Calcium Dynamics: Spatio-Temporal Organization from the Subcellular to the Organ Level

Geneviève Dupont,* Laurent Combettes,† and Luc Leybaert‡
*Theoretical Chronobiology Unit, Université Libre de Bruxelles, Faculté des Sciences, 1050 Brussels, Belgium
†Institut National de la Santé et de la Recherche Médicale, Unité U757, Université de Paris-Sud, Orsay, France
‡Department of Physiology and Pathophysiology, Faculty of Medicine and Health Science, Ghent University, 9000 Ghent, Belgium

Many essential physiological processes are controlled by calcium. To ensure reliability and specificity, calcium signals are highly organized in time and space in the form of oscillations and waves. Interesting findings have been obtained at various scales, ranging from the stochastic opening of a single calcium channel to the intercellular calcium wave spreading through an entire organ. A detailed understanding of calcium dynamics thus requires a link between observations at different scales. It appears that some regulations such as calcium-induced calcium release or PLC activation by calcium, as well as the weak diffusibility of calcium ions play a role at all levels of organization in most cell types. To comprehend how calcium waves spread from one cell to another, specific gap-junctional coupling and paracrine signaling must also be taken into account. On the basis of a pluridisciplinar approach ranging from physics to physiology, a unified description of calcium dynamics is emerging, which could help understanding how such a small ion can mediate so many vital functions in living systems.

KEY WORDS: Ca^{2+} oscillations, Ca^{2+} waves, Puffs, Intercellular communication, $InsP_3$, Gap junctions, Fertilization, Hepatocytes.

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I. Introduction

The essential role of Ca^{2+} in the maintenance of life has been well known for about 120 years (Ringer, 1883). More than being required for the ionic balance of the cell, this ion is ubiquitously used as an intracellular messenger, from plants to mammals and from eggs to highly specialized neurons (Berridge *et al.*, 2000). An increase in the level of free Ca^{2+} is indeed a key step in the onset of vital intracellular processes such as fertilization, gene expression, or secretion. Surprisingly, in a given cell type, Ca^{2+} can induce a whole spectrum of different physiological responses. At the genetic level, about 300 genes and 30 transcription factors which are regulated by intracellular Ca^{2+} have been identified (Feske *et al.*, 2001; Lanahan and Worley, 1998). At the metabolic level too, many responses are mediated by Ca^{2+}. In hepatocytes, for instance, a hormone-induced rise in Ca^{2+} can lead to the production of glucose, but also to bile secretion, cell division, or even to apoptosis or necrosis (Dupont *et al.*, 2000a; Gaspers and Thomas, 2005). How does the cell discriminate between these various types of processes inducible by Ca^{2+}? Many years of research in the field of Ca^{2+} signaling have led to an answer: the signal-induced Ca^{2+} increases are very well organized, both in time and space. Interestingly, this is a quite common strategy in cellular signaling. Besides the well-known oscillatory and wavelike character of electrical signals in excitable cells, cAMP increases also possess a precise spatio-temporal organization in some cell types (Goldbeter, 2006).

In the field of Ca^{2+} signaling, the term temporal organization means that the rise in cytosolic Ca^{2+} does not occur as a monotonous rise, but as repetitive spikes. These spikes mainly result from the release of Ca^{2+} from the endoplasmic reticulum (ER). This release is initiated by inositol 1,4,5-trisphosphate ($InsP_3$), generated in response to the external stimulus through a well-characterized signaling cascade (Berridge and Irvine, 1989). The $InsP_3$ receptor ($InsP_3R$) located in the ER membrane is a Ca^{2+} channel that, when open, allows for the flux of Ca^{2+} down its electro-chemical gradient. The concentration of Ca^{2+} in the ER ($[Ca^{2+}]_{ER} \approx 500\ \mu M$) is indeed considerably higher than in the cytosol ($[Ca^{2+}]_i = 0.1\ \mu M$). As will be much emphasized in this review, the specific regulatory properties of the $InsP_3R$ are largely responsible for the oscillatory nature of the Ca^{2+} signal. Oscillations in $[Ca^{2+}]_{ER}$ are thus observed in antiphase to $[Ca^{2+}]_i$ oscillations (Ishii *et al.*, 2006). As summarized in Table I, other messengers than $InsP_3$ can be involved in this periodic exchange, mainly cyclic ADP ribose (cADPR) that activates the ryanodine receptor (RyR) and nicotinic acid adenine dinucleotide (NAADP). Moreover, Ca^{2+} dynamics are not limited to Ca^{2+} exchanges between the cytosolic and ER compartments. First, increasing evidences point to an active role of mitochondria (Ishii *et al.*, 2006; Jouaville *et al.*, 1995;

TABLE I

Summary of the Main Processes Involved in the Ca^{2+} Fluxes in and from the Cytosol in Nonexcitable Cells

Cytosolic Ca^{2+} exchanges[a]	Entry in or exit from the cytosol	Channels	Main physiological regulators[b]
ER	Into the cytosol	$InsP_3R$	$InsP_3$, Ca^{2+}
		RyR	Ca^{2+}, cADPR
		NAADPR[c]	NAADP
	Into the ER	SERCA	ATP, Mg^{2+}
Mitochondria	Into the cytosol	PTP	Ca^{2+}, H^+, $\Delta\Psi$
	Into the mito	Ca^{2+}-sensitive uniporter	Ca^{2+}
Golgi	Into the cytosol	$InsP_3R$	$InsP_3$, Ca^{2+}
	Into the Golgi	SPCA	ATP, Mg^{2+}
External medium	Into the cytosol	TRP channels	*Largely unknown*
		I_{ARC}	AA
		ROC	Extracellular ligands
	Out of the cell	PMCA	ATP, Mg^{2+}

[a]Note that Ca^{2+} handling by Ca^{2+} buffers also much affect the cytosolic Ca^{2+} dynamics.

[b]The regulation of nuclear Ca^{2+}, also affected by most of these regulators, is not considered in this Table (Gerasimenko *et al.*, 2003).

[c]Some reports suggest that these receptors are located on Ca^{2+} stores that are distinct from the ER (Patel, 2004).

$InsP_3R$, Inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor.; NAADPR, nicotinic acid adenine dinucleotide phosphate receptor; SERCA, SR/ER Ca^{2+} ATPase; PTP, permeability transition pore; $\Delta\Psi$: electrical potential across the mitochondrial membrane; SPCA, secretory pathway Ca^{2+} ATPase; I_{ARC}, arachidonic acid (AA)-regulated Ca^{2+} flux; TRP channels, transient receptor potential channels; ROC, receptor-operated Ca^{2+} channels; PMCA, plasma-membrane Ca^{2+} ATPase.

Szabadkai *et al.*, 2003) in the generation of Ca^{2+} oscillations. In some cell types, another intracellular organelle, the Golgi apparatus, is able to release Ca^{2+} in response to an increase in $InsP_3$ (Vanoevelen *et al.*, 2004). Some evidence also suggests that NAADP receptors are distinct from $InsP_3$- and cADPR-sensitive ones, and reside on different Ca^{2+} stores that remain to be fully identified (Patel, 2004). Finally, a poorly defined secondary Ca^{2+} store, related to the lysosome, has been reported as well (Lee, 2004).

Ca^{2+} exchanges with the external medium play a crucial role either in the onset of Ca^{2+} oscillations or, at least, in their long-term maintenance. Entry of Ca^{2+} can be a direct consequence of hormonal activation of a receptor-operated Ca^{2+} channel (ROC), or can be induced by the emptying of internal stores. Although the latter mechanism is far from being fully understood, it most probably involves specific transient receptor potential (TRP) channels (Wissenbach *et al.*, 2004) and/or a Ca^{2+} influx pathway through an

arachidonic acid regulated channel (I_{ARC}) (Shuttleworth, 2004). The complexity arising from the interplay between these various Ca^{2+} fluxes, together with the difficulty to intuitively reason on oscillatory phenomena, may explain why modeling is so often associated with the experimental studies to gain a clear understanding of the molecular mechanisms of Ca^{2+} oscillations. This synergistic approach between theory and experiments will be particularly emphasized in this review.

The shape and frequency of Ca^{2+} oscillations depend on the nature and concentration of the extracellular signal (hormone or neurotransmitter). Thus, in hepatocytes, vasopressin and phenylephrine most often induce regular Ca^{2+} spikes whereas stimulation by other agonists such as ATP or cAMP leads to complex oscillations corresponding to a large peak, followed by smaller amplitude oscillations superimposed on a plateau phase (Dixon *et al.*, 2000). In all cases, the frequency of the Ca^{2+} spikes rises with the concentration of the hormonal signal, a phenomenon which is known as frequency coding. Thus, the amplitude of the various Ca^{2+}-mediated intracellular responses, such as mitochondrial metabolism (Robb-Gaspers *et al.*, 1998), CaMKII activity (De Koninck and Schulman, 1998), or secretion (Malgaroli and Meldolesi, 1991) increases with the frequency of Ca^{2+} oscillations. Other intracellular responses are selectively activated by Ca^{2+} oscillations at precise frequencies. Such frequency selectivity has been observed, for example, for gene expression (Dolmetsch *et al.*,1998) or for neuronal differentiation (Spitzer, 2003). Another elegant demonstration of this phenomenon is provided by the simultaneous observation of Ca^{2+} increases and contraction of smooth muscle cells in living lung slices (Perez and Sanderson, 2005a,b). Airway and blood vessel smooth muscle cells contract in response to specific but distinct frequencies of Ca^{2+} oscillations. It is thus of key importance to understand the molecular mechanisms responsible for these Ca^{2+} oscillations and waves and for the precise control of their frequency.

Signal-induced Ca^{2+} increases are also spatially organized. Intracellular waves of Ca^{2+} often accompany Ca^{2+} oscillations (Berridge, 1993); in most cases, the Ca^{2+} concentration first increases locally, and this local rise then propagates in the whole cell as a wave, traveling at a speed of 10–50 μms^{-1} (Berridge and Dupont, 1994). The appearance of the Ca^{2+} waves varies greatly from one cell type to another. The front can be planar as in cardiac or smooth muscle cells, elliptical as in hepatocytes or in eggs, or even adopt a spiral shape (Berridge and Dupont, 1994; Jaffe, 1993). The exact physiological role of intracellular Ca^{2+} waves remains difficult to establish, mainly because they are most often observed under nonphysiological conditions. For example, Ca^{2+} waves in cardiac myocytes are initiated by overloading the Ca^{2+} stores (Lipp and Niggli, 1993). In the same manner, spiral waves in *Xenopus* oocytes are only observed in immature oocytes after stimulation by ACh. Interesting exceptions are the cases of some polarized cells in which intracellular Ca^{2+} waves are clearly related to secretion (Kasai and

Augustine, 1990) or eggs at fertilization. In the latter case, the direction of the wave determines the antero-posterior axis of the future animal. The precise localization of the initiation point of the first few Ca^{2+} increases in response to sperm attachment is thus a key factor that remains to be fully understood (Dumollard *et al.*, 2002).

In some cases, the Ca^{2+} wave does not die out when reaching the cell membrane but somehow propagates to an adjacent cell, coupled to the first one by gap junctions. Such intercellular waves, first observed in airway epithelial cells (Sanderson *et al.*, 1990), also occur in a large number of organs or tissues. In the liver, they propagate in an entire lobule and can stimulate liver growth or bile flow (Nathanson and Schlosser, 1996; Nicou *et al.*, 2003). They are also associated with secretion in the pancreas (Yule *et al.*, 1996). In the brain, Ca^{2+} signals even communicate between different cell types: astrocytes, endothelial cells of the vessel wall, and smooth muscle cells surrounding the blood vessel (Braet *et al.*, 2004) in *in vitro* cell culture models.

The spatio-temporal organization of Ca^{2+} signals in the form of oscillations; intracellular and intercellular Ca^{2+} waves all tightly depend on the subcellular properties of the Ca^{2+} releasing entities. In particular, the arrangement of the $InsP_3$-sensitive Ca^{2+} channels on the surface of the ER considerably affect the global Ca^{2+} signal (Marchant *et al.*, 1999; Thomas *et al.*, 1998). Thus, much technical effort has been made to visualize elemental events of Ca^{2+} release. In nonexcitable cells, these events have been separated into blips (the Ca^{2+} increases caused by the opening of a single $InsP_3R/Ca^{2+}$ channel) and puffs (the Ca^{2+} increases caused by the opening of a small group of channels). These events occur in a random manner and at a very low level of stimulation. The rise time of the associated increase in cytosolic Ca^{2+} is less than 100 ms and the spatial spreading of a few micrometers. How these Ca^{2+} increases, highly localized in time and space, interact to give a coordinated cytosolic Ca^{2+} signal at a slightly higher level of stimulation is one of the fascinating questions in the field of Ca^{2+} dynamics that remains to be answered.

In this review, we are particularly interested in this multiscale aspect of Ca^{2+} signaling. Thus, we present in more details one aspect of Ca^{2+} dynamics associated with each of the different scales described previously: oscillations at the cellular level (Section II), elemental Ca^{2+} increases (Section III), propagation of intracellular (Section IV) waves, and intercellular (Section V) waves. Interesting reviews can be found in the literature and provide a complete state of the art of these fields (Berridge *et al.*, 2000, 2003; Combettes *et al.*, 2004; Dupont *et al.*, 2000; Falcke, 2004; Gaspers and Thomas, 2005; Rizzuto and Pozzan, 2006; Schuster *et al.*, 2002; Sneyd, 2005). Our aim is to treat here particularly new or illustrative problems in each category. In all cases, particular emphasis is put on the synergy provided by an experimental and modeling approach to gain a deeper understanding of the complex, but fascinating phenomena related to Ca^{2+} dynamics.

II. Oscillations

A. Mechanism Based on the Regulatory Properties of the $InsP_3$ Receptor

From physics to biology, oscillatory phenomena often rely on the interplay between regenerative and declining processes. The $InsP_3R$ can serve for both functions at the same time. Indeed, reconstituted in lipid bilayers and stimulated by a constant dose of $InsP_3$, it is both activated and inhibited by Ca^{2+}, with the maximum open probability at about 0.2 μmol Ca^{2+} (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). Thus, activation at low cytosolic Ca^{2+} provides the regenerative, autocatalytic mechanism—often referred to as CICR for Ca^{2+}-induced Ca^{2+} release—and inhibition at high cytosolic Ca^{2+} can supply the required declining process. As expected from these observations, cycles of Ca^{2+} release and reuptake can be reproduced in permeabilized cells with $InsP_3$ clamped at a submaximal concentration (Hajnoczky and Thomas, 1997; Zimmermann, 2000).

