Ca^{2+} oscillations in hepatocytes do not require the modulation of $InsP_3$ 3-kinase activity by Ca^{2+}

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Abstract Receptor-mediated production of inositol 1,4,5-trisphosphate ($InsP_3$) initiates Ca^{2+} release and is responsible for cytosolic Ca^{2+} oscillations. $InsP_3$ oscillations have also been observed in some cells. One of the enzymes controlling $InsP_3$ catabolism, the $InsP_3$ 3-kinase, is stimulated by Ca^{2+}; this regulation is presumably part of the reason for $InsP_3$ oscillations that have been observed in some cells. Here, we investigate the possible role of Ca^{2+}-activated $InsP_3$ catabolism on the characteristics of the $InsP_3$-induced Ca^{2+} oscillations. Numerical simulations show that if it is assumed that the Ca^{2+}-independent $InsP_3$ catabolism is predominant, Ca^{2+} oscillations remain qualitatively unchanged although the relative amplitude of the oscillations in $InsP_3$ concentrations becomes minimal. We tested this prediction in hepatocytes by masking the Ca^{2+}-dependent $InsP_3$ catabolism by 3-kinase through the injection of massive amounts of $InsP_3$ 5-phosphatase, which is not stimulated by Ca^{2+}. We find that in such injected hepatocytes, Ca^{2+} oscillations generated by modest agonist levels are suppressed, presumably because of the decreased dose in $InsP_3$, but that at higher doses of agonist, oscillations reappear, with characteristics similar to those of untreated cells at low agonist doses. Altogether, these results suggest that oscillations in $InsP_3$ concentration due to Ca^{2+}-stimulated $InsP_3$ catabolism do not play a major role for the oscillations in Ca^{2+} concentration.

Key words: Calcium oscillation; Inositol 1,4,5-trisphosphate; $InsP_3$ 3-kinase; $InsP_3$ 5-phosphatase; Hepatocyte; $InsP_3$ oscillation

1. Introduction

Inositol 1,4,5-trisphosphate ($InsP_3$) is a widespread second messenger inducing Ca^{2+} release from endoplasmic reticulum (ER) stores. In the vast majority of cell types, the resulting rise of Ca^{2+} in the cytosol takes the form of repetitive Ca^{2+} oscillations, whose period ranges from seconds to minutes [1,2]. One of the most accepted explanations for Ca^{2+} oscillations rests on the biphasic regulation of the $InsP_3$ receptor ($InsP_3R$), which can be activated by low concentrations of Ca^{2+} and inactivated by higher concentrations of this messenger. Within the framework of this explanation, Ca^{2+} oscillations can be generated in the presence of a constant level of $InsP_3$, with the level of $InsP_3$ controlling the presence and the frequency of Ca^{2+} oscillations [3,4].

In some cell types, it has recently become possible to detect changes in $InsP_3$ levels in single cells [5–7]. The observation that in these cases Ca^{2+} and $InsP_3$ oscillate in synchrony suggests that feedbacks at the level of $InsP_3$ synthesis or/and catabolism might play a role in the regulation of Ca^{2+} dynamics. Pathways of $InsP_3$ synthesis and degradation have been well characterized [8] and are schematized in Fig. 1. Upon binding to its specific membrane receptor, the external stimulus (A) triggers the activation of receptor-associated G-proteins. This in turn stimulates a phospholipase C (PLC) which catalyzes the hydrolysis of membrane-bound phosphatidyl inositol 4,5-bisphosphate (PIP2) to form $InsP_3$ and diacylglycerol (DAG). Already at this level, $InsP_3$ oscillations could arise either through regulation of protein kinase C (PKC), a Ca^{2+}- and DAG-dependent kinase that could exert a negative feedback on the receptor–G-protein complex [9,10], or through a Ca^{2+} stimulation of PLC activity [10–12] (the latter effect does not seem to occur in hepatocytes [13,14]). These two mechanisms could generate $InsP_3$ oscillations due to negative or positive regulation of $InsP_3$ synthesis; Ca^{2+} oscillations would thus be driven by $InsP_3$ oscillations.

