Investigation of the roles of Ca^{2+} and InsP_3 diffusion in the coordination of Ca^{2+} signals between connected hepatocytes

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SUMMARY

Glycogenolytic agonists induce coordinated Ca^{2+} oscillations in multicellular rat hepatocyte systems as well as in the intact liver. The coordination of intercellular Ca^{2+} signals requires functional gap-junction coupling. The mechanisms ensuring this coordination are not precisely known. We investigated possible roles of Ca^{2+} or inositol 1,4,5-trisphosphate (InsP_3) as a coordinating messengers for Ca^{2+} spiking among connected hepatocytes. Application of ionomycin or of supra-maximal concentrations of agonists show that Ca^{2+} does not significantly diffuse between connected hepatocytes, although gap junctions ensure the passage of small signaling molecules, as demonstrated by FRAP experiments. By contrast, coordination of Ca^{2+} spiking among connected hepatocytes can be favored by a rise in the level of InsP_3, via the increase of agonist concentrations, or by a shift in the affinity of InsP_3 receptor for InsP_3. In the same line, coordination cannot be achieved if the InsP_3 is rapidly metabolized by InsP_3-phosphatase in one cell of the multiplet. These results demonstrate that even if small amounts of Ca^{2+} diffuse across gap junctions, they most probably do not play a significant role in inducing a coordinated Ca^{2+} signal among connected hepatocytes. By contrast, coordination of Ca^{2+} oscillations is fully dependent on the diffusion of InsP_3 between neighboring cells.

Key words: Calcium oscillations, Waves, Liver, Gap junctions

INTRODUCTION

Fifteen years ago, it was shown in hepatocytes that the Ca^{2+} signals in response to hormonal stimulation generally consist of a series of peaks, also called oscillations, in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Woods et al., 1986). The period of such oscillations varies between a few seconds and a few minutes, depending on the agonist concentration. Shortly after, it was observed that such sustained oscillations occur in isolated rat liver perfused with inositol 1,4,5-trisphosphate (InsP_3)-dependent agonists, suggesting some coordination between billions of hepatocytes constituting the liver (Graf et al., 1987). Since then, progress in fluorescence microscopy has revealed that each Ca^{2+} peak is spatially organized at the single cell level: Ca^{2+} concentration first increases locally, then the increase propagates throughout the whole cell as a kind of wave, travelling at a speed of 10-20 μm s^{-1} (Rooney et al., 1990; Thomas et al., 1996). Moreover, studies in more highly integrated systems that preserved the functional integrity of the intact tissue, such as sections of cerebral tissue, preparations of intact retina, intestinal crypts and isolated intact perfused livers, showed that these intracellular movements of Ca^{2+} may be propagated from cell to cell, creating an apparent intercellular wave (Dani et al., 1992; Newman and Zahs 1997; Lindqvist et al., 1998; Nathanson et al., 1995; Robbgaspers and Thomas 1995; Motoyama et al., 1999; reviewed by Tordjmann et al., 2000). Thus, the propagation of InsP_3-dependent intercellular Ca^{2+} waves provides a mechanism to coordinate the activity of a large number of cells (Eugenin et al., 1998).

In the liver, coordinated intercellular Ca^{2+} waves were first observed in doublets or triplets of freshly isolated hepatocytes (Nathanson and Burgstahler, 1992; Combettes et al., 1994), and have more recently been demonstrated in the isolated intact perfused liver (Nathanson et al., 1995; Robbgaspers and Thomas 1995; Motoyama et al., 1999; Patel et al., 1999). It is well known that cells in a tissue may communicate directly via gap junctions (the 'junctional coupling' pathway) or indirectly via a chemical messenger that is released by the cell into the extracellular medium, where it stimulates a target cell (paracrine pathway). Intercellular Ca^{2+} waves may be propagated via one or both of these pathways. For example, hepatocytes can communicate both through gap junctions (Saez et al., 1989) and via paracrine factors such as ATP (Schlosser et al., 1996). However, numerous results obtained on isolated multicellular systems of rat hepatocytes (doublets