In vivo, synthesis of $InsP_3$ results from a series of biochemical reactions (Berridge and Irvine, 1989). Shortly, the binding of the extracellular agonist to its specific receptor leads, via a G-protein activation cascade, to phospholipase C (PLC) activation. PLC catalyzes the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and $InsP_3$. $InsP_3$ is a soluble, low–molecular-weight molecule (486 Da) that easily diffuses inside the cell and binds to receptors located on the ER surface and roughly evenly distributed across the cytoplasm (at least in nonpolarized cells). The Ca^{2+} release initiated by this binding can trigger successive cycles of activation/inhibition of the $InsP_3R$, resulting in sustained Ca^{2+} oscillations. Many mathematical models have demonstrated that such a mechanism can explain the generation and the main properties of cytosolic Ca^{2+} oscillations (Falcke, 2004; Keener and Sneyd, 1998; Schuster *et al.*, 2002). The physiological relevance of this scenario is moreover corroborated by experimental observations that are basically of two types. First, activation of the $InsP_3Rs$ by injecting into the cell substances other than $InsP_3$ can induce Ca^{2+} oscillations very similar to the ones observed in physiological conditions, confirming the assumption that the oscillatory mechanism is located after the step of $InsP_3$ synthesis. These substances are nonmetabolizable analogs of $InsP_3$ (Dumollard and Sardet, 2001; Wakui *et al.*, 1989) or a compound (thimerosal) that sensitizes the $InsP_3R$ to basal $InsP_3$ levels (Swann, 1991). The second type of experiments confirms that the Ca^{2+} sensitivity of the $InsP_3R$ is crucial to get oscillations: DT40 cells expressing a mutant $InsP_3R$ with a reduced sensitivity to Ca^{2+} do not exhibit Ca^{2+} oscillations upon stimulation of cross-linked B-cell receptors (Miyakawa *et al.*, 2001). Despite

these convincing observations, no one would nowadays state with confidence that $InsP_3$ remains constant during Ca^{2+} oscillations in all nonexcitable cells. Since a few years, a group of arguments even seems to emerge to deny this assumption (Taylor and Thorn, 2001).

Before going further, it might be worth adding a few words about the interest of the problem. Whether $InsP_3$ oscillates in synchrony with Ca^{2+} may indeed be considered as a secondary issue. However, referring to other oscillatory phenomena (Goldbeter, 2002) such as circadian rhythms, pulsatile hormone secretion, or the dynamics of the cell cycle, it is well established that periodicity always originates from a subtle interplay between diverse regulatory mechanisms that control the dynamics of living systems. An approximate, qualitative understanding of Ca^{2+} oscillations could thus bypass important regulatory pathways that need to be uncovered to be able to control the many details of Ca^{2+} oscillations. Given that the spatio-temporal organization of Ca^{2+} signals plays such a crucial role to selectively activate the appropriate Ca^{2+}-mediated cellular process, it would be a mistake to deliberately ignore any of these regulations.

B. Possible Involvement of $InsP_3$ Dynamics

Monitoring $InsP_3$ changes in single cells has long remained a challenging technical problem. It is now feasible by following translocation of green fluorescent protein (GFP) tagged to the pleckstrin homology (PH) domain of PLC, from the plasma membrane to the cytoplasm. In such a cell indeed, a rise of $InsP_3$ in the cytosol results in a transfer of fluorescence from the plasma membrane to the cytoplasm, because the PH domain has a greater affinity for $InsP_3$ than for PIP_2. Hirose *et al.* (1999), the pioneers of this method, have shown in this manner that ATP-induced Ca^{2+} oscillations in epithelial cells are accompanied by periodic variations in the level of $InsP_3$. Similar experiments performed in CHO cells lead to the conclusion that depending on the type of stimulation, Ca^{2+} oscillations are accompanied by an oscillatory or a constant level of $InsP_3$ (Nash *et al.*, 2001a,b; Young *et al.*, 2003). The increase of cytoplasmic fluorescence could, however, be due to the consumption of PIP_2 in the membrane (Xu *et al.*, 2003), and other methods for imaging $InsP_3$ concentration leads to opposite conclusions (Bartlett *et al.*, 2005; Matsu-ura *et al.*, 2006; Tamarina *et al.*, 2005). Those studies strongly point to a need for the reconsideration of the role of $InsP_3$ dynamics in Ca^{2+} oscillations.

In parallel to these experimental observations, diverse modeling studies converge on a similar conclusion. We have seen in the previous section that simple mathematical models can describe Ca^{2+} oscillations as the result of successive cycles of activation/inhibition of the $InsP_3R$. In this framework,

the delay between two successive Ca^{2+} spikes is dictated by the time needed by the $InsP_3R$ to recover from Ca^{2+}-induced inhibition. The characteristic time of this biochemical step has been estimated to ~10s *in vitro* (Combettes *et al.*, 1994; Finch *et al.*, 1991) and to a few seconds *in vivo* (Fraiman *et al.*, 2006). This time is much smaller than the period of Ca^{2+} oscillations in most cell types. This discrepancy could be explained by the existence of an additional control of the $InsP_3R$ activity, such as an agonist-induced PKA-dependent phosphorylation (Le Beau *et al.*, 1999).

Interplay between Ca^{2+} dynamics and $InsP_3$ metabolism is theoretically another, and probably more widespread, mechanism affecting the period of Ca^{2+} oscillations. Basically, Ca^{2+} levels can influence both synthesis and degradation of $InsP_3$ (Fig. 1). Membrane-bound phospholipase C (PLC) is responsible for $InsP_3$ synthesis, via hydrolysis of PIP_2. All PLC isoforms (β, γ, δ, ε, ζ) require Ca^{2+} for activity (Rhee, 2001). That such positive-feedback may occur for Ca^{2+} concentration in the physiological range of 0.1–1 μmol has been shown for γ (Renard *et al.*, 1987), δ (Allen *et al.*, 1997), and ζ isoforms (Kouchi *et al.*, 2004). Theoretically, stimulation of PLC activity by Ca^{2+} in the appropriate concentration range can lead to concomitant Ca^{2+} and $InsP_3$ oscillations, with a period that now depends on the rate of $InsP_3$ synthesis by PLC (Meyer and Stryer, 1991). At the level of $InsP_3$ degradation, $InsP_3$ can be either dephosphorylated by the $Ins(1,4,5)P_3$ 5-phosphatase to yield Ins(1,4) bisphosphate or phosphorylated by the $Ins(1,4,5)P_3$ 3-kinase into Ins(1,3,4,5) tetrakisphosphate (Shears, 1992).

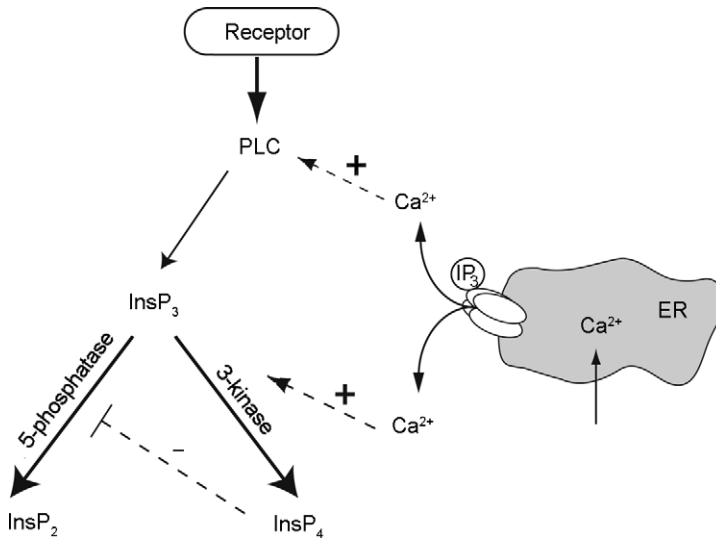

FIG. 1 Schematic representation of the main interplays between Ca^{2+} and $InsP_3$ metabolism.

This 3-kinase is Ca^{2+}-sensitive as binding of Ca^{2+}/calmodulin enhances its activity. The stimulation factor varies from 2 to 10 depending on the isoform (Sims and Allbritton, 1998; Takazawa *et al.*, 1990). Importantly, the product of $InsP_3$ phosphorylation by 3-kinase, $InsP_4$, is a competitive inhibitor of the other $InsP_3$-metabolizing enzyme, the 5-phosphatase. Much care should thus be taken when interpreting experiments in which the levels of 3-kinase have been modified, as these changes are, at least in part, counteracted by the changes of activity of 5-phosphatase. As expected intuitively, the incorporation of such a regulation in a mathematical model can also lead to $InsP_3$ oscillations as each Ca^{2+} spike provokes an enhanced degradation of $InsP_3$ (Dupont and Erneux, 1997). However, in contrast to the stimulation of PLC activity by Ca^{2+}, in this case, $InsP_3$ oscillations passively follow Ca^{2+} oscillations (i.e., this regulation cannot on its own give rise to sustained oscillations and the peak of $InsP_3$ slightly follows that of Ca^{2+}). Before turning to the question of the relevance of these regulations of $InsP_3$ metabolism by Ca^{2+} in real cells, it should be mentioned that it is not possible to exclude that $InsP_3$ oscillations result from other regulatory influences on $InsP_3$ synthesis, more specifically at a step located upstream of PLC activation. For hepatocytes, for example, a specific regulation at the level of the Gα subunit of the G-protein activation cascade leading to PLC activation might play such a role (Kummer *et al.*, 2000).

Although it is since long known that $InsP_3$ 3-kinase, one of the $InsP_3$ metabolizing enzymes (Fig. 1), is activated by Ca^{2+}, it took some time to clearly state that this regulation leads to $InsP_3$ oscillations. However, based on a careful quantitative evaluation of the kinetics of $InsP_3$ metabolism by the kinase and the phosphatase, modeling suggests $InsP_3$ oscillations resulting from this regulation have a tiny amplitude (Dupont and Erneux, 1997). Cellular conditions are indeed such that the major part of the $InsP_3$ pool is catabolized by 5-phosphatase. This conclusion holds with the observation performed in CHO cells that overexpression of 5-phosphatase clearly abolishes any Ca^{2+} oscillatory activity in response to stimulation, whereas 3-kinase overexpression has a negligible effect on internal Ca^{2+} mobilization (De Smedt *et al.*, 1997).

Despite this observation, one might imagine that in another cell type, the 3-kinase might play a more important role and, more specifically, allow for a rather long time interval between two Ca^{2+} spikes. If each Ca^{2+} spike provokes the catabolism of a significant amount of $InsP_3$, it could take some time for $InsP_3$ to reach a level sufficient to trigger Ca^{2+} release. In this view, the long period would correspond to the time necessary to rebuild the level of $InsP_3$ necessary to activate Ca^{2+} release through the receptor. This possibility has been indirectly tested in hepatocytes (Dupont *et al.*, 2003b). The strategy used in this study aimed at masking Ca^{2+}-dependent $InsP_3$ catabolism by 3-kinase through the injection of massive amounts of 5-phosphatase,

which is not stimulated by Ca^{2+}. In such injected hepatocytes, Ca^{2+} oscillations generated by modest agonist doses are suppressed because of the resulting low level of $InsP_3$. At higher doses of agonist, oscillations reappear. Importantly, the characteristics of these oscillations are similar to those of untreated cells at low agonist dose, despite the fact that $InsP_3$ oscillations due to 3-kinase stimulation by Ca^{2+} do not occur (nearly all the $InsP_3$ is metabolized by the 5-phosphatase, which is much more abundant because of the injection). This study thus confirms that the oscillations of $InsP_3$ that would result from Ca^{2+}-regulation of the $InsP_3$ 3-kinase do not play an active role in the control of Ca^{2+} oscillations.

We have seen from the previous explanation that Ca^{2+}-stimulation of PLC activity is another mechanism whereby $InsP_3$ oscillations could occur. In contrast to 3-kinase-induced $InsP_3$ oscillations, this regulation is an oscillatory mechanism on its own: Ca^{2+} increases PLC activity, which in turn induces the synthesis of $InsP_3$ responsible for a further Ca^{2+} rise in the cytosol. This type of mechanism is known as *cross-catalysis*. The declining process necessary to avoid an infinite increase of Ca^{2+} and $InsP_3$ can be Ca^{2+} elimination from the cytosol and/or $InsP_3$ catabolism. Thus, in such a scheme, the Ca^{2+} regulation of the $InsP_3R$, although always present, would only modulate the shape of the oscillations. Figure 2 (upper panel) shows in a schematic way Ca^{2+} and $InsP_3$ oscillations generated by such a mechanism. $InsP_3$ and Ca^{2+} rise concomitantly up to a point where pumping into the ER exceeds Ca^{2+} release from the ER, leading to the decreasing part of the Ca^{2+} spike. The level of $InsP_3$ then falls down because PLC is not activated any longer. Due to the basal leak through the $InsP_3R$ and to Ca^{2+} entry from the outside, Ca^{2+} slowly rises again in the cytosol, and the whole sequence of events can reproduce itself. Thanks to a better understanding of this mechanism, Sneyd *et al.* (2006) have suggested a very elegant method to detect whether PLC stimulation by Ca^{2+} is responsible for Ca^{2+} oscillations in a given cell type. The basis of the method is to perturb agonist-induced Ca^{2+} oscillations by a direct, artificial release of $InsP_3$ in the cytoplasm (flash photolysis of caged $InsP_3$). As shown in Fig. 2 (upper panel), in such an oscillatory mechanism, a sudden increase in $InsP_3$ will provoke a delay in Ca^{2+} oscillations, which corresponds to the time required for the level of $InsP_3$ to go back to its normal values during the oscillatory cycles. Once this is done, the situation is similar to the prepulse one, and no change in frequency is observed. This effect of an $InsP_3$ pulse on agonist-induced Ca^{2+} oscillations shown in the upper panel of Fig. 2 drastically differs from the effect such a pulse would have on Ca^{2+} oscillations occurring in the presence of a constant level of $InsP_3$. In the framework of such a mechanism indeed, it is well known that the frequency of oscillations directly depends on the amplitude of the $InsP_3$ signal (Atri *et al.*, 1993). Thus, a sudden increase in $InsP_3$ during agonist-induced Ca^{2+} oscillations provokes a transient rise in frequency