However, if Ca^{2+} regulates $InsP_3$ catabolism, $InsP_3$ oscillations could also be a consequence, rather than a cause, i.e. they could simply follow Ca^{2+} oscillations, themselves produced by the above-mentioned biphasic regulation of the $InsP_3R$. $InsP_3$ can be transformed either by $InsP_3$ 5-phosphatase-mediated dephosphorylation to yield inositol 1,4-bisphosphate, or by $InsP_3$ 3-kinase-mediated phosphorylation to yield inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) [15]. Note that $InsP_4$ is also a substrate for 5-phosphatase and thus acts as a competitive inhibitor of $InsP_3$ dephosphorylation. The binding of Ca^{2+}/calmodulin (CaM) to 3-kinase enhances its activity at variable extents: the A isoform of the enzyme is stimulated 2- to 3-fold by Ca^{2+}/CaM, whereas the B isoform is stimulated up to 10-fold [16,17].

Mathematical modelling [10,18,19] has confirmed the intuitive prediction that this well-characterized Ca^{2+} stimulation of $InsP_3$ catabolism can generate $InsP_3$ oscillations. Basically, one could conceive two effects of these catabolism-induced $InsP_3$ oscillations. First, an active role of these oscillations in the pacemaker mechanism of Ca^{2+} oscillations was suggested by studies performed in hepatocytes [20] and smooth

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; DAG, diacylglycerol; PKC, protein kinase C

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muscle cells [21]. It was there shown that the Ca^{2+} signal following uncaging of poorly-metabolized $InsP_3$ analogs decayed more slowly than the signal following $InsP_3$ uncaging. The interpretation of Fink et al. [21] was that $InsP_3$ degradation was a prerequisite for Ca^{2+} recovery. The control of $InsP_3$ removal by a Ca^{2+}-stimulated 3-kinase would provide an ideal mechanism for this. Second, even if $InsP_3$ oscillations are not strictly required for Ca^{2+} oscillations, one could argue that the enhanced degradation of $InsP_3$ following a Ca^{2+} spike (due to Ca^{2+}-enhanced activity of the $InsP_3$ 3-kinase) plays a role in determining the relatively low frequency of Ca^{2+} oscillations, which cannot be explained on the basis of the kinetic properties of the $InsP_3R$ [22]. In this view, each Ca^{2+} spike would provoke a decrease in $InsP_3$ so that the level of this messenger becomes too low to allow Ca^{2+} release through the $InsP_3R$. Consequently, 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.

In the present study, we investigate both theoretically and experimentally the possible role of the Ca^{2+}-controlled catabolism of $InsP_3$ in the triggering and characteristics of Ca^{2+} oscillations. We first use a previously developed theoretical model [18,22] to analyze the effect of masking the Ca^{2+}-sensitive pathway of $InsP_3$ catabolism by a Ca^{2+}-insensitive one. The model predicts that Ca^{2+} oscillations remain qualitatively unchanged, although $InsP_3$ oscillations practically disappear. This prediction is then corroborated by experiments of $InsP_3$ 5-phosphatase injection in hepatocytes.

2. Materials and methods

2.1. Preparation of hepatocytes

Isolated rat hepatocytes were prepared from fed female Wistar rats by limited collagenase (from Boehringer) digestion of rat liver, as previously described [23]. Under these conditions, about 20% of the cells recovered were associated by two (doublet) or three (triplet), and were ascertained not to be non-specific aggregates of non-connected cells by conventional light screening for dilated bile canaliculi, indicators of maintained functional polarity. After isolation, rat hepatocytes were maintained (5×10^5 cells/ml) at 4°C in Williams' medium E (Gibco) supplemented with 10% fetal calf serum, penicillin (100 000 units/ml) and streptomycin (100 μg/ml). Cell viability, assessed by trypan blue exclusion, remained greater than 96% for 4–5 h.

2.2. Measurement of intracellular Ca^{2+} in individual cells

2.2.1. Loading of hepatocytes with fura2. Hepatocytes were loaded with fura2 (Molecular Probes Inc.) by injection (see below). Small aliquots of the suspended hepatocytes (5×10^5 cells) were diluted in 2 ml of Williams' medium E modified as described above, then plated onto dish glass coverslips coated with collagen I, and incubated for 60 min at 37°C under an atmosphere containing 5% CO_2. After cell plating, the coverslips were then washed twice with a saline solution (20 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.96 mM NaH_2PO_4, 5 mM $NaHCO_3$, and 1 g/l glucose, pH 7.4). Dish coverslips were put onto a thermostated holder (36°C) on the stage of a Zeiss Axiovert 35 microscope set up for epifluorescence microscopy.

