging. These data suggest that the subcellular distribution of IP3 receptor populations plays a key role in determining the initiation of Ca\(^{2+}\) signaling in response to hormones in hepatocytes. However, the combination of the class 1 IP3R CICR mechanism with the class 2 positive feedforward of Ca\(^{2+}\) on PLC to generate sufficient IP3 yields a more robust signal that ensures that Ca\(^{2+}\) release is not spatially restricted in the presence of hormone and gives rise to the properties of propagating intracellular Ca\(^{2+}\) waves in hepatocytes. Thus, the combination of two types of interacting Ca\(^{2+}\) oscillation mechanisms give rise to a uniform intracellular Ca\(^{2+}\) signal with fixed kinetics and relatively low frequency and limits stochastic behavior between Ca\(^{2+}\) transients.

Limitations of the Study

In this study we challenge the dogma that different cell types elicit Ca\(^{2+}\) oscillations via class 1 or class 2 mechanisms and clearly demonstrate that both phenomena can be observed in primary rat hepatocytes. It is likely that other cell types, particularly those with functional polarization such as epithelial cells, may also generate Ca\(^{2+}\) oscillations via a hybrid class 2 mechanism, but examining different cell types was beyond the scope of the present study. A further test of our proposed mechanism would be to demonstrate unequivocally that IP3 levels oscillate with hormone and not with photorelease of caged IP3. However, it was not possible to make single-cell IP3 measurements during IP3 uncaging and cytosolic Ca\(^{2+}\) recording. We and others have shown previously that hormone-induced oscillations in IP3 occur concurrently with Ca\(^{2+}\) oscillations using Forster resonance energy transfer (FRET)-based IP3 sensors (Gaspers et al., 2014; Tanimura et al., 2009). However, combining flash photolysis of caged IP3 with detection of IP3 with FRET probes is technically challenging, because the UV excitation used for uncaging can quench the FRET fluorophore,
and the required blue light excitation may elicit uncontrolled IP3 uncaging. As an alternative approach we utilized an IP3 buffer to perturb IP3 dynamics and demonstrated that fast Ca2+ oscillations still occur when IP3 is photoreleased in the presence of IP3 buffering (Figure 2), whereas hormone-induced Ca2+ oscillations are suppressed by the expression of the IP3 buffer (Gaspers et al., 2014).

SUPPLEMENTAL INFORMATION
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METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

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AUTHOR CONTRIBUTIONS
P.J.B., A.P.T, and J.S. designed the research. P.J.B. and I.C. performed the research. P.J.B. and I.C. analyzed the data. P.J.B. and A.P.T. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

IP$_3$-Dependent Ca$^{2+}$ Oscillations Switch into a Dual Oscillator Mechanism in the Presence of PLC-Linked Hormones

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Figure S1. Related to Figure 2. Slow continuous release of caged IP$_3$ elicits [Ca$^{2+}$]$_c$ oscillations. Hepatocytes were transfected with RGECO1, cultured overnight and then loaded with caged IP$_3$ (2 μM; 1 h). Representative traces of slow monophasic (A) and oscillatory (B) [Ca$^{2+}$]$_c$ increases (22 cells exhibited oscillatory behavior; total of 45 cells; n=6 experiments). Gray area shows duration of slow IP$_3$ uncaging with low-intensity UV illumination (50 ms exposures from microscope xenon lamp at 2 Hz). Panels C and D show data only from oscillating cells. Peak width (FWHM) and ISI of [Ca$^{2+}$]$_c$ oscillations induced by photorelease of caged IP$_3$ were shorter than with VP (5 nM added subsequently to the same cells) (C), but there was no difference in the rate of [Ca$^{2+}$]$_c$ rise (D). Data are mean±SEM (10 cells from 6 independent experiments). ** p<0.01, ***p<0.001 paired Student’s t-test.
Figure S2. Related to Figure 3. Vasopressin-induced Ca^{2+} oscillations in naïve cells. Hepatocytes were transfected with GCaMP3, cultured overnight and then loaded with caged IP_3 (2 μM; 1 h) for comparison with uncaging studies, but were not exposed to UV light. Traces of \([\text{Ca}^{2+}]_c\) from regions of interest at the Ca^{2+} wave initiation site, the distal pole of the cell and the whole cell are shown. Asynchronous local fluctuations in Ca^{2+} during the cell-wide oscillations are not as apparent when the integrated Ca^{2+} response of the whole cell is plotted. Similar VP responses with spatial heterogeneity were observed in 4 independent experiments.
Figure S3. Related to Figure 5. Periodic release of caged IP$_3$ does recapitulate VP-induced Ca$^{2+}$ oscillations.