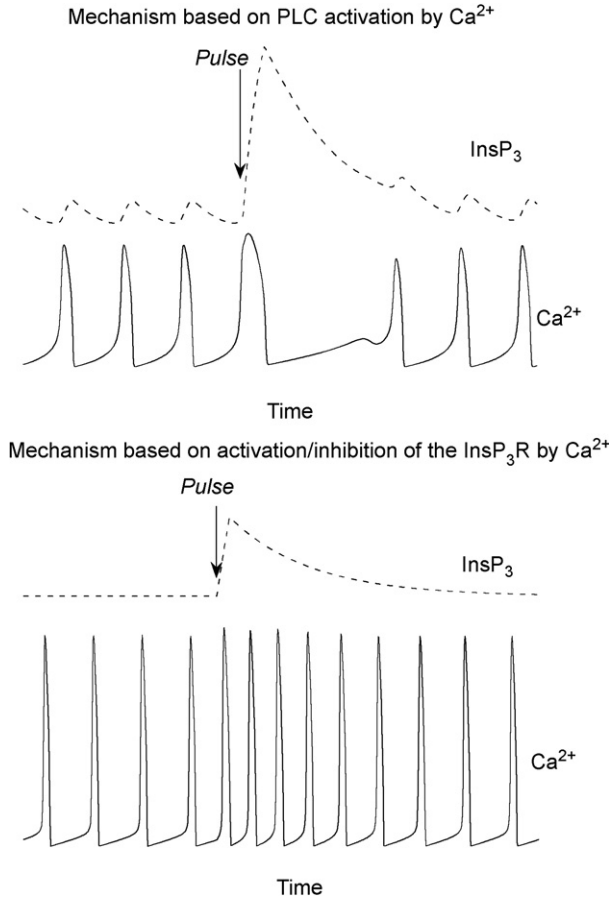

FIG. 2 Schematic representation of the protocol proposed by Sneyd *et al.* (2006) to discriminate between the two most plausible mechanisms for Ca^{2+} oscillations. If phospholipase C (PLC) activity is stimulated by Ca^{2+} (upper panel), oscillations in $InsP_3$ must accompany Ca^{2+} oscillations. If some $InsP_3$ is exogenously added during Ca^{2+} oscillations, it will delay the next Ca^{2+} spikes, without significant change in the frequency of Ca^{2+} oscillations. In contrast (lower panel), if Ca^{2+} oscillations rely on successive cycles of activation/inhibition of the $InsP_3$ receptor ($InsP_3R$), Ca^{2+} oscillations can occur with a constant level of $InsP_3$. In this case, the addition of $InsP_3$ during oscillations will provoke a transient rise in the frequency of Ca^{2+} oscillations, with a progressive return to the original frequency. This protocol, originally based on modelling, has been tested on pancreatic acinar cells (where a delay has been observed) and in smooth muscle cells (where a rise in frequency has been observed). See text and Sneyd *et al.* (2006) for details.

(Fig. 2, lower panel). Interestingly, the number of spikes necessary for the resettlement of the prepulse periodicity depends on the amount of $InsP_3$ injected into the cell (not shown).

In the same study, Sneyd *et al.* (2006) have applied this protocol to two different cell types. In pancreatic acinar cells, liberation of $InsP_3$ during agonist-induced Ca^{2+} oscillations provokes a delay similar to the one shown in the upper panel of Fig. 2, suggesting that PLC stimulation by Ca^{2+} plays a predominant role in the oscillatory mechanism in this cell type. In contrast, the release of $InsP_3$ during methacholine-induced Ca^{2+} oscillations in airway smooth muscle provokes a transient increase in the frequency of Ca^{2+} oscillations, as illustrated in the lower panel of Fig. 2. Thus, in this cell type, Ca^{2+} oscillations are presumably regulated by the $InsP_3R$ dynamics. It should be interesting to perform these experiments in other cell types as well, particularly in MDCK or epithelial cells where $InsP_3$ oscillations have been visualized using the GFP technique (see Section II.B).

Finally, a related indirect method to assess the involvement of $InsP_3$ dynamics in the core oscillatory mechanism has been tested in CHO cells (Politi *et al.*, 2006). The main concept here is that slowing down $InsP_3$ dynamics would significantly affect Ca^{2+} dynamics only if Ca^{2+} oscillations rely on activation of $InsP_3$ synthesis by Ca^{2+}—and not stimulation of $InsP_3$ catabolism by Ca^{2+}. Although this conclusion results from modeling studies and cannot be easily apprehended from an intuitive point of view, one can roughly understand that $InsP_3$ can drive Ca^{2+} oscillations only if it is able to vary fast enough. Following this reasoning, the authors of this study have transfected CHO cells with an $InsP_3$-binding protein, whose only effect is to slow down the $InsP_3$ changes. As the latter cells show a dose-dependent quenching of ATP-induced Ca^{2+} oscillations, they conclude that these are driven by PLC-induced $InsP_3$ oscillations.

C. Effect of the Different Isoforms of the $InsP_3$ Receptor

In those cell types in which the Ca^{2+} oscillatory mechanism resides at the level of the $InsP_3R$, it is highly relevant to consider in detail the regulations of this receptor/channel. Three isoforms of $InsP_3R$ have been identified ($InsP_3R1$, $InsP_3R2$, and $InsP_3R3$) and their levels of expression are largely tissue-specific. Experiments in which the levels of expression of these isoforms have been modified (overexpress or knockdown) clearly indicate that their proportions significantly affect the time course of cytosolic Ca^{2+} concentration (Hattori *et al.*, 2004; Miyakawa *et al.*, 1999; Morel *et al.*, 2003). The inhomogeneous spatial distribution of the three receptor subtypes inside a given cell also indicates that the latters play different roles in the regulation of Ca^{2+} dynamics. For example, in hepatocytes, $InsP_3$-induced Ca^{2+} signals begin sooner in the apical region where $InsP_3R2$ are concentrated (Pusl and Nathanson, 2004).

On the other side, *in vitro* experiments on these three receptor subtypes indicate they are regulated differently by Ca^{2+} and $InsP_3$ (Patterson *et al.*, 2004; Taylor and Laude, 2002). In particular, different open probabilities at steady state have been reported. Although consistent experimental data are lacking, the classical bell-shaped curve most probably corresponds to type 2 $InsP_3R$. This curve directly originates from the activation/inhibition of channel activity at increasing Ca^{2+} concentrations. Another subtype, presumably type 3, displays activation at low Ca^{2+} concentration but apparently lacks inhibition, at least for physiological Ca^{2+} levels. Finally, a displacement of the bell-shaped curve with $InsP_3$ has also been reported. In this case, that relates to the $InsP_3R1$, the maximum of the bell-shaped curve shifts to the right when increasing the $InsP_3$ concentration. Differences among the subtypes concerning regulation by $InsP_3$ are much less controversial: type 2 has the greatest affinity for $InsP_3$, followed by types 1 and 3 successively.

Based on these results about the steady state open probabilities, modeling strongly suggests the changes in the overall Ca^{2+} dynamics observed after modification of the levels of expression of the various subtypes can be ascribed to their different regulations by $InsP_3$ and Ca^{2+} (Dupont and Combettes, 2006). Thus, type 2 receptor, which shows the sharpest dependence on cytosolic Ca^{2+} and has the highest affinity for $InsP_3$, is the main oscillatory unit as shown in DT40 cells (Miyakawa *et al.*, 1999) and myocytes (Morel *et al.*, 2003). Stimulation of type 1 receptor can also lead to oscillations, but most often damped rather than sustained (Hattori *et al.*, 2004; Miyakawa *et al.*, 1999). In contrast, type 3 receptor tends to suppress oscillations. This surprising result is because $InsP_3R3$, which is not inhibited by Ca^{2+}, provides a constant flux of Ca^{2+} without providing the feedback necessary for oscillations to occur. Simply stated, in the presence of $InsP_3$, type 3 receptor constantly releases Ca^{2+}, which inhibits types 1 and 2. Modeling, however, predicts this is only the case at relatively high receptor density. In a cell type in which the total receptor density ($InsP_3R1$+$InsP_3R2$ +$InsP_3R3$) is low, the constant Ca^{2+} flux provided by type 3 receptors could activate types 1 and 2 and thereby favor Ca^{2+} oscillations (Dupont and Combettes, 2006). This prediction might be related to the observation performed in pancreatic acinar cells that both $InsP_3R2$ and $InsP_3R3$ support Ach- and CCK-induced Ca^{2+} oscillations (Futatsugi *et al.*, 2005).

D. Physiological Impacts of the Oscillatory Dynamics and Frequency Coding

It is well established that Ca^{2+} is a key regulator of diverse cellular responses such as secretion, contraction, neuronal differentiation, or fertilization (Berridge *et al.*, 2000). The impact of an *oscillatory* Ca^{2+} signal is less clear, although it

has led to many speculations. Soon after the discovery of Ca^{2+} oscillations, it has been proposed that agonist-induced Ca^{2+} oscillations provide a cellular example of frequency coding. This concept is illustrated in Fig. 3. Upon increasing the stimulation level, the frequency of Ca^{2+} oscillations increases, as well as the physiological response of the cell (e.g., in the form of secretion or gene expression). Because Ca^{2+} is known to be responsible for the activation of the signaling cascade leading to the cellular response of the agonist, it is logical to assume the level of response is encoded in the frequency of Ca^{2+} oscillations. Frequency coding is well known to be more resistant to noise than amplitude coding (Prank *et al.*, 2000; Rapp, 1987).

The question, however, arises as how a cellular response can be sensitive to the frequency of Ca^{2+} oscillations. A survey of the literature suggests there is no general mechanism for this, and there may be other physiological advantages to an oscillatory Ca^{2+} signal than the frequency coding per se. An obvious possibility is to induce a long-lasting Ca^{2+} increase that does not provoke the possible deleterious effects of Ca^{2+}, such as apoptosis or phosphate precipitation. This seems to be the case for fertilization in mammals in which a wide range of oscillatory Ca^{2+} signals of various amplitude, duration, and frequency are able to activate the egg and initiate development (Toth *et al.*, 2006). The egg somehow integrates the biological effects of all

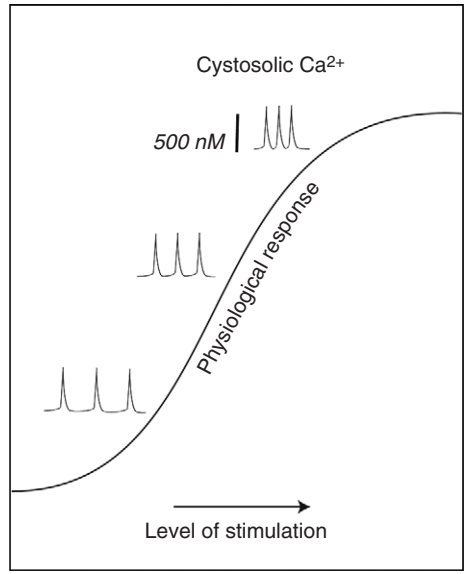

FIG. 3 Schematic representation of the frequency coding of Ca^{2+} oscillations. Increasing the level of stimulation leads to higher frequency Ca^{2+} oscillations and thereby to a larger physiological response.

Ca^{2+} signals to which it is submitted, whatever their precise temporal characteristics. The detailed molecular mechanisms leading to such an integration effect remain to be understood. A similar phenomenon has been reported in the liver, where the mitochondrial metabolic output is optimized by an oscillatory level of Ca^{2+}. Cytosolic Ca^{2+} increases are indeed rapidly transmitted into the mitochondrial matrix. This increase in mitochondrial Ca^{2+} in turn stimulates various mitochondrial dehydrogenases. As the uptake mode of mitochondria is short-lived, a sustained Ca^{2+} increase in the cytosol only induces a transient increase in oxidative metabolism. In contrast, in the presence of cytoplasmic Ca^{2+} oscillations, the resulting mitochondrial Ca^{2+} oscillations are integrated and produce a sustained increase in NADH (Hajnoczky *et al.*, 1995; Robb-Gaspers *et al.*, 1998).

Another advantage of an oscillatory signal is to increase the sensitivity of the target of the Ca^{2+} signal. In B-lymphocytes, gene expression is optimized by an oscillatory signal: a constant level of Ca^{2+} induces less expression than an oscillatory signal of the same average value (Dolmetsch *et al.*, 1998; Li *et al.*, 1998). In pancreatic acinar cells, intermediate levels of acetylcholine (Ach) that trigger Ca^{2+} oscillations are a more potent stimulus of secretion than higher concentrations of Ach that lead to a high and steady level of Ca^{2+} (Kasai and Augustine, 1990).

A spectacular case of frequency sensitivity is that of the ubiquitous Ca^{2+}-calmodulin kinase II (CaMKII). This enzyme is subject to a complex mode of regulation; subunits that have previously bound the Ca^{2+}/CaM complex can phosphorylate other subunits of the same holoenzyme. After this intramolecular reaction, the autophosphorylated subunits can remain active (as kinases) even after dissociation of Ca^{2+}/CaM (Rongo, 2002). This acquisition of a Ca^{2+}-independent activity largely depends on the temporal pattern of the Ca^{2+} signal activating the enzyme. *In vitro* experiments performed on isolated CaMKII attached to PVC tubing indeed reveal the autonomous activity increases in a roughly exponential manner with the frequency of Ca^{2+} spikes (De Koninck and Schulman, 1998). When the concentration of CaM is fixed at 100 nM and the duration of the Ca^{2+} pulses at 200 ms, the frequency leading to half-maximal autonomy is shown to be approximatively 2.5 Hz. If CaMKII is exposed to lower frequencies, it fails to integrate them, and the activity remains low whatever the total number of spikes. The Ca^{2+}-CaM complex indeed dissociates between two spikes, preventing the intersubunit phosphorylation. By contrast, for higher frequencies of stimulation by Ca^{2+}, there is no time for a significant dissociation of the Ca^{2+}-CaM complex, and the autonomous activity progressively increases during the stimulatory protocol. Modeling studies suggest this sensitivity of CaMKII to the temporal pattern of the Ca^{2+} spikes could be shifted to lower frequencies—as generally observed for agonist-induced Ca^{2+} oscillations—when increasing the duration of the spikes and/or the CaM concentrations

(Dupont *et al.*, 2003a). This effect can also been obtained, or enhanced, when taking into account the presence of phosphatases (Kubota and Bower, 2001).