2.2.2. Microinjection. Microinjection was performed using an Eppendorf microinjector (5242), as described previously [24]. Micropipettes with an internal tip diameter of 0.5 μm (Femtotips, Eppendorf) were filled with test agents and 5 mM fura2 in a buffer solution containing 100 mM KCl, 20 mM NaCl and 10 mM HEPES adjusted to pH 7.1. After microinjection, cells were allowed to recover for at least 10 min. The success of microinjection was assessed by monitoring the morphology of cells and their ability to retain injected fura2 and to display a low $[Ca^{2+}]_i$. Cells were microinjected either with inactivated $InsP_3$ 5-phosphatase or with $InsP_3$ 5-phosphatase (activity: 120 nmol/min/ml in the pipette). Purification and determination of activity of the recombinant type I $InsP_3$ 5-phosphatase (19 μmol/min/ml in this study) were performed as described previously [25]. $InsP_3$ 5-phosphatase was inactivated at 90°C for 20 min.

Ca^{2+} imaging was described previously [24].

3. Theoretical prediction as to the role of the Ca^{2+}-stimulated $InsP_3$ catabolism

The model for Ca^{2+} oscillations [18,22] relies on the biphasic regulation of the $InsP_3R$ by Ca^{2+}, with $InsP_3$ synthesized at a constant rate (proportional to the level of stimulation) and degraded by both 3-kinase and 5-phosphatase. The 3-kinase is stimulated by Ca^{2+}, and its product, $InsP_4$, competes with $InsP_3$ for 5-phosphatase (Fig. 1). When the concentration of agonist (and thus of $InsP_3$) increases, cells typically display:

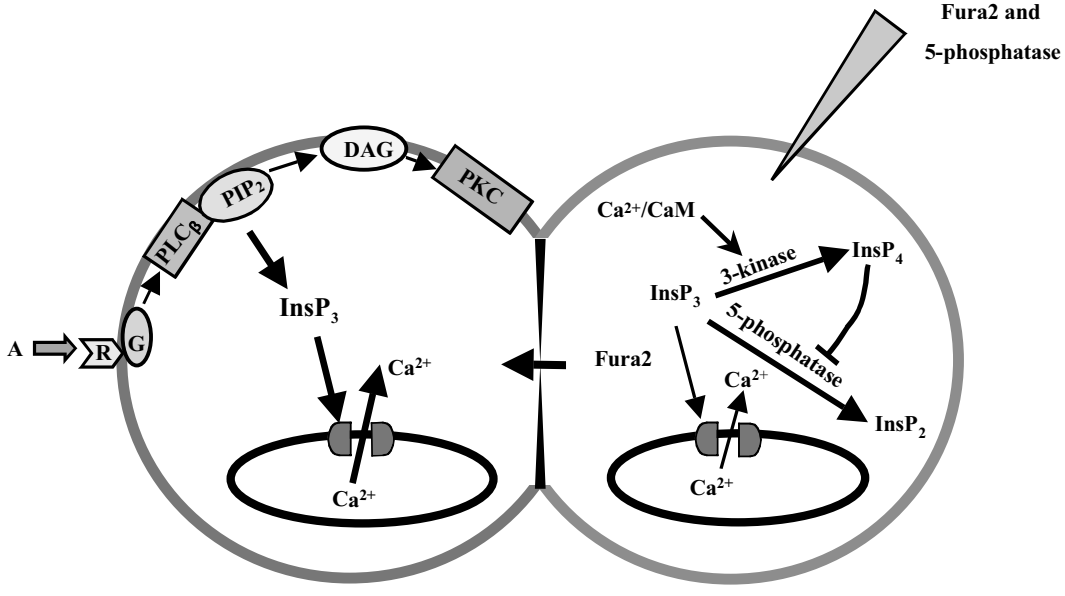

Fig. 1. Schematic representation of the intracellular Ca^{2+} dynamics and of the $InsP_3$ signalling pathway in a doublet of connected hepatocytes.

(1) low constant levels of Ca^{2+}, (2) sustained Ca^{2+} oscillations, the frequency of which increases with the agonist concentration, and (3) high sustained levels of Ca^{2+} [1–4].