or triplets of cells tightly connected by gap junctions) argue against a role for the paracrine pathway in the propagation and the coordination of the intercellular Ca^{2+} waves. Indeed, propagation of Ca^{2+} waves between isolated hepatocytes or among connected hepatocytes within multiplets following mechanical stimulation of one cell disappears as soon as the preparation is perfused (Schlosser et al., 1996; Tordjmann et al., 1997). By contrast, perfusion of InsP_3-dependent agonists such as vasopressin or noradrenaline induces coordinated intercellular Ca^{2+} waves that are inhibited by selectively blocking gap-junction coupling (Nathanson and Burgstahler, 1992; Tordjmann et al., 1997). Thus, it is clear that functional gap junctions are fundamental to ensure the coordination of these Ca^{2+} signals. However, the mechanisms ensuring this coordination are not precisely known and are still a matter of debate (Höfer, 1999; Dupont et al., 2000).

Ca^{2+} and InsP_3 are the two most likely candidates for the intercellular messenger involved in the propagation and coordination of intercellular Ca^{2+} waves in connected hepatocytes. It has been shown, especially in doublets of hepatocytes, that Ca^{2+} ions and InsP_3 can spread from one cell to another through gap junctions (Saez et al., 1989; Niessen and Willecke, 2000; reviewed by Sanderson et al., 1994). Moreover, both second messengers are thought to induce an 'autocatalytic' increase in $[Ca^{2+}]_i$ (reviewed by Taylor, 1998). In fact, in a large number of different cultured cell types, it has been shown that propagation of Ca^{2+} waves induced by mechanical stimulation of one cell probably relies on the progressive diffusion of the InsP_3, massively synthesized in the stimulated cell, to its neighbors (Sanderson, 1995; Sneyd et al., 1995). However, experimental results obtained in multiplets of connected hepatocytes suggest that such a mechanism cannot account for the coordination of apparent intercellular Ca^{2+} waves in these cells. The main difference between hepatocytes and the other tissues is that, although gap-junction permeability is essential for coordinating Ca^{2+} oscillations in the coupled cells, each hepatocyte needs to be stimulated to display Ca^{2+} oscillations. Focal stimulation of one cell within a doublet by submaximal concentrations of InsP_3-dependent agonists, such as vasopressin or noradrenaline, does not induce a Ca^{2+} increase in the adjacent cells (Tordjmann et al., 1997). Two recent independent studies using different mathematical models propose that intercellular Ca^{2+} waves arise because each individual hepatocyte in multiplets displays repetitive Ca^{2+} spikes with a slight phase-shift with respect to neighboring cells; both studies emphasize the crucial role of gap-junction coupling to coordinate these Ca^{2+} spikes (Höfer, 1999; Dupont et al., 2000). However, whereas it is suggested in the first model that synchronization requires gap-junctional diffusion of cytosolic Ca^{2+} (Höfer, 1999), it is proposed in the other model that Ca^{2+} spikes are coordinated by the junctional diffusion of small amounts of InsP_3 (Dupont et al., 2000). In the present experimental study, the purpose was to determine the respective roles of Ca^{2+} and InsP_3 in the coordination of Ca^{2+} oscillations in hepatocytes. Experiments were performed on doublets and triplets of hepatocytes, which are real fragments of the liver cell plate. Our results indicate that gap-junctional diffusion of InsP_3 plays a key role in the coordination of Ca^{2+} signals among connected hepatocytes.

MATERIALS AND METHODS

BAPTA-Dextran, calcein/AM, Fura2, Fura2/AM and Ca^{2+}-orange/AM were obtained from Molecular Probes Inc. and Fura2PE3 from Teflab. William's medium E was from GIBCO, ionomycin was from Calbiochem, and collagenase from Boehringher. All other chemicals were purchased from Sigma and were of the highest grade available commercially.

Preparation of hepatocytes

Isolated rat hepatocytes were prepared from fed female Wistar rats by limited collagenase digestion of rat liver, as previously described (Combettes et al., 1994). In these conditions, about 20% cells were associated by two (doublet) or three (triplet) and were distinguished from aggregates of non-connected cells in conventional light microscopy by screening for dilated bile canaliculi, indicators of maintained functional polarity (Gautam et al., 1987). After isolation, rat hepatocytes were maintained (5×10^5 cells/ml) at 4°C in Williams' medium E supplemented with 10% foetal 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 hours.