III. Elementary Aspects of Ca^{2+} Signaling

A. Random Opening of a Few Ca^{2+} Channels: Blips and Puffs

Ca^{2+} oscillations result from the synchronized and periodic opening of a large number of $InsP_3R$ throughout the cytoplasm. At low levels of $InsP_3$, channels open and close randomly, leading to unsynchronized, small-amplitude Ca^{2+} increases called blips and puffs (in nonexcitable cells) or sparks (in electrically excitable cells). These events, highly localized both in time and space, have been much studied in HeLa cells and *Xenopus* oocytes (Marchant *et al.*, 1999; Thomas *et al.*, 1999). Thus, the study of Ca^{2+} dynamics offers the fascinating possibility to make the link between microscopic, stochastic events and cellular, highly organized ones.

To give some idea about the scales of these so-called elementary events, recall that a Ca^{2+} blip supposed to correspond to the opening of a single $InsP_3R$ for about 3 ms would correspond to the release of ~1000 Ca^{2+} ions. Given the high buffering capacity of the cytosol, only ~30 of them would remain free and participate to the observed Ca^{2+} signal. This provokes a Ca^{2+} rise of about 40 nmol above baseline in a volume of 1 fL that lasts ~70 ms. From the weakness of the signal, it follows that it will not be able to activate by diffusion another $InsP_3R$ located a few micrometers away. Thus, logically, $InsP_3Rs$ are most often assembled in clusters (Katayama *et al.*, 1996). As a consequence, slightly larger, but still random Ca^{2+} increases can be observed at submaximal $InsP_3$ concentrations. These events, called puffs, represent Ca^{2+} increases of around 170 nM above basal level, lasting ~500 ms on average. On the basis of numerical simulations, one can estimate that puff sites consist of ~20–30 closely packed channels (Swillens *et al.*, 1999). Inside such a cluster, all $InsP_3$ receptors are exposed to the same Ca^{2+} concentration. This arrangement allows for an optimal communication between the channels of the cluster via Ca^{2+}, while providing an increase in cytosolic Ca^{2+} that could be high enough to provoke the activation of an adjacent puff site (Shuai and Jung, 2003).

The properties of these blips and puffs have been studied in many details and reviewed (Bootman *et al.*, 1995, 2002). Interestingly, these properties are highly similar in *Xenopus* oocytes (Callamaras *et al.*, 1998) and in HeLa cells (Thomas *et al.*, 1998), despite the huge differences in the dimensions of the cells. Most of these properties can be adequately reproduced by models based on the one hand on the regulatory properties of the $InsP_3Rs$ determined by

electrophysiological measurements and, on the other hand, on the characteristics of Ca^{2+} buffering and diffusion in the cytosol (Falcke, 2004; Shuai and Jung, 2003; Swillens *et al.*, 1999). An interesting exception is the observed mean duration of the Ca^{2+} puffs, which is larger than expected. To explain this discrepancy, new hypotheses need to be further investigated.

It has been shown theoretically that puff durations in agreement with experiments can be obtained when assuming that Ca^{2+} binding to the $InsP_3R$ obeys to saturable kinetics (Sneyd and Dufour, 2002; Ullah and Jung, 2006). This implies that activation at high Ca^{2+} levels is somehow limited, thus preventing the simultaneous activation of all the channels of the puff site. In this model moreover, $InsP_3$-bound $InsP_3Rs$ are sequentially activated and inactivated by Ca^{2+}. This assumption prevents the possible inactivation of $InsP_3$-bound receptors due to the high level of ambient Ca^{2+} in the puff site, even if they have not opened before; thus the global duration of activity can be longer (Ullah and Jung, 2006).

B. Possible Involvement of Mitochondria in Ca^{2+} Dynamics

Another intriguing possibility that might explain the unexpectedly long duration of puffs could be that mitochondria are somehow involved in the generation of these Ca^{2+} signals. It is long known that mitochondria can rapidly take up Ca^{2+} (Rizzuto and Pozzan, 2006). However, as the affinity of the mitochondrial Ca^{2+} uniporter is low, it has long been assumed that the latter organelles do not play an active role under physiological conditions. Later, this assumption was questioned after the observation that oxidizable substrates that energize mitochondria increase the amplitude and velocity of Ca^{2+} waves in *Xenopus* oocytes (Jouaville *et al.*, 1995). A key fact suggesting the participation of mitochondria to cytosolic Ca^{2+} dynamics is the observed close apposition between $InsP_3$-gated Ca^{2+} channels on the ER and mitochondria. These organelles are thus exposed to Ca^{2+} concentrations that are much higher than those measured in the bulk cytoplasm. In HeLa cells, for example, it was clearly shown that ER and mitochondria form a finely intertwined network (Rizzuto *et al.*, 1998). As a direct consequence of this arrangement, agonist-induced Ca^{2+} oscillations are paralleled by rapid spiking of mitochondrial Ca^{2+}. Frequency decoding in mitochondria has even been observed in hepatocytes, cardiomyocytes, and HeLa cells (Rizzuto and Pozzan, 2006).

The question, however, remains as to how Ca^{2+} handling by mitochondria affects the shape of the cytosolic Ca^{2+} signals and, more specifically, of Ca^{2+} puffs. In the case of global cytosolic Ca^{2+} spikes, this possibility has been tested by Babcock *et al.* (1997) who have monitored simultaneously cytosolic and mitochondrial Ca^{2+} after stimulation of rat chromaffin cells. Their

results clearly demonstrate that both Ca^{2+} uptake and Ca^{2+} release by mitochondria affect the shape of the depolarization-induced or bradykinin-induced Ca^{2+} spikes. Thus, inhibiting Ca^{2+} entry into mitochondria using a substance that collapses their membrane potential (CCCP) leads to a cytosolic Ca^{2+} peak of much larger temporal extent. As a consequence, mitochondrial Ca^{2+} uptake is responsible for the rapid decay of the cytosolic Ca^{2+} spike produced by Ca^{2+} entry or mobilization from the ER. On the other hand, inhibition of the Na^{+}/Ca^{2+} exchanger responsible for Ca^{2+} extrusion from mitochondria hastens the final recovery of cytosolic Ca^{2+} (Babcock *et al.*, 1997). Thus, one can conclude that, under normal conditions (i.e., in the absence of any inhibitor), mitochondria sequester Ca^{2+} during the phase of substantial release from the ER, and then release it back, accounting thereby for the slowness of the final decrease in cytosolic Ca^{2+}. This release might be related to the so-called mitochondrial Ca^{2+}-induced Ca^{2+} release that has been clearly demonstrated in Ehrlich ascites tumor cells (Ichas *et al.*, 1994). Two other studies confirm the important role of Ca^{2+} handling by mitochondria to determine the shape and characteristics of the Ca^{2+} spikes. First, using a preparation of permeabilized blowfly salivary glands, Zimmerman (2000) has clearly shown that the frequency of the $InsP_3$-induced repetitive changes of Ca^{2+} concentration in the ER is affected by the mitochondrial membrane potential. Second, it was shown in agonist-stimulated HeLa cells that there is a phase shift between Ca^{2+} oscillations in the ER and in mitochondria (Ishii *et al.*, 2006); thus, the Ca^{2+} released by mitochondria, once Ca^{2+} release by the ER has stopped, allows for priming the subsequent cytosolic Ca^{2+} spike. This would correspond in the cytosol to the "pacemaker Ca^{2+} rise" that has long been observed in many cell types during agonist-induced Ca^{2+} oscillations (Berridge and Dupont, 1994).

In contrast to these many studies related to the effect of Ca^{2+} handling by mitochondria on Ca^{2+} oscillations, there is to our knowledge no report on the impact of mitochondria on elementary Ca^{2+} signals. This might, however, be an important issue to understand the exact temporal shape of Ca^{2+} blips and puffs and thereby the transition from puffs to Ca^{2+} waves when increasing the concentration of $InsP_3$ (see Section IV.B).

IV. Intracellular Ca^{2+} Waves

A. General Aspects

The elementary aspects of Ca^{2+} release (blips, puffs, sparks) are only observed at rest, or at very low level of stimulation (i.e., when the concentration of $InsP_3$ is so weak that only a few receptors are activated). At higher

stimulation levels, Ca^{2+} is seen to increase either simultaneously in the whole cell or as a wave. In the latter case, Ca^{2+} first increases locally, and this Ca^{2+} rise then invades the whole cell at an approximately constant rate. These spectacular waves can adopt a variety of shapes depending on various factors, but the patterns are deterministic and reproducible, in contrast with the random character of the elementary events. Depending on the size of the cell and on the kinetics of the various Ca^{2+} fluxes, the waves can appear as a sharp front (cardiac cells, neutrophils, astrocytes, *Xenopus* oocytes, etc.) or as a tide (hepatocytes, mammalian eggs, pancreatic acinar cells, endothelial cells, etc.). In the latter case, the Ca^{2+} increase propagates through the whole cell before a roughly homogeneous return to the basal level, whereas in the former case, a sharp band of high Ca^{2+} passes through the cell. Both types of waves can, however, be accounted for by the same mechanism. In most cases, it seems that the Ca^{2+} waves are due to the propagation in an excitable cytosol of a Ca^{2+} pulse that is emitted periodically and locally (Lechleiter and Clapham, 1992). In other words, one specific region of the cell acts as an oscillator (governed by the regulations listed in Section II), and this local Ca^{2+} rise sequentially activates the release of Ca^{2+} from adjacent regions. This type of mechanism of wave propagation is classical, both in chemistry and in biology, and has been extensively investigated to understand the propagation of electrical excitation in nerve cells (Keener and Sneyd, 1998). Thus, features such as the annihilation of fronts propagating in opposite directions or the simultaneous decrease in period and propagation rate can be ascribed to the generic properties of waves propagating in excitable media.

One basic assumption underlying this hypothesis is that one specific region of the cell acts as the oscillator and is thus more sensitive to $InsP_3$ than the rest of the cell. Following this hypothesis, the Ca^{2+} wave must always originate from the same locus inside the cell. This reproducibility of the wave initiation site has, however, only been reported in a limited number of cases and certainly depends on the experimental conditions. Circumventing this discrepancy, other studies have proposed that Ca^{2+} waves correspond to phase waves: in this case, the whole cell is in an oscillatory (and not just excitable) state, but due to some spatial heterogeneity, which may be random, the different regions of the cell are slightly phase-shifted with respect to one another. This results in the appearance of a phenomenon of wave propagation although no matter (neither Ca^{2+} nor $InsP_3$) needs to be transported (Jafri and Keizer, 1994). It is most likely that both mechanisms can be found, depending on the cell type and on the level of stimulation. Low levels of stimulation would correspond to the propagation of fronts in an excitable medium (because only the most sensitive region are oscillatory), whereas higher levels of stimulation would induce phase waves.

B. From Ca^{2+} Puffs to Ca^{2+} Waves

From a mechanistic point of view, oscillations are linked to waves. Temporal and spatial organizations indeed rely on the same regulatory phenomena (Keener and Sneyd, 1998). Thus, in the case of Ca^{2+} dynamics, the basic mechanisms that can account for oscillations, whatever they are (Ca^{2+}-induced Ca^{2+} release, PLC stimulation by Ca^{2+}, G-protein activation, etc.), can also account for the existence of Ca^{2+} waves if diffusion is taken into account.

The understanding of the link between microscopic Ca^{2+} dynamics (blips and puffs) and Ca^{2+} waves represents a much more difficult task. In experiments, it is clear that an increase in the level of external stimulation (HeLa cells; Thomas *et al.*, 1999) or of $InsP_3$ (*Xenopus* oocytes; Sun *et al.*, 1998) transforms a Ca^{2+} signal resembling asynchronous flickering (blips and puffs) into coordinated oscillations and waves. It looks as if once the number of $InsP_3Rs$ that have bound $InsP_3$ and are thus susceptible to release Ca^{2+} becomes sufficient, their Ca^{2+} releasing activity becomes coherent and, even more surprisingly, periodic.

The molecular mechanisms allowing for this puff to wave transition have been extensively investigated by M. Falcke in a very innovative way (Falcke, 2003a,b, 2004; Falcke *et al.*, 2000; Thule and Falcke, 2004). This work has led to rather new hypotheses as to the mechanisms of propagation of biological waves. This analysis is based on experimental observations performed in *Xenopus* oocytes (Sun *et al.*, 1998). One basic observation is that a single Ca^{2+} puff is not able to trigger a Ca^{2+} wave and that initiation of such a wave depends on an increase in the frequency of Ca^{2+} puffs rather than in their amplitude. It looks as if several, nearby clusters need to become active together to create a local elevated average Ca^{2+} around these clusters, which can in turn initiate a wave of activation of the $InsP_3$-bound $InsP_3Rs$ within the cell. Based on stochastic numerical simulations of the De Young-Keizer model (De Young and Keizer, 1992), Falcke was able to describe the transition from puffs to low frequency, large amplitude waves and further to fast oscillations on an elevated background when increasing stimulation. One crucial assumption to get this sequence of events is that of the existence of so-called focal sites made of ~15 puff sites very close to one another (mean distance ~3 μm), each puff site containing 25 $InsP_3Rs$. Diffusion is not simulated explicitly but, to save computing time, is taken into account by the assumption of the superposition of single cluster profiles (Falcke, 2003a; Falcke *et al.*, 2000).