Numerical simulations also show that the level of $InsP_3$ oscillates in phase with Ca^{2+} (Fig. 2A). As these oscillations rely on the Ca^{2+} stimulation of $InsP_3$ catabolism by 3-kinase, it can be expected that their amplitude would be much reduced if the relative importance of the other degradation pathway was increased. Thus, we simulated the effect of 5-phosphatase injection and assumed that the concentration of this enzyme is increased by a factor of 25. If all the other parameters of the model are kept constant, oscillations are abolished and a low constant level of Ca^{2+} is predicted, consistent with the observed reduction in the level of $InsP_3$ (Fig. 2B). If the external stimulation is then increased, Ca^{2+} oscillations are recovered, but now occur in the presence of a nearly constant level of $InsP_3$ (Fig. 2C). In this case, the average activity of the phosphatase exceeds that of the kinase by a factor of 30, while both activities were roughly the same in the normal situation corresponding to Fig. 2A.

Interestingly, these results predict that the characteristics of the repetitive Ca^{2+} spikes (shape, amplitude and order of magnitude of the period) remain similar to those obtained in response to submaximal stimulation of a cell that was not supposed to be injected with the enzyme. Thus, the model suggests that $InsP_3$ oscillations driven by Ca^{2+}-activated $InsP_3$ degradation are not essential for $InsP_3$-induced Ca^{2+} oscillations. Detailed examination of the behavior of the model shows that this lack of effect is due to (1) receptor inactivation