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

Loading of hepatocytes with fura2

Hepatocytes were loaded with fura2, as previously described (Tordjmann et al., 1997), either by injection (see below) or by incubation with the dye. 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 minutes at 37°C under an atmosphere containing 5% CO_2. After cell plating, the medium was removed and replaced with a medium containing 3 μM fura2/AM or fura2PE3/AM for 30 minutes at 37°C under an atmosphere containing 5% CO_2. 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 glucose 1 g/l, pH 7.4). Dish coverslips were put onto a thermostatted holder (34°C) on the stage of a Zeiss Axiovert 35 microscope set up for epifluorescence microscopy.

Microinjection

Microinjection was performed using an Eppendorf microinjector (5242), as described previously (Tordjmann et al., 1997). Micropipettes with an internal tip diameter of 0.5 μm (Femtotips, Eppendorf) were filled with test agents together with 5 mM fura2 in a buffer solution containing 100 mM KCl, 20 mM NaCl, 10 mM Hepes adjusted to pH 7.1. After microinjection, cells were allowed to recover for at least 10 minutes. The success of microinjection was assessed by monitoring the morphology of cells before and after manipulation and checking the ability of the cell to retain injected fura2 and low $[Ca^{2+}]_i$. Purification and determination of activity of the recombinant type I InsP_3 5-phosphatase (19 μmol/minute/ml in this study) was performed as described previously (Communi et al., 1996; De Smedt et al., 1997). InsP_3 5-phosphatase was inactivated at 90°C for 20 minutes. Cells were microinjected either with inactivated InsP_3 5-phosphatase or with InsP_3 5-phosphatase (activity: 120 nmol/min/ml in the pipette) as described above.

Determination of $[Ca^{2+}]_i$ changes in hepatocytes

Ca^{2+} imaging was as described previously (Tordjmann et al., 1997). Briefly, the excitation light was supplied by a high pressure xenon arc lamp (75 watt), and the excitation wavelengths were selected by 340 and 380 nm filters (10 nm bandwidth) mounted in a processor-controlled rotating filter wheel (Sutter, Novato, CA) between the UV

lamp and the microscope. Fluorescence images were collected either by a low-light level ISIT camera (Lhesa Électronique, Cergy Pontoise, France) or a CCD camera (Princeton Instruments, Evry, France), digitized and integrated in real time by an image processor (Metafluor, Princeton Instruments).

Simultaneous determination of gap-junction permeability and $[Ca^{2+}]_i$ changes in hepatocytes multiplets

Gap-junction permeability was determined in hepatocyte multiplets using fluorescence recovery after photobleaching (FRAP). These experiments were performed on an inverted confocal microscope (Zeiss, LSM510) using the fluorescent tracer calcein, a low molecular weight (623 Da) fluorescent dye that has been previously reported to be an effective marker for gap-junction communication (Tomasetto et al., 1993; Jacobi et al., 1998). To avoid cell injury, this fluorescent compound can be loaded into cultured cells in its esterified form, calcein/AM. $[Ca^{2+}]_i$ changes were followed with the fluorescent Ca^{2+}-sensitive dye Ca^{2+}-orange. Because the excitation and emission characteristics of calcein (λex=494 nm; λem=520 nm) do not significantly overlap with those of Ca^{2+}-orange (λex=546 nm; λem=580 nm), both the $[Ca^{2+}]_i$ and the spread of calcein can be simultaneously evaluated. Thus, hepatocytes were incubated with calcein/AM (3 μM) and Ca^{2+}-orange/AM (5 μM) for 20 minutes at 37°C. Cells were then washed three times and incubated for 10-20 minutes to allow complete ester hydrolysis. Calcein and Ca^{2+}-orange fluorescence were then observed at 520 nm and 580 nm, respectively, after excitation was provided by the 488 nm and the 546 nm lines of a krypton/argon laser. The AOTF system of the Zeiss confocal microscope allows use of the same Argon-Krypton laser to both image and bleach the fluorescence in numerous cells of interest. After baseline fluorescence images were obtained at each wavelength, agents were added and change in $[Ca^{2+}]_i$ was monitored on line by following the change in Ca^{2+}-orange fluorescence (see Fig. 3). Then, the fluorescence emitted by calcein was bleached by exposure of a given cell to high (100%) intensity laser light (488 nm) close to the calcein excitation maximum (494 nm). Complete or almost complete photobleaching was achieved after 30 scans for a total duration of 15 seconds (Fig. 3). Recovery of fluorescence in the bleached cell was monitored for 8 minutes. After correction and normalization, fluorescence was plotted over time to generate fluorescence recovery curves which were fitted to an exponential function using a scientific plotting program (Origin, MicroCal). Analysis allow us to obtain two values: the degree of recovery (%) and the rate of recovery.