In this context, a wave is initiated when, by chance, a few clusters in or close to a focal site switch on (nearly) simultaneously, so that the resulting local Ca^{2+} increase is high enough to sequentially activate the other release sites (or puff sites) inside the cell. This process has been called nucleation. As expected, the nucleation probability is much smaller than the puff probability, and

the curvature of the nucleus determines the probability that it will grow. The relative regular periodicity of the waves arises from the large number of puff sites: statistically, in a distribution of $InsP_3Rs$ clusters mimicking that of a large cell such as a *Xenopus* oocyte, the opening of about 10–20 nearby puff sites will occur at roughly regular intervals. The regularity increases with the number of sites susceptible to becoming active and strongly depends on the strength of the spatial coupling, that is, on the distance between the clusters, the diffusion properties of the buffers, and so forth. This completely new type of description of the Ca^{2+} dynamics also allows for a plausible explanation for the long periodicity of the oscillatory waves as compared to the intrinsic time scales of the $InsP_3R$ (see Section II.B). As the time interval between two successive waves largely consists in the delay before the simultaneous opening of a sufficient number of neighboring puff sites, it is unrelated to the kinetics of the $InsP_3R/Ca^{2+}$ channel.

If one goes a step further, these results (Falcke, 2004) draw attention to the fact that the deterministic description used in most models (and in fact in most verbal analysis) is not valid for many aspects of Ca^{2+} dynamics. The latter description indeed ignores fluctuations, which play a primary role in the generation of Ca^{2+} oscillations from Falcke's point of view. A different theoretical approach converges toward the same conclusion. Using a standard tool to convert ordinary differential equations into stochastic equations (known as the Gillespie method), Kummer *et al.* (2005) have shown that the transition between a deterministic and a stochastic behavior for Ca^{2+} dynamics occurs within a range of particular numbers, which roughly correspond to the typical number of receptors and channels in a cell. The robustness of the deterministic oscillations, however, depends on various parameters as, for example, the Ca^{2+} buffer capacity.

C. Fertilization Ca^{2+} Waves

In eggs, the fertilizing sperm evokes a Ca^{2+} increase essential for egg activation and embryonic development. The precise factor, responsible for triggering this Ca^{2+} increase, called sperm factor, remains to be established in most species (Kurokawa *et al.*, 2004). In mammals, it has been shown to be a specific isoform of the phospholipase C, namely PLCζ (Saunders *et al.*, 2002). The first wave, called the fertilization wave, has the highest amplitude and longest duration. It is necessary for the block to polyspermy as well as for egg activation and resumption of the cell cycle. In some species (among which an important category is mammals), it is followed by repetitive Ca^{2+} waves of lower amplitude and shorter duration. The fertilization Ca^{2+} wave emanates from the point of sperm-egg fusion and traverses the entire egg at a rate of the order of 5–10 μms^{-1} in nearly all species.

The shape of this wave has been extensively studied in *Xenopus* oocytes where it presents a number of intriguing features (Fontanilla and Nuccitelli, 1998). In particular, the front is initially concave, suggesting that the wave moves faster around the cortex than through the center of the egg. This observation implies that there is some heterogeneity in the mature egg at the level of either ER distribution or $InsP_3$ production (Bugrim *et al.*, 2003; Fall *et al.*, 2004; Hunding and Ipsen, 2003; Yi *et al.*, 2005). Moreover, it is difficult to conceive that the Ca^{2+} wave can cross over the whole egg (whose diameter is 1 mm) without a regenerative production of $InsP_3$. As the $InsP_3$ diffusion coefficient equals 220 $\mu m^2/s^{-1}$ and as the wave takes a few minutes to cross the egg, one can estimate that a significant increase of $InsP_3$ would arise in only 1/6 of the egg if passive diffusion were the only mechanism transporting $InsP_3$. Thus, it is highly probable that a Ca^{2+}-activated production of $InsP_3$ occurs in the cortex of the egg. A wave of $InsP_3$ would thus accompany the fertilization Ca^{2+} wave (Fall *et al.*, 2004), a hypothesis that remains to be confirmed experimentally.

An even more complex situation is encountered at fertilization of ascidian eggs. Ascidians share with mammals, starfishes, molluscs, and a few other species the property to display repetitive cytosolic Ca^{2+} transients at fertilization (Dumollard *et al.*, 2004; Stricker, 1999). The first Ca^{2+} wave drives egg activation, but the following ones are required for completion of the meiotic cell cycle and for embryo development (Ozil, 1998). Ascidian eggs moreover have the unique property of displaying two series of Ca^{2+} waves. Series I Ca^{2+} waves consist of the large amplitude fertilization wave, followed by a few smaller spikes. These last about 8 min and drive meiosis I up to the extrusion of the first polar body. After a pause of about 5 min, Ca^{2+} waves then resume with a progressively increasing and then decreasing amplitude. Series II lasts for 15–20 min, drives meiosis II, and stops just before the extrusion of the second polar body. Distinct subcellular regions that repetitively initiate these diverse types of Ca^{2+} waves have been identified (Dumollard and Sardet, 2001; Dumollard *et al.*, 2002; McDougall and Sardet, 1995). Three such regions, called calcium wave pacemakers, have been reported. The fertilization wave initiates at the point of sperm entry, while the initiation sites of the subsequent waves progressively migrate with the sperm aster toward the vegetal contraction pole, a cortical constriction of 15–20 μm in diameter. It is a region of dense ER and mitochondria accumulation. Actually, an artificial pacemaker located in the animal hemisphere is visible when stimulating the egg directly with $InsP_3$. It probably corresponds to a region rich in ER clusters, present around the meiotic spindle in the mature unfertilized egg (Dumollard and Sardet, 2001).

A careful analysis of these experimental data, together with numerical simulations, can lead to some precise predictions as to the ER distribution and the characterization of the sperm factor in this cell type (Dupont and Dumollard, 2004). Thus, it appears there are most probably two gradients of

ER density in the ascidian egg: one along the animal–vegetal axis and one from the cortex to the center of the egg. This could explain the precise shape of the fertilization wave, which propagates faster underneath the membrane than in the center of the egg. Moreover, it accounts for the artificial pacemaker observed after the direct release of $InsP_3$ or its nonmetabolizable analog $gPIP_2$ into the egg. Whatever the locus of $gPIP_2$ increase, the Ca^{2+} wave generated by this stimulus always originates from the same subcortical region of the animal pole. As to the sperm factor (whose nature is still unknown in the ascidian egg), the study (Dupont and Dumollard, 2004) shows that it needs to be Ca^{2+}-sensitive, for reasons similar to those evoked here in the case of the regenerative production of $InsP_3$ in *Xenopus* oocytes. Otherwise, simulations would predict the existence of spatially restricted Ca^{2+} waves (i.e., propagating solely in the regions where $InsP_3$ is high because the sperm factor has significantly diffused), which are never observed experimentally. That the sperm factor would be the same as in mammalian eggs, namely, the Ca^{2+}-sensitive PLCζ (Saunders *et al.*, 2002), is, however, doubtful given the rather low diffusion coefficient of the latter protein. Given that the diameter of an ascidian egg (~150 μm) is about twice as large as that of a mammalian egg, a similar Ca^{2+} wave pattern requires a larger diffusibility of the sperm factor.

A very interesting problem that still needs to be solved is that of the relocalization of the pacemaker during the first series of Ca^{2+} waves, that is, the movement of the origin of the waves from the animal to the vegetal pole. Interestingly, although the temporal pattern of Ca^{2+} release seen at fertilization can be exactly reproduced by the injection of $gPIP_2$, the spatial characteristics of both Ca^{2+} responses are very different: in the case of $gPIP_2$ release, the second and successive Ca^{2+} waves emanate from the artificial pacemaker, whereas, as previously mentioned, in the case of fertilization, the initiation site of the successive Ca^{2+} waves progressively relocate toward the vegetal pole. There is thus obviously a fertilization-related event—presumably not mediated by Ca^{2+}—that dictates the spatial characteristics of the late fertilization Ca^{2+} spikes of series I and that needs to be discovered.

V. Intercellular Ca^{2+} Waves

A. General Aspects

In organs or in isolated multicellular systems, Ca^{2+} waves are not restricted to the cytosol of one cell but propagate, via an appropriate Ca^{2+} signal communication pathway, toward other cells as intercellular Ca^{2+} waves (Rottingen and Iversen, 2000). Intercellular Ca^{2+} waves propagate over a time scale of several seconds and may involve tens to hundreds of cells,

depending on the stimulus strength and the propagation mechanism involved (Sanderson *et al.*, 1994). They have been observed in a broad variety of cell types, for example in glial cells (Newman and Zahs, 1997; Scemes and Giaume, 2006), bone cells (Jorgensen, 2005), hepatocytes (Nathanson *et al.*, 1995; Robb-Gaspers and Thomas, 2005), pancreatic cells (Yule *et al.*, 1996), various types of epithelia (Isakson *et al.*, 2003; Nihei *et al.*, 2003), endothelia (Burdyga *et al.*, 2003; Gomes *et al.*, 2006; Vandamme *et al.*, 2004) and smooth muscle cells (Hennig *et al.*, 2002; Young *et al.*, 2002). Ca^{2+} waves not only propagate between cells of the same type (homotypic Ca^{2+} waves) but also (at least in culture systems) between different cell types (heterotypic Ca^{2+} waves), such as between meningeal cells and astrocytes (Grafstein *et al.*, 2000), astrocytes and endothelial cells (Leybaert *et al.*, 1998), astrocytes and Müller cells (Newman, 2001), and osteoblasts and osteoclasts (Jorgensen, 2005) to name some examples. In the brain, the electrically nonexcitable astrocytes communicate Ca^{2+} signals among each other, a signaling cascade that is activated by neuronally released glutamate acting on metabotropic glutamate receptors located on astrocytes (Fiacco and McCarthy, 2006). The importance of astrocytic calcium signals in the brain is related to their central position between the information processing neurons and the vascular cells that control energy substrate delivery to the parenchyma. First, astrocytic Ca^{2+} signals may influence the synaptic signal transmission because astrocytes can release glutamate in response to a cytoplasmic Ca^{2+} increase (Haydon and Carmignoto, 2006). Second, astrocytic Ca^{2+} signals may influence brain energy metabolism that is compartmentalized and divided between astrocytes and neurons (Charles, 2005). Third, astrocytic Ca^{2+} signals may influence the vascular cells (i.e., smooth muscle and endothelial cells) to modulate blood vessel diameter and transports across the blood-brain barrier (Leybaert, 2005; Takano *et al.*, 2006). In this section, we will first consider the two fundamental mechanisms of Ca^{2+} wave propagation, via gap junctions (Section V.B) or paracrine signaling (Section V.C), followed by considerations on signal regeneration in both modes of wave propagation (Section V.D) (see Fig. 4). Table II gives an overview of modeling studies that have contributed to improving our insights in the various modes of Ca^{2+} wave propagation. Finally, Section V.E describes selected findings on Ca^{2+} wave propagation in *in situ* and *in vivo* systems, while the last section discusses intercellular Ca^{2+} wave propagation and function in the liver (Section V.F).

B. Intercellular Ca^{2+} Waves and Gap Junctions

Gap junctions offer the most direct connection between the cytoplasm of two adjacent cells, and the finding at the end of the 1980s that second messengers such as $InsP_3$ (molecular weight 486 Da) permeate through gap junction

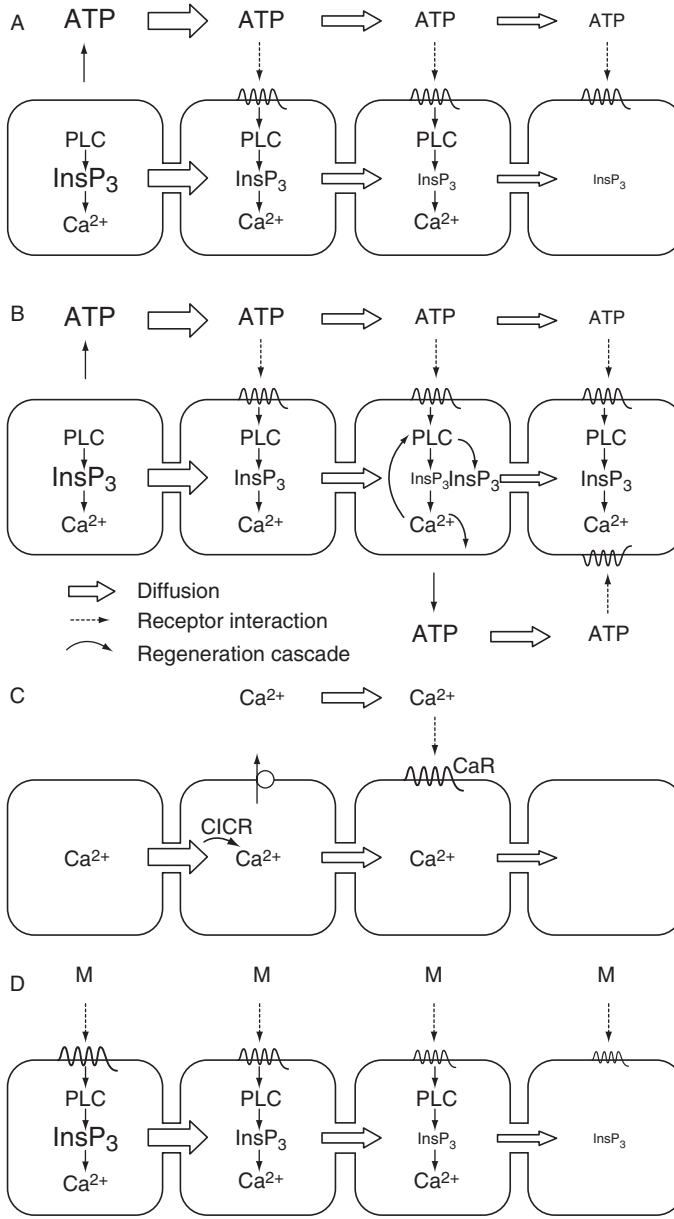

FIG. 4 Overview of possible mechanisms for intercellular Ca^{2+} wave propagation. (A) Passive waves can be communicated via intracellular or extracellular messengers such as $InsP_3$ or ATP diffusing via gap junctions (the connections between cells) or in the extracellular space. They are based on a concentration gradient that decreases away from the stimulated cell (left cell). (B) Passive waves can be supported by messenger regeneration that increases the propagation distance. Ca^{2+} can activate Ca^{2+}-dependent PLC isoforms to generate more $InsP_3$ and can also

channels has instigated extensive research on the mechanisms of Ca^{2+} wave propagation (Saez *et al.*, 1989). One of the early observations on intercellular Ca^{2+} waves came from experiments on airway epithelial cells (Sanderson *et al.*, 1988). These cells are equipped with cilia that change their beat frequency in function of the cytoplasmic Ca^{2+} concentration (Lansley and Sanderson, 1999), thus offering an elegantly simple system to monitor Ca^{2+} changes inside the cells. Careful mechanical stimulation of the plasma membrane of a single airway epithelial cell in a confluent monolayer culture, with a small glass pipette, triggered an increase of the ciliary beat frequency that radially spread from the stimulation point and propagated several cell diameters away (typically in the range of 1–10 cells). Further work with fluorescent Ca^{2+} reporter dyes, various experimental conditions, and pharmacological antagonists demonstrated a pathway involving activation of PLC in the stimulated cell, production of $InsP_3$, its diffusion in the cytoplasm, permeation of gap junction channels, diffusion in the cell at the *trans* side of the junction, and finally the triggering of Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores (Sanderson, 1995, 1996) (Fig. 4A). Intercellular Ca^{2+} waves that rely on $InsP_3$ diffusion are sometimes called diffusive Ca^{2+} waves. They involve the installation of an $InsP_3$ concentration gradient in the cytoplasm of rows of connected cells that decreases away from the stimulation point and provokes Ca^{2+} changes (and wave propagation) as long as the $InsP_3$ concentration is above the threshold for $InsP_3$-triggered Ca^{2+} release. Waves that exclusively rely on the movement of $InsP_3$ via gap junctions propagate over a limited distance, generally not much more than 2–4 cells (Braet *et al.*, 2001, 2003; Fry *et al.*, 2001), although this obviously depends on the amount of $InsP_3$ generated and the degree of gap junctional coupling. Diffusive Ca^{2+} waves will be called here *passive* waves, analogous to the passive spread of electrotonic subthreshold electrical signals.