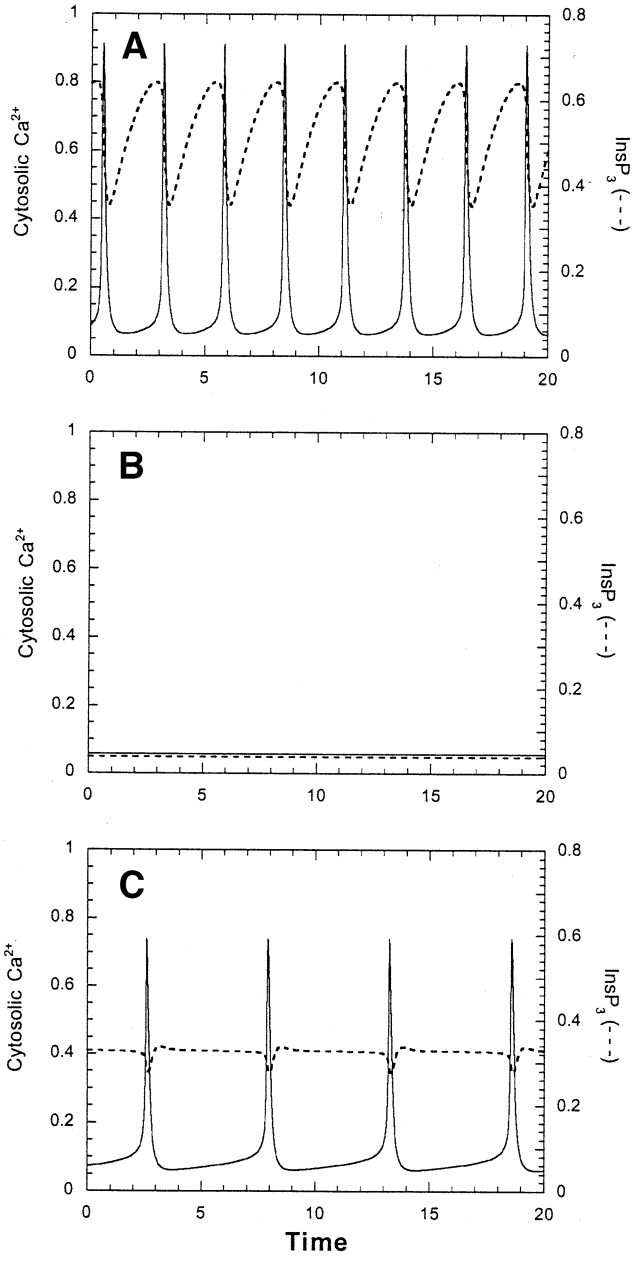

Fig. 2. Theoretical prediction as to the possible role of the Ca^{2+}-stimulated $InsP_3$ catabolism. A: Oscillations in Ca^{2+} (solid line) and $InsP_3$ (dashed line) in a cell stimulated with a submaximal dose of agonist. B: The amount of 5-phosphatase in the simulated cell has been multiplied by 25 as compared to its value in A. C: Ca^{2+} oscillations can reappear if the cell is stimulated with a high dose of agonist. Curves have been obtained by numerical simulations of the model [20] with: $K_{act} = 0.5$ μM, $n_a = 3$, $K_{inh} = 0.17$ μM, $n_i = 4$, $k_- = 0.5$ s^{-1}, $k_1 = 2.57$ s^{-1}, $b = 0.0007$ s^{-1}, $K_{IP} = 1$ μM, $V_{MP} = 6$ μM s^{-1}, $K_P = 0.35$ μM, $\alpha = 0.1$, $Ca_{tot} = 80$ μM, $V_{PLC} = 4$ μM s^{-1}, $V_k = 5$ μM s^{-1}, $K_k = 1$ μM, $K_d = 0.3$ μM, $n_d = 2$. For A: $\gamma = 0.12$, $V_{p1} = 5$ μM s^{-1}, $V_{p2} = 0.2$ μM s^{-1}, for B: $\gamma = 0.12$, $V_{p1} = 125$ μM s^{-1}, $V_{p2} = 5$ μM s^{-1}, and for C: $\gamma = 1$, $V_{p1} = 125$ μM s^{-1}, $V_{p2} = 5$ μM s^{-1}. These parameters only aim at qualitatively representing the situation encountered in hepatocytes, as most parameters are experimentally unknown. Scales are in s and μM.

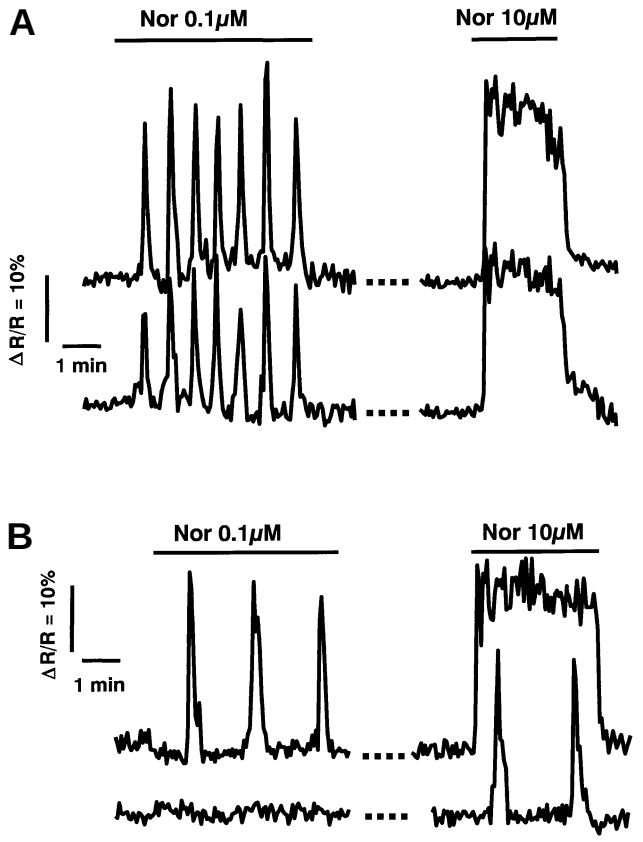

Fig. 3. Effect of 5-phosphatase on $InsP_3$-dependent agonist-induced $[Ca^{2+}]_i$ oscillations. One cell of the doublet (lower trace in each panel) was microinjected with fura2 and either with inactive (A) or active $InsP_3$ 5-phosphatase (B). Then, hepatocyte doublets were challenged with noradrenaline (Nor, 0.1 μM or 10 μM) for the time shown by the horizontal bar. Results are representative of those obtained using four (A) and five (B) doublets. For technical convenience, tracings were interrupted (the gap represents 3 min).

being much faster than $InsP_3$ removal, and (2) minimal levels of $InsP_3$ during the course of oscillations being still above the threshold required for an oscillatory behavior.

4. Experimental results: effect of injecting $InsP_3$ 5-phosphatase into one cell of a hepatocyte doublet

In liver, hepatocytes are tightly coupled by gap junctions [26]. Ca^{2+} increases induced by agonists activating the $InsP_3$ cascade, such as vasopressin or noradrenaline, are highly coordinated within multiplets when gap junctions are functional (e.g. Fig. 3A and see [27] for review). Previous work suggests that calcium spikes are coordinated by the diffusion of small amounts of $InsP_3$ between cells that slightly differ in their sensitivity to the hormonal stimulus [28] (but see also [29]).