RESULTS AND DISCUSSION

Investigation of the possible role of Ca^{2+} as a coordinating messenger for Ca^{2+} spiking among connected hepatocytes

Previous reports using microinjected Ca^{2+} suggest that this messenger diffuses through gap junctions in hepatocyte doublets (Saez et al., 1989). However little information is available on the ability of Ca^{2+} to pass through gap junctions under less invasive techniques and/or more physiological stimulation. In a previous study, we have shown that focal stimulation of one hepatocyte of a doublet with a low agonist concentration induced Ca^{2+} oscillations that were restricted to the stimulated cell (Tordjmann et al., 1997). The latter results suggest either that Ca^{2+} was not able to diffuse through gap junctions under these conditions or that the change in Ca^{2+} associated fluorescence was too low to be detected. In the present study, we first aimed to further investigate if Ca^{2+} can flow through hepatic gap junctions under physiological conditions. For this purpose, single-cell stimulation was performed either by a focal application from a glass micropipette filled with ionomycin (500 nM) or by a global perfusion of maximal agonists concentrations. In both types of experiments, fura2 loading was performed by microinjection of the dye in one cell, fura2 diffusion via gap junctions ensuring that the two cells were efficiently coupled.

Focal application of ionomycin

Focal application of ionomycin was achieved by positioning a glass micropipette close to the cell of interest and applying a constant pressure via the Eppendorf injector, delivering picoliter quantities of ionomycin-containing solution (500 nM). As shown in Fig. 1 (left panel), the ionomycin-microperfused cell exhibited a rapid and high $[Ca^{2+}]_i$ rise; by contrast, $[Ca^{2+}]_i$ remained at a basal low level in the non-microperfused cell. However, after ionomycin was washed away, global perfusion of noradrenaline (1 μM) induced well coordinated $[Ca^{2+}]_i$ oscillations within the two cells (right panel).

Global application of supra-maximal agonists concentrations

In these experiments, one of two connected hepatocytes was injected with fura2 and heparin. Heparin inhibits both InsP_3 binding and the resulting InsP_3-induced Ca^{2+} release (Worley et al., 1987; Cullen et al., 1988). As shown in Fig. 2, which is representative of 10 doublets, perfusion of maximal concentrations of vasopressin (10 nM) or noradrenaline (10 μM) elicited an immediate Ca^{2+} rise in the non-injected cells, which was maintained at a high level for at least 3 minutes, especially in the presence of vasopressin (Fig. 2B), whereas no change of fluorescence was observed in the heparin-injected cell. By contrast, addition of thapsigargin or ionomycin induced a $[Ca^{2+}]_i$ increase in the two connected cells (data not shown).

Gap-junctional conductivity during agonist stimulation

The two previous results (sections A and B) show that $[Ca^{2+}]_i$ remains at a basal level even when Ca^{2+} is high in any of the connected hepatocytes. Why did Ca^{2+} not significantly diffuse from one cell to the other? Is it because diffusion of Ca^{2+} within the cytosol is weak (Allbritton et al., 1992) or rather to a weak gap-junctional communication under the conditions of stimulation used in the latter experiments? It is well known that gap junctions are sensitive to Ca^{2+} increases (Lowenstein, 1981; De Mello, 1994; Spray et al., 1994), and it has even been suggested that, in an hepatoma cell line, gap-junction permeability is attenuated at Ca^{2+} concentrations as low as 500 nM (Lazrak and Peracchia, 1993). Moreover, it has been shown in different cells types, such as astrocytes, hippocampal neurons, the ciliary epithelium of the eye or pancreatic acinar cells, many different agonists can modulate gap-junction permeability (Giaume and McCarthy, 1996; Yule et al., 1996; Stelling and Jacob, 1997; Chanson et al., 1999). Thus, InsP_3-dependent Ca^{2+} agonists could affect gap-junction permeability in hepatocytes.

To investigate this possibility, gap-junction permeability was quantified in dying hepatocyte doublets using FRAP