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activate Ca^{2+}-triggered ATP release via vesicular or hemichannel pathways. ATP-triggered ATP release is also possible (not shown). (C) Ca^{2+} movement via gap junctions can sustain Ca^{2+} wave propagation if the intracellular space is excitable enough to support Ca^{2+}-induced Ca^{2+} release (CICR), which effectively regenerates the Ca^{2+} signal. Recovery from a cellular Ca^{2+} increase involves Ca^{2+} reuptake in the stores but also the pumping of Ca^{2+} out of the cell. This may locally increase the extracellular Ca^{2+} concentration, thereby activating Ca^{2+}-sensing receptors (CaR) on adjacent cells. CaR are G-protein–coupled receptors that on their turn activate Ca^{2+} release from the stores. This cascade is likely to proceed in the intercellular narrow clefts that separate the cells but is illustrated for clarity in the bulk of the extracellular space. (D) Phase waves are based on an $InsP_3$ concentration gradient installed over several coupled cells that trigger Ca^{2+} oscillations with slightly different frequencies in each cell. The $InsP_3$ concentration gradient is brought about by a receptor density gradient on the plasma membranes, depicted as progressively smaller serpentine receptors. The extracellular messenger (M) is not specified here and is assumed to be applied at a constant concentration for simplicity.

TABLE II
Overview of Simulation Studies on Intercellular Ca^{2+} Waves

Communication pathway	Fig. 3 Panel	Insights	Model D or S	References
$InsP_3$ and GJ	A	$InsP_3$ passage via GJ, indications for the necessity of $InsP_3$ regeneration	D	(Sneyd *et al.*, 1994)
$InsP_3$ and GJ	A	Considerations on the amount of $InsP_3$ necessary to obtain waves and on the minimal GJ permeability to sustain waves	D	(Sneyd *et al.*, 1995a,b)
$InsP_3$ and GJ	A	Considerations on the diffusion of $InsP_3$ or Ca^{2+} via GJ, interplay between Ca^{2+} waves and Ca^{2+} oscillations	D	(Sneyd *et al.*, 1998)
$InsP_3$ and GJ	A + B	Role of $InsP_3$ regeneration, importance of intracellular Ca^{2+} store loading, considerations on $InsP_3$ permeability of GJ	D	(Hofer *et al.*, 2002)
$InsP_3$ and GJ + PC via ATP	A + B	Long-range Ca^{2+} waves obtained without messenger regeneration	S	(Iacobas *et al.*, 2006)
GJ, various messengers		Electrical coupling is faster and stronger than Ca^{2+} coupling in pancreatic β-cells; also applies for other cell types (Dora *et al.*, 2003)	D	(Tsaneva-Atanasova *et al.*, 2006)
PC via ATP	B	Role of Ca^{2+}-dependent and Ca^{2+}-independent ATP release	D	(Stamatakis and Mantzaris, 2006)
PC via ATP	B	ATP-triggered ATP release via Ca^{2+}-independent mechanisms	D	(Bennett *et al.*, 2005)
Ca^{2+} and GJ	C	GJ Ca^{2+} permeability necessary to obtain Ca^{2+} waves decreases when cytoplasmic Ca^{2+} diffusitivity decreases	D	(Hofer *et al.*, 2001)
PC via extracellular Ca^{2+}	C	Entrainment of Ca^{2+} oscillations in neighbor cells with Wenckebach-like phenomena	D + S	(Gracheva and Gunton, 2003)
PC via extracellular Ca^{2+}	C	Entrainment of Ca^{2+} oscillations in neighbor cells	D	(Kepseu and Woafo, 2006)
$InsP_3$ and GJ	D	Interplay of cell surface receptor stimulation and GJ to obtain phase waves in hepatocytes	D	(Dupont *et al.*, 2000b)

*D, Deterministic model; S, Stochastic model; GJ, gap junctions; PC, paracrine communication.

Intercellular Ca^{2+} waves relying on gap junctional communication have now been demonstrated in multiple cell types, including epithelial, endothelial, glial, bone, liver, pancreas, and cancer cells, next to many other mainly electrically nonexcitable cells. Mechanical stimulation has been used as an easy trigger for intercellular Ca^{2+} waves, but mechanistically, it is more interesting to locally apply biochemically defined triggers. Microinjection is one possibility, but localized photo-activation of inactive precursors by flash photolysis is a more elegant approach. Microinjection or flash photolysis of $InsP_3$ has convincingly demonstrated the role of $InsP_3$ in Ca^{2+} wave propagation between cells (Boitano *et al.*, 1992; Churchill and Louis, 1998; Fry *et al.*, 2001; Leybaert *et al.*, 1998; Niessen and Willecke, 2000). Other possible messengers include cyclic ADP ribose that has a molecular weight of 541 Da and is able to trigger small intercellular Ca^{2+} waves in lens cells (Churchill and Louis, 1998) and astrocytes, although with slower kinetics as compared to $InsP_3$-based waves (Leybaert and Sanderson, 2001). The role of calcium ions diffusing themselves via gap junctions is discussed further in Section V.D. Gap junctions also contribute to another type of waves that have been called phase waves. In this case, the junctional diffusion helps to establish a concentration gradient over multiple rows of cells, thereby tuning each cell to a slightly different oscillation frequency, according to the coupled oscillator model (Dupont *et al.*, 2000b). If these cells are equipped with a gradient of decreasing G-protein–coupled receptor density, then the global exposure to a single agonist concentration may rapidly install the $InsP_3$ concentration gradient (equilibrated between cells by the gap junctions) and give the impression of a propagating wave because of the slight phase difference between neighboring oscillating cells (Fig. 4D). This kind of intercellular wave propagation has been thoroughly investigated in hepatocytes and is discussed in Section V.F.

C. Intercellular Ca^{2+} Waves and Paracrine Signal Communication

Next to junctional communication, Ca^{2+} waves may also be communicated between cells by paracrine signaling, involving the release of a messenger, its diffusion in the extracellular space, binding of the substance to a receptor, and activation of a signaling cascade that ultimately increases cytoplasmic Ca^{2+} (Fig. 4B). These waves are also diffusive in nature, given that the messenger diffuses outside the cells. The extracellular messenger is in most cases ATP or glutamate (Arcuino *et al.*, 2002; Guthrie *et al.*, 1999; Hassinger *et al.*, 1996; Newman, 2001; Parpura *et al.*, 1994; Scemes, 2000; Schlosser *et al.*, 1996; Verderio and Matteoli, 2001; Ye *et al.*, 2003), but other messengers such as nitric oxide (NO) (Willmott *et al.*, 2000) have also been

implicated. ATP and glutamate liberation by the cells has been best documented to occur via vesicular mechanisms, that is, via the process of exocytosis (Bezzi *et al.*, 2004; Bodin and Burnstock, 2001b; Knight *et al.*, 2002; Montana *et al.*, 2006), but alternative pathways also exist, such as by diffusion through large pore channels formed by either connexin hemichannels, pannexin hemichannels, or P2X7 receptor channels (Evans *et al.*, 2006; Locovei *et al.*, 2006a; Suadicani *et al.*, 2006; Tran Van Nhieu *et al.*, 2003).

The downstream effects of these messengers leading to cytoplasmic Ca^{2+} changes may involve Ca^{2+} entry via plasma membrane channels (Cornell-Bell *et al.*, 1990), but activation of G-protein–coupled receptors with activation of PLC and subsequent generation of $InsP_3$ is the more common and best-characterized mode (Gallagher and Salter, 2003; Gomes *et al.*, 2005; Piazza *et al.*, 2006; Scemes, 2000; Scemes *et al.*, 2000). Just like passive Ca^{2+} waves based on $InsP_3$ permeation through gap junctions, paracrine communicated Ca^{2+} waves rely on the release of the messenger and the installation in the extracellular space of a concentration gradient away from the stimulation point. ATP is currently the messenger that appears to be involved in most cell types, including hepatocytes, keratinocytes, mast cells, bone cells, and various types of epithelial, endothelial, and glial cells (Arcuino *et al.*, 2002; Frame and de Feijter, 1997; Gomes *et al.*, 2005; Henriksen *et al.*, 2006; Koizumi *et al.*, 2004; Newman, 2001; Osipchuk and Cahalan, 1992; Scemes *et al.*, 2000; Schlosser *et al.*, 1996; Vandamme *et al.*, 2004; Zhang *et al.*, 2003). Obviously, the receptor repertoire is important in shaping the characteristics of the Ca^{2+} wave, and waves based on P2Y1 or P2Y2 receptors differ in their propagation distance because of different sensitivities to ATP (Scemes *et al.*, 2000; Suadicani *et al.*, 2004). In addition to the receptor subtype, other factors determine in a complex manner the wave characteristics: the possible sensitivity of the downstream receptors to ATP degradation products like ADP (acting mainly on P2Y1 receptors), the kinetics of ATP and ADP formation and degradation (Iacobas *et al.*, 2006), and last but not least, the interaction with the gap junctional pathway (Suadicani *et al.*, 2004). In most cases, wave propagation is actually sustained by both the gap junctional and paracrine pathway (Fam *et al.*, 2000; Frame and de Feijter, 1997; Henriksen *et al.*, 2006; Jorgensen *et al.*, 1997; Scemes *et al.*, 2000; Suadicani *et al.*, 2004), with $InsP_3$ not only diffusing via gap junctions, but also triggering ATP release via Ca^{2+}-dependent or independent mechanisms (Braet *et al.*, 2003; Wang *et al.*, 2000).

Ca^{2+} waves mediated by gap junctions or paracrine signaling differ in several ways. The propagation velocity of both propagation modes is often in the same range (around 10–20 μm/s), presumably because the molecular weights (and thus diffusion constants) of $InsP_3$, ATP, and glutamate are all in the same range: 486, 507, and 292 kDa, respectively. Gap junction-based waves typically experience some delay when passing from one cell to another,

in the order of 0.5–1 s, related to the time it takes to build up enough $InsP_3$ at the *trans* side of the gap junction to reach the threshold for $InsP_3$ receptor activation. It has been elegantly demonstrated that these waves cross the cell border exactly at the location of the gap junction plaque (an array of gap junction channels) connecting the two cells (Paemeleire *et al.*, 2000). Gap junction-based Ca^{2+} waves sometimes follow tortuous pathways (even in confluent cultures) because of heterogeneities in the degree of coupling between cells. Paracrine communicated Ca^{2+} waves propagate in a more homogenous manner (at least in the *in vitro* setting) that may, however, be altered by heterogeneities in plasma membrane receptor densities. Individual cells in the preparation sometimes even lack the appropriate receptors and thus do not exhibit any Ca^{2+} increase. Waves based on paracrine communication are able to cross cell-free zones and can be deflected by convective flows (Hassinger *et al.*, 1996; Paemeleire and Leybaert, 2000). If recorded at high temporal and spatial resolution, differences may also be observed at the subcellular level: in the case of gap junction waves, the Ca^{2+} signal in the cell at the *trans* side of the junction is generated almost "at the mouth" of the junctional channels where $InsP_3$ flows in from a neighbor cell at the *cis* side, whereas in the case of paracrine communication, the first Ca^{2+} signal activity can be observed in the perinuclear endoplasmic reticulum (Paemeleire *et al.*, 2000).

It should be noted that a less classical mode of Ca^{2+} wave propagation based on paracrine signaling has also been reported. This mechanism relies on the pumping of Ca^{2+} out of the cell during the off-phase of the cytoplasmic Ca^{2+} transient (Hofer *et al.*, 2000). The resulting local elevation of the extracellular Ca^{2+} concentration on its turn acts on Ca^{2+}-sensing receptors in the plasma membranes of neighbor cells, triggering activation of PLC, formation of $InsP_3$ and release of Ca^{2+} (Fig. 4C). This kind of communication was observed in HEK293 cells transfected with the Ca^{2+}-sensing receptor, but given that this receptor is present in various cell types, this propagation mode might be involved in other cell types as well.

Passive intercellular Ca^{2+} waves, mediated either by an intracellular or extracellular messenger, may also involve an *active* component, meaning that the intracellular or extracellular messenger may be regenerated by the cells. Aspects related to messenger regeneration are discussed in the next section.