Hepatocytes were transfected with GCaMP3, cultured overnight and then loaded with caged IP$_3$ (2 μM; 1 h). Hepatocytes were exposed to single UV flashes at 2 min intervals. A: Traces of [Ca$^{2+}$]$_c$ from regions of interest at the puff site and the distal pole of the cell (representative of three independent experiments). B: Summary data to show proportion of cells responding to each individual UV pulse with no response, local responses at the puff site, partial propagation with intermittent Ca$^{2+}$ waves, propagation of Ca$^{2+}$ waves consistently across the cell, and global Ca$^{2+}$ responses across the entire cell (summary of 10 cells from 3 independent experiments, note 1 experiment only contained 4 UV pulse events).
**Transparent Methods**

**Primary cell culture.** Isolated hepatocytes were prepared by collagenase perfusion of livers obtained from male Sprague-Dawley rats. Cells were transfected with GCaMP3 (Tian et al., 2009) R-GECO1 (Zhao et al., 2011), GFP or GFP-LBD (Gaspers et al., 2014) cDNA using nucleofection (Alonza) then maintained in Williams E media for 16-24 h, as described previously (Rooney et al., 1989). Animal studies were approved by the Institutional Animal Care and Use Committee at Rutgers, New Jersey Medical School.

**Flash Photorelease of caged IP3.**

Overnight cultured hepatocytes were incubated in medium composed of (in mM): 121 NaCl, 25 HEPES, 5 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.3 CaCl2, 5.5 glucose, Where required, cells were loaded with the membrane permeant form of caged IP3 (2 µM, D-2, 3-O-isopropylidene-6-O-(2-nitro-4,5-dimethoxy)benzyl-myoinositol 1,4,5-trisphosphate-hexakis(propionoxymethyl) ester; Sichem GmbH) for 1 h at room temperature. Cells were transferred to the microscope chamber of a spinning disk confocal microscope. GCaMP3 images (excitation, 488 nm; emission 510 nm long band pass filter) were acquired at 10 Hz. Photorelease of caged IP3 was achieved by light pulses from a nitrogen charged UV laser (Photon Technology International). The cell permeant caged IP3 is synthesized with the 2- and 3-hydroxyl groups of myo-inositol protected by an isopropylidene group to ensure that the phosphate groups remain in the 1,4 and 5 positions (Dakin and Li, 2007). Of note, once released from the cage this modified form of IP3 is metabolized at a slower rate, in the order of minutes, compared to natural IP3 which is metabolized in seconds (Dakin and Li, 2007). Cell viability was assessed by the addition of maximal hormone concentrations at the end of each experiment. Only cells responsive to hormone stimulation are included in the presented data.

**Slow photorelease of caged IP3**

Coverslips were prepared as described above and transferred to the stage of an epifluorescence microscope coupled to a Xeon lamp with high speed wave length switching capabilities (DG4 Sutter). Cells expressing recombinant proteins were selected by screening for GFP fluorescence (excitation 488 nm, emission 525 nm). R-GECO1 images (excitation 548 nM, emission 600 nm)
were acquired at 2 Hz. Continuous photorelease of caged IP\textsubscript{3} was achieved with 50 ms exposure to 380 nm light at 2 Hz.

**Mathematical model**

We model only the apical region of the hepatocyte which is assumed to be spatially homogeneous. Let $c = [\text{Ca}^{2+}]$, $c_e = [\text{Ca}^{2+}]_{\text{ER}}$, $p = [\text{IP}_3]$, $c_t = c + \gamma c_e$, where $\gamma$ is the ratio of the ER to the cytoplasmic volume. Also, let $h$ be a variable that controls the rate at which IPR can be activated by $\text{Ca}^{2+}$, as described in (Sneyd et al., 2017). Then, conservation gives $\frac{dc}{dt} = J_{\text{IPR}} - J_{\text{serca}} + \delta(J_{\text{in}} - J_{\text{pm}})$, $\frac{dc_t}{dt} = \delta(J_{\text{in}} - J_{\text{pm}})$, $\frac{dp}{dt} = \tau_p(p_s - p)$, while the dynamics of the IPR result in the equation $\tau_h \frac{dh}{dt} = h_\infty - h$. Expressions and notation for each flux are as in (Sneyd et al., 2017), the only differences being the parameters $t_{\text{max}} = 80$, $K_t = 0.09$, $K_f = 4$, $\bar{R} = 1.5e-5$, $V_{\text{pm}} = 0.07$, $a_0 = 0.004$, $\alpha_1 = 0.01$, $K_{\text{ce}} = 14$, $d = 2.5$, $k_b = 0.4$ (all units the same as in (Sneyd et al., 2017)), and in the expression for $p_s$. In the absence of VP, $p_s = 0$, while, in the presence of VP, $p_s = \frac{V_{\text{PLC}} c^2}{(K_{\text{PLC}}^2 + c^2)}$, where $V_{\text{PLC}} = 0.16 \text{ mM/s}$, $K_{\text{PLC}} = 0.1 \text{ mM}$.
Supplemental References

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