The fact that these coupled cells show very similar Ca^{2+} oscillations provides an ideal tool to evaluate the role of $InsP_3$ metabolism in the regulation of Ca^{2+} dynamics (see Fig. 1 for a schematic representation of the experiments). Indeed, injection of an enzyme that acts specifically on $InsP_3$ catabolism in the injected cell but cannot diffuse through gap junctions makes it possible to observe the effect of $InsP_3$ metabolism on Ca^{2+} oscillations, while the non-injected cell provides a natural control for unperturbed Ca^{2+} oscillations. Moreover, 3-kinase B has been isolated from rat hepatocytes [30] and shown to be stimulated by Ca^{2+} [31], while the activity of $InsP_3$ 5-phosphatase has been shown to be unaffected by changes in $[Ca^{2+}]$ in this cell type [32]. Thus, we have injected type I $InsP_3$ 5-phosphatase in only one cell of hepatocyte doublets. This isoform is the most widespread of $InsP_3$ 5-phosphatases and it is not stimulated by Ca^{2+} [33].

Together with $InsP_3$ 5-phosphatase, fura2 was microinjected; diffusion of this dye via gap junctions revealed that the two cells were indeed coupled. As shown in Fig. 3A, control injection in one cell of $InsP_3$ 5-phosphatase that had been previously inactivated did not result in any difference between the two cells as regards noradrenaline-induced Ca^{2+} oscillations. The two cells showed similar Ca^{2+} responses both at low (0.1 μM) and maximal (10 μM) noradrenaline concentration. In contrast, Ca^{2+} signals in the two cells were different when active $InsP_3$ 5-phosphatase had been injected into one cell of the doublet, whatever the concentration of the agonist (Fig. 3B), consistent with the reduction of the $InsP_3$ concentration in the injected cell anticipated by the model (Fig. 2B).

In contrast, at supra-maximal concentrations of noradrenaline (10 μM), the non-injected cell shows a high sustained level of Ca^{2+}, reflecting a very high level of $InsP_3$, but the injected cell displays low-frequency Ca^{2+} oscillations, typical of an intermediate level of $InsP_3$ (Fig. 3B, right panel). Thus, as predicted by the model (Fig. 2C), a hepatocyte that has been made silent by injection of 5-phosphatase can become responsive again by increasing the concentration of the agonist. The critical observation is that oscillatory Ca^{2+} signals can be observed at high enough agonist concentrations, despite the massive Ca^{2+}-independent $InsP_3$ catabolism induced by the injection of 5-phosphatase.

5. Discussion

The present results show that, although $InsP_3$ oscillations probably arise in intact cells due to the stimulation of 3-kinase activity by Ca^{2+}, these oscillations do not play a predominant role neither in the triggering nor in the main characteristics of Ca^{2+} oscillations. However, they do not exclude the possibility that $InsP_3$ oscillations generated by another mechanism, for example by a PKC-mediated feedback at the level of the receptor-coupled G-protein, might play a crucial role for hepatic Ca^{2+} oscillations [10]. Yet the observation [34] that $InsP_3$-dependent cycles of Ca^{2+} release and re-uptake can be reproduced in permeabilized hepatocytes with $InsP_3$ clamped at submaximal concentration, suggests that the Ca^{2+} feedback on the $InsP_3$ ($InsP_3R$) might well be the central oscillatory mechanism in this cell type. This suggestion is corroborated in a more indirect manner by two other studies. The first one shows that type 2 $InsP_3R$, which is the most abundant in hepatocytes [35], is required for the normal Ca^{2+} oscillations, while types 1 and 3 do not sustain Ca^{2+} oscillations on their own [36]. Although both type 1 and type 2 display a bell-shaped dependence on Ca^{2+}, type 2 is known to be more sensitive to cytosolic Ca^{2+}, which may explain its observed predominant role in the generation of Ca^{2+} oscillations. The other study also strongly suggests that the $InsP_3R$ is the driving force of Ca^{2+} oscillations: it shows that Ca^{2+} oscillations (but not Ca^{2+} release) are abolished in DT40 cells in which the sensitivity of the $InsP_3R$ to cytosolic Ca^{2+} has been decreased by substitution of the appropriate residues [37].

Nevertheless, even in the hypothesis of a primary role of the $InsP_3R$ in the generation of Ca^{2+} oscillations, the origin of the long periods observed in hepatocytes and other cells still remains unsolved. The present study demonstrates that the clue for these long periods can probably not be found in the Ca^{2+} dependence of $InsP_3$ catabolism, and thus emphasizes the necessity for investigating alternative mechanisms.

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