D. Intercellular Ca^{2+} Waves and Messenger Regeneration

Intercellular Ca^{2+} waves communicated via gap junctions or extracellular messengers are both based on passive (diffusive) mechanisms. In some cases, however, these waves may be supported by regenerative steps that can substantially alter the wave propagation characteristics. We have discussed

in Sections II.A and IV.A that intracellular Ca^{2+} oscillations and waves rely on a regenerative process known as Ca^{2+}-induced Ca^{2+} release (CICR). At the level of intercellular signaling, the fact that CICR increases Ca^{2+} excitability opens up the possibility that Ca^{2+} itself, instead of $InsP_3$, becomes the messenger that brings over the Ca^{2+} signal via gap junctions (Hofer *et al.*, 2001) (Fig. 4C). Ca^{2+} can indeed directly permeate gap junction channels, although in various models its permeability is chosen to be 100 times less than the $InsP_3$ permeability of gap junctions (Hofer *et al.*, 2002). In addition, its diffusion in the cytoplasm is, under normal circumstances, much slower than for $InsP_3$ because of binding to immobile or slowly mobile Ca^{2+}-binding proteins (effective diffusion constant of Ca^{2+} is $\sim$20 $\mu m^2/s$ as compared to $\sim$300 $\mu m^2/s$ for $InsP_3$ [Allbritton *et al.*, 1992]; see also Section IV). In line with these considerations, several studies have indicated that under normal conditions, increasing Ca^{2+} in a single cell does not trigger substantial Ca^{2+} waves (Braet *et al.*, 2001, 2003; Clair *et al.*, 2001; Fry *et al.*, 2001; Leybaert *et al.*, 1998). Limited propagation may ensue if the amount of focally injected Ca^{2+} is large enough (Churchill and Louis, 1998). Manifest intercellular Ca^{2+} waves based on Ca^{2+} passage via gap junctions can be triggered if the basal level of $InsP_3$ is slightly elevated, which renders the cells (and $InsP_3$ receptors) more sensitive to CICR. Such conditions have been reported in pancreatic acinar cells and blowfly salivary gland cells (Yule *et al.*, 1996; Zimmermann and Walz, 1999). In principle, a high density of $InsP_3$ receptors or a very close apposition of these receptors to the gap junctions may also promote this particular mode of wave propagation (Hofer *et al.*, 2001), but clear experimental evidence is currently not available concerning this possibility. Finally, gap junctions offer a pathway for the entrainment of oscillations in adjacent cells (see references in Table II), but if the Ca^{2+} permeability of the junctions is increased, the entrainment is lost (resulting in oscillator death) (Tsaneva-Atanasova *et al.*, 2006).

A second regeneration mechanism that has already been mentioned for intracellular Ca^{2+} dynamics is the Ca^{2+}-triggered regeneration of $InsP_3$ (see Section II.B). PLCε is a Ca^{2+}-activated PLC isoform that has been implicated in gap junction-mediated waves, such that $InsP_3$ flowing in via gap junctions triggers a Ca^{2+} increase that on its turn generates Ca^{2+}-activated $InsP_3$ synthesis that boosts the build-up of $InsP_3$ concentration (Hofer *et al.*, 2002) (Fig. 4B). This results in faster and more extensive wave propagation: faster because of the more rapid $InsP_3$ diffusion due to the larger concentration difference between connected cells and more extensive because $InsP_3$ regenerates itself in subsequent cells participating in the Ca^{2+} wave. If regeneration would be complete, that is, if it elevates $InsP_3$ in each cell to the same supra-threshold level, wave propagation over infinite distances would ensue, a condition not yet experimentally encountered. In any case, regeneration results in a more uniform amplitude and propagation velocity

over the wave trajectory (fully uniform in cases of complete regeneration), in contrast to passive waves in which wave velocity decreases when the distance from the stimulation point increases. Partial $InsP_3$ regeneration via PLCε (limited regenerative signaling) has been included in some Ca^{2+} wave models, to increase the (limited) propagation distance of gap junction-based waves and thus improve the match with the experimental data (Hofer *et al.*, 2002).

Not only intracellular but also extracellular messengers can be regenerated. Hassinger *et al.* (1996) were the first to suggest extracellular messenger regeneration based on the observation that the ability of a Ca^{2+} wave to cross a cell-free lane did not depend on the location of the trigger stimulus with respect to the border of the cell-free zone (Hassinger *et al.*, 1996). The most obvious pathway for extracellular messenger regeneration is that the cytoplasmic Ca^{2+} signal produced by that particular messenger triggers its own liberation (Fig. 4B). ATP and glutamate are released by vesicular mechanisms in many cell types including astrocytes and endothelial cells (Bezzi *et al.*, 2004; Bodin and Burnstock, 2001a,b), but the Ca^{2+}-dependency of this process has not unequivocally been demonstrated and is definitely not a universal feature. ATP and glutamate liberation via hemichannels, composed of either connexins or pannexins, has also been reported to be triggered by cytoplasmic Ca^{2+} changes (De Vuyst *et al.*, 2006; Evans *et al.*, 2006; Leybaert *et al.*, 2006; Locovei *et al.*, 2006a,b). Messenger regeneration is also possible without cytoplasmic Ca^{2+} changes, and ATP-induced ATP release has been clearly demonstrated in astrocytes (Anderson *et al.*, 2004). A direct signaling loop that may underlie ATP-induced ATP release is the binding of ATP to P2X7 or P2X4 receptors with the subsequent opening of large pores that allow ATP to leave the cell (Duan and Neary, 2006). Just like PLCε-mediated $InsP_3$ regeneration, extracellular messenger regeneration is expected to result in faster and larger waves. A paper by Stamatakis and Mantzaris (2006) has quantitatively investigated the effect of ATP regeneration, for both Ca^{2+}-dependent and Ca^{2+}-independent release pathways. Ca^{2+}-dependent ATP release resulted in oscillatory behavior, spiral waves, and infinite propagation distances, whereas Ca^{2+}-independent ATP release was characterized by finite propagation (Stamatakis and Mantzaris, 2006). Bennett *et al.* (2006) have also incorporated ATP regeneration in a very elegant experimental model consisting of a linear array of astrocytes and reported propagation distances up to 600 μm (Bennett *et al.*, 2006). In addition to signal regeneration, signal degradation may also occur, and this is especially true for ATP that is prone to rapid degradation to ADP, AMP, and adenosine via ectonucleotidases (200 ms half-time for conversion to adenosine) (Dunwiddie *et al.*, 1997; Fields and Burnstock, 2006).

A special case of interest is the concept of trigger cells, a concept first demonstrated by luminometric extracellular ATP imaging that showed ATP was released as a point-source burst originating from a single cell

subsequently identified by its uptake of hemichannel-permeable fluorescent reporters (Arcuino *et al.*, 2002). Opening of connexin hemichannels that release ATP only occurs in a subpopulation of the cells, which range in the order of 10–25 % of the total cell number, depending on the cell type, culture confluence, and connexin subtype (De Vuyst *et al.*, 2006; L. Leybaert, unpublished observation). The concept of trigger cells, combined with the fact that hemichannel opening is a cytoplasmic Ca^{2+}-dependent process, opens up the possibility of hybrid Ca^{2+} waves propagation, namely, Ca^{2+} waves that involve passive propagation in zones free of trigger cells and messenger regeneration taking place if the wave encounters a trigger cell, initiating a new wave event from that point on. Such a scenario predicts saltatory Ca^{2+} wave progression, with the wave jumping between subsequent trigger cells, analogous to saltatory action potentials jumping between Ranvier nodes in myelinated axon fibers. Saltatory progression of Ca^{2+} waves has been described for intracellular waves (Keizer *et al.*, 1998) and has also been concluded for intercellular waves based on heterogeneities in P2Y1 receptor density (e.g., cells missing these receptors as already mentioned) (Iacobas *et al.*, 2006; Suadicani *et al.*, 2004).

E. Intercellular Ca^{2+} Waves: From Cultures to Living Tissues

In principle, intercellular Ca^{2+} waves allow for the transmission of local information to a global level, thereby amplifying the signal and possibly coordinating and synchronizing the function of a large group of cells. It should, however, be realized that most evidence comes from simple *in vitro* cultures of adherent cell monolayers. The question is whether Ca^{2+} waves can also be observed in preparations somewhat closer to the *in vivo* situation or at least under conditions that allow some conclusions on the existence, mechanisms, and role of Ca^{2+} waves *in vivo*. This question not only involves the cell or tissue model, but also the triggers, which may be applied at too strong an intensity not relevant for the *in vivo* situation. In addition, investigations on the role or function of intercellular Ca^{2+} waves are hampered by the fact that it is often very difficult to determine whether the communicated Ca^{2+} signal itself or other messengers, diffusing in via gap junctions or produced as a result of paracrine signaling, are involved in the responses. An interesting example is a study investigating Ca^{2+} wave propagation between the supporting cells of the organ of Corti, the structure that contains the auditory receptors. The most common form of genetic nonsyndromic deafness is associated with mutations in the gene that encodes for connexin 26, the building block of gap junctions that connect these cells. In an elegant series of experiments, Beltramello *et al.* (2005) demonstrated that cells containing the mutated connexin 26 did not propagate Ca^{2+} waves because of a

selective disturbance in the permeability of the gap junctions to $InsP_3$ (Beltramello *et al.*, 2005). The fact that neither the electrical properties, nor the passage of reporter dyes (with MW similar to $InsP_3$) via the gap junctions was affected by this mutation, strongly suggests that deficient Ca^{2+} signal communication is at the basis of this kind of deafness (Beltramello *et al.*, 2005). This study, performed in HeLa cells and isolated cochleae, illustrates that investigations on simple cell expression systems have great potential to explore the role and function of Ca^{2+} waves. Tissue slices isolated from the living brain is another preparation that allows investigation of intercellular Ca^{2+} waves in a more or less preserved tissue arrangement. Loading slices acutely prepared from various mouse brain regions with Ca^{2+} dyes shows most prominent staining in the astrocytes, allowing easy observations of the Ca^{2+} dynamics in these cells (Nimmerjahn *et al.*, 2004). Slices of rat thalamus display spontaneous astrocyte Ca^{2+} activity (oscillations) that propagates to a limited number of surrounding astrocytes (Parri *et al.*, 2001). Electrical stimulation of mouse cortical slices with a locally positioned micropipette triggers astrocytic Ca^{2+} waves characterized by a very rapid propagation because of the contribution of the neuronal network. When synaptic communication is silenced with TTX or by applying nominally Ca^{2+}-free extracellular conditions (to block presynaptic neurotransmitter release), the Ca^{2+} waves propagate more slowly (12–14 μm/s) (Haas *et al.*, 2006), a velocity that is comparable to waves recorded in astrocyte cultures. Propagation extends several 100 μm away from the stimulation point, often going beyond the field imaged with a × 20 objective lens. In neocortical slices, these waves are predominantly propagated by the gap junctional pathway, based on their significant reduction with the gap junction blocker carbenoxolone and in slices prepared from mouse with conditionally deleted connexin 43 expression in astrocytes (Haas *et al.*, 2006). The importance of gap junctional coupling was confirmed in this study by dye injection experiments that showed extensive dye spread up to 600 μm away from the injected cell. By contrast, in the CA1 region of hippocampal slices, Ca^{2+} waves were unaffected by carbenoxolone but significantly reduced by suramin, a blocker of purinergic receptors, indicating that the waves are predominantly mediated by the paracrine purinergic pathway (Haas *et al.*, 2006). This study thus confirms that the two propagation pathways observed in cultures also exist in slices, although the electrical trigger applied was strong and cannot be considered as physiological stimulation. The study furthermore demonstrated that the neocortical gap junction-dependent Ca^{2+} waves were accompanied by a wave of ATP release that propagated independently from the Ca^{2+} wave, a fact illustrating that the situation in slices is much more complex than in culture systems. An additional layer of methodological sophistication can be achieved with two-photon imaging, which allows Ca^{2+} imaging in the cortex of living anesthetized small laboratory animals up to a depth of ~400 μm (Ohki *et al.*, 2005). Several studies that have more recently become available

are directed at understanding the role of astrocytic calcium signaling in relation to neuronal function and vascular responses (Takano *et al.*, 2006; Tian *et al.*, 2005; Wang *et al.*, 2006). Long-range Ca^{2+} wavelike activity has been reported in cortical neurons and astrocytes (Adelsberger *et al.*, 2005; Garaschuk *et al.*, 2000), but the propagation is much more complex and involves the active contribution of the neuronal network and its influence on surrounding astrocytes via glutamate spillover from synapses. Manifest intercellular Ca^{2+} waves solely sustained by astrocytes as observed in culture models have not yet been described *in vivo* (Fiacco and McCarthy, 2006). Mulligan and MacVicar (2004) and Takano *et al.* (2006) have reported limited propagation of Ca^{2+} changes over several rows of astrocytic endfeet located in close apposition to the blood vessels, following focused photoactivation of Ca^{2+} in astrocytes with two photon technology. Another interesting study comes from the isolated and blood-perfused lung preparation (Parthasarathi *et al.*, 2006). Photo-activation of Ca^{2+} in a single endothelial cell of alveolar capillaries triggered Ca^{2+} changes up to 150 μm away from the stimulation site and propagated from capillaries to venules in a bidirectional manner. Propagation of the Ca^{2+} changes was absent in mouse lungs lacking connexin 43 and inhibited by peptide blockers of gap junctions. These are remarkable findings as photo-activation or micro-injection of Ca^{2+} in cultured cells, including endothelial cells, does not generally trigger propagating Ca^{2+} changes unless special conditions are applied as already discussed in Section V.D. (Braet *et al.*, 2001, 2003; Fry *et al.*, 2001; Leybaert *et al.*, 1998). This study is quite interesting for another reason: the authors addressed the question of the possible function of endothelially communicated Ca^{2+} signals. They found that the Ca^{2+} signal communicated a proinflammatory signal, characterized by increased P-selectin expression and potentiation of thrombin-induced microvascular permeability increases (Parthasarathi *et al.*, 2006). To date, this study, together with those by Mulligan and MacVicar (2004) and Takano *et al.* (2006), is one of the few to clearly demonstrate a functional role of communicated Ca^{2+} signals in the *in vivo* situation. The liver is another promising model organ to investigate the functional impact of Ca^{2+} waves and is discussed next.

F. Intercellular Ca^{2+} Waves Through Connected Hepatocytes: Implication for Liver Function

Hormonal stimulation of a group of hepatocytes, whose connections through gap junctions remain intact, induces Ca^{2+} increases that are tightly connected through the different cells. The Ca^{2+} oscillations have the same frequency, but are slightly phase-shifted with respect to one another, giving the appearance of an intercellular Ca^{2+} wave (Combettes *et al.*, 1994;

Nathanson and Burgstahler, 1992) that has been denoted as a phase wave (Fig. 4D). Such coordination was also observed in intact, perfused livers (Nathanson *et al.*, 1995; Robb-Gaspers and Thomas, 1995). Strikingly, the order in which the cells respond is always the same for a given agonist; the sequence of cellular responses is maintained when stimulation is repeated and does not depend on agonist concentration. Thus, these Ca^{2+} waves always propagate in one direction. In the intact liver, however, the nature of the starting area remains a matter of controversy (Motoyama *et al.*, 1999; Nathanson *et al.*, 1995; Robb-Gaspers and Thomas, 1995). The unidirectionality of Ca^{2+} waves can be ascribed to the gradient in cellular sensitivity to glycogenolytic agonists such as vasopressin or noradrenaline (Jungermann and Katz, 1989). In agreement with this, perivenous hepatocytes are more sensitive to vasopressin, noradrenaline and angiotensin II than periportal hepatocytes (Clair *et al.*, 2003; Tordjmann *et al.*, 1996, 1998). As these differences in Ca^{2+} responses are located upstream from the G-protein and that the binding properties of these specific extracellular receptors do not differ in the various regions of the liver lobule, it has been concluded that the gradient in sensitivity relies on a gradient in receptor density. This heterogeneity is in fact not surprising as it has also been reported for other properties of liver cells as, for example, the metabolism of carbohydrates, amino acids, and ammonia (Jungermann and Katz, 1989; Jungermann and Thurman, 1992).

The gradient of sensitivity is responsible for the existence of a constant initiation point of the intercellular wave, but not for the coordination of the Ca^{2+} signals. Indeed, Ca^{2+} peaks appear at different frequencies in a group of cells when gap junctions have been chemically inhibited, suggesting that the diffusion of an intercellular messenger may sensitize adjacent cells, thereby accelerating their response. This messenger indeed acts as an accelerator, and not as a trigger, as no Ca^{2+} increase can be obtained in an unstimulated cell, even if this is functionally connected to cells where the level of Ca^{2+} is oscillating or constantly high (Clair *et al.*, 2001; Koukoui *et al.*, 2006; Niessen and Willecke, 2000b; Tordjmann *et al.*, 1996, 1998). In other words, hormonal stimulation is absolutely required to induce Ca^{2+} *spiking* and functional gap junctions for *coordination*. The two most likely candidates that may flow through the gap junctions and coordinate the spikes are Ca^{2+} and $InsP_3$. At present, experiments do not allow a way to fully discriminate between the two plausible messengers, and the question has been approached by modeling (Dupont *et al.*, 2000b; Hofer, 1999). One of these models favors the hypothesis that $InsP_3$ would be the primary coordinating messenger. An essential role is ascribed to the gradient of hormonal sensitivity, which determines both the direction of the wave and the propagation velocity. In fact, in this model, Ca^{2+} does not really flow from one cell to the other, and

the intercellular propagation is only apparent. The different levels of $InsP_3$ induce different latencies of response; although these differences are reduced by the passage of $InsP_3$ through gap junctions, there is a slight phase-shift between the Ca^{2+} spikes among the hepatocytes of the multiplet. This sequential spiking giving the appearance of a phenomenon of wave propagation is known as phase wave. Simulations of this model allowed us to predict that coordination should be much reduced when stimulation is very low or that some level of coordination should be observed for the few first spikes even when gap junctions are inhibited. These predictions have been confirmed experimentally (Clair *et al.*, 2001; Dupont *et al.*, 2000b), thus supporting the still controversial idea that $InsP_3$ is the most important messenger diffusing through gap junctions in hepatocytes.

The liver provides an ideal system to investigate the link between the Ca^{2+} waves and the physiological response. Indeed, Ca^{2+} waves have been associated with two major functions of the liver: glucose output and bile flow. Since 1992, it is known that cell-to-cell communication via gap junctions is needed for efficient glucose release triggered by electrical stimulation of sympathetic liver nerves (Seseke *et al.*, 1992). This was confirmed soon after by the use of connexin 32-deficient mice (Nelles *et al.*, 1996). Glucose release induced by perfusion of submaximal concentrations of noradrenaline or glucagon was also significantly smaller in Cx32-deficient than in wild-type liver, showing that intercellular signal propagation through gap junctions can, at least partially, compensate the heterogeneity of hepatocytes from the periportal to the perivenous area (Stumpel *et al.*, 1998). At the same time, using a very different technical approach, Eugenin *et al.* (1998) reached a similar conclusion.

Communication via gap junctions is also involved in bile secretion. In contrast with basal bile flow which is not affected by the inhibition of gap junctional coupling, bile flow in vasopressin-stimulated liver is significantly modified (Nathanson *et al.*, 1999). It is well known that vasopressin acts on hepatocytes via Ca^{2+} increase (see earlier). However, inhibition of gap junctional coupling had no effect on bile flow in liver perfused with t-BuBHQ, a cell permeant agent which increases $[Ca^{2+}]_i$ by inhibiting ER Ca^{2+} pumps, independently from the $InsP_3$. Interestingly, when gap junction permeability was inhibited, the effect of vasopressin on bile secretion was reduced to the level observed in the presence of t-BuBHQ. Contrary to vasopressin, whose receptors are distributed heterogeneously along the liver cell plate, t-BuBHQ probably increased $[Ca^{2+}]_i$ similarly among all hepatocytes within the entire lobule. These observations thus strongly suggest that the dependency of bile secretion on gap junction is due to the gradient of the V1a vasopressin receptors and thereby, most probably, to the existence of intercellular Ca^{2+} waves.

An even more direct demonstration of the importance of Ca^{2+} waves for bile secretion has been given by Serriere *et al.* (2001). They first showed that periportal to perivenous gradient in sensitivity to vasopressin was abolished when this agonist is administered for 24 h, via intraperitoneal osmotic minipumps. This is due to a selective decrease in sensitivity to vasopressin in the perivenous hepatocytes. As a consequence, intercellular Ca^{2+} waves were impaired and increase in vasopressin-induced bile flow was much smaller in these animals, showing *without ambiguity* that receptor distribution controls intercellular Ca^{2+} wave propagation and bile flow.

Another fascinating aspect of Ca^{2+} signaling in the liver is that it could be associated with liver regeneration. Although this possible link is much more speculative, it is worth mentioning in such a review. After hepatectomy (that can reduce the organ up to one third of its volume), a rat liver can restore its initial mass within a few days. It is believed that the wounding procedure itself induces propagating Ca^{2+} waves, which are in turn responsible for the induction of a set of signaling pathways (Sung *et al.*, 2003). The exact processes occurring between wounding and Ca^{2+} increases remain to be elucidated, but it has been shown that hepatectomy is rapidly followed by vasopressin (Vp) secretion by the hypothalamus (Nicou *et al.*, 2003). Moreover, this release of hormone is involved both in the control of liver growth (leading to regeneration) and in the stimulation of bile flow (allowing the remaining part of the liver to perform a nearly normal hepatic function). On the other hand, as emphasized in the preceding section, Vp is a well-known Ca^{2+} mobilizing agent in hepatocytes that can induce repetitive Ca^{2+} waves in multicellular systems or in isolated organs. Given the well-known role of Ca^{2+} as a regulator of gene transcription (Cruzalegui and Bading, 2000) and cell cycle progression (Baran, 1996), it is logical to assume a primary role to intracellular and intercellular Ca^{2+} waves in liver regeneration.

Surprisingly, the Ca^{2+} signaling pathway in hepatocytes desensitizes 24 h after partial hepatectomy. After a temporary recovery of sensitivity, desensitization reoccurs 5 days after surgery. In both cases, desensitization relies on a decrease in the number of external AVP receptors and internal $InsP_3$ receptors. Moreover, the relative proportions of $InsP_3R$ isoforms also change (Magnino *et al.*, 2000; Nicou *et al.*, 2007). In normal conditions, hepatocytes possess about 80% of type 2 receptors and 20% of type 1. One day after hepatectomy, type 2, but not type 1, receptors are downregulated, thus altering the relative proportions of both isoforms. Interestingly, these changes in receptor densities are accompanied by a significant change in the shape of Vp-induced Ca^{2+} oscillations (Nicou *et al.*, 2007). By a mechanism that remains to be identified, this global desensitization of the Ca^{2+}-signaling pathway is related with progression through the cell cycle toward the S phase, thereby promoting liver regeneration.

VI. Concluding Remarks

This chapter was devoted to the overview of the molecular mechanisms underlying Ca^{2+} dynamics in nonexcitable cells. We have considered both temporal and spatial aspects, as well as the different scales at which Ca^{2+} signaling has been described: from less than 1 μm around a single channel to macroscopic distances related to a whole organ.

Following the chronological order of discoveries, the first section of this review was devoted to Ca^{2+} oscillations and, more especially, to the discussion of their molecular mechanism (Section II). As proposed very early (Berridge, 1993; Goldbeter *et al.*, 1990), the autocatalytic regulation by which cytosolic Ca^{2+} activates its own release from the ER, known as CICR, plays a major role in the onset of $InsP_3$-induced Ca^{2+} oscillations. Compelling evidence, however, suggests $InsP_3$ metabolism also influences Ca^{2+} oscillations in a dynamical way. Both synthesis and degradation of the former messenger are controlled by Ca^{2+} and have been shown in experiments and in models to affect the existence and characteristics of Ca^{2+} oscillations. At a more detailed level, the precise regulatory properties of the $InsP_3$ receptors—that differ between the various isoforms—also shape the global Ca^{2+} response of a given cell type to external stimulation. At the present stage, we feel that our understanding of the detailed mechanism of Ca^{2+} oscillations would benefit greatly from the long duration and concomitant imaging of various major actors of the phenomenon, such as $InsP_3$, ER, and mitochondrial Ca^{2+}. Not only the variations of these concentrations, but also the phase relationship between them is indeed highly dependent on the underlying oscillatory mechanism (Ishii *et al.*, 2006).

If the level of stimulation is too low to generate Ca^{2+} oscillations, asynchronous, small-amplitude Ca^{2+} increases can be observed (Section III). The analysis of these events, called blips or puffs depending on their amplitude, reveals information about the activity of single $InsP_3$ receptors *in vivo*. As expected from the CICR regulation, these channels are coupled via diffusion of Ca^{2+} through the cell. Because of the high buffering capacity of the cytoplasm, coupling is short-ranged. Stochastic modeling (Falcke, 2004; Swillens *et al.*, 1999) can account for most experimental observations (Bootman *et al.*, 1997; Marchant *et al.*, 1999), except for the puff duration. Alternative explanations, such as the involvement of mitochondria, need to be quantitatively investigated. We remain intrigued by the fact that only a limited number of cell types seem to exhibit Ca^{2+} blips and puffs. One might speculate that the existence of elementary events is somehow related to the arrangement of the $InsP_3$ receptors in clusters (instead of a roughly homogenous distribution) or to their possible apposition to mitochondria.

In the next section (IV), we approached one of the most challenging question related to Ca^{2+} dynamics: the link between elementary events and global Ca^{2+} signals such as oscillations and waves. Ca^{2+} signaling is one of the few examples in cell physiology in which the link between stochastic and deterministic behaviors appears so clearly in the experiments. The transition between the two regimes is seen upon an increase in $InsP_3$ concentration. Stochasticity originates from the fact that fluctuations play a primary role in systems characterized by a small number of particles. If the level of $InsP_3$ is low, most of the $InsP_3$ receptors do not participate in the Ca^{2+} dynamics. In a small cell such as a HeLa cell or a hepatocyte, the number of $InsP_3$ receptors and Ca^{2+} ions precisely lies in the range where the transition between a stochastic and a deterministic behavior is expected to occur (Kummer *et al.*, 2005). Much work remains to be done to fully understand this transition. As stated by Falcke (2004) who has extensively investigated this question, the clarification of the link between Ca^{2+} puffs and waves would also largely benefit other fields of sciences, particularly physics, where the role of fluctuations on the global behavior of the system is presently a challenging topic.

From a physiological point of view, fertilization waves represent one of the most spectacular examples of the intracellular, spatial organization of Ca^{2+} signaling. These waves, starting at the point of sperm attachment, set up the antero-posterior axis of the future organism (Dumollard *et al.*, 2004). In addition to the general principles of Ca^{2+} signaling common to other cell types, some peculiar features such as the arrangement of the organelles in the egg (Stricker, 1999) and the Ca^{2+} sensitivity of the sperm factor (Saunders *et al.*, 2002) play a major role in the regulation of these vital waves.

The last section of this review (Section V) was devoted to intercellular Ca^{2+} waves. To allow the passage of the waves from one cell to the other, organs rely either on gap junctional coupling, or on paracrine signaling or even both modes of propagation. The most visible difference between these two types of waves relates to the possible delay when passing from one cell to the other, which is only observed in gap junction-based waves. The messenger flowing through gap junction is most often $InsP_3$. The role of calcium ions diffusing themselves through these connections is more controversial. For paracrine communication, ATP is the most frequent extracellular messenger. Once released by a trigger cell, it binds to purinergic receptors of adjacent cells, where it activates the phosphoinositide pathway. If these cells in turn secrete ATP, the mechanism is said to be regenerative, and the Ca^{2+} wave could in principle propagate over infinite distances without any loss of amplitude or velocity.

That in living organisms, intercellular Ca^{2+} waves allow for the propagation of local information to a global level is confirmed by experiments performed *in vivo*. Thus, Ca^{2+} waves have been observed in entire livers

(Robb-Gaspers and Thomas, 1995) and are a main trigger for liver regeneration after hepatectomy (Nicou *et al.*, 2007). Astrocytic Ca^{2+} waves have also been reported in acutely isolated brain slices (Haas *et al.*, 2006), but their appearance and role under *in vivo* conditions is less clear. It should be realized that the *in vivo* situation is much different from the slice condition, both in terms of triggering stimuli and the washout of substances in the interstitial environment. Large Ca^{2+} waves like those observed with cortical spreading depression (Peters *et al.*, 2003) will without a doubt soon be reported *in vivo* as well, offering interesting opportunities to investigate their impact on the neighboring neurons and vascular cells. At the physiological level, Ca^{2+} signal communication may, however, well be limited to a couple of cells instead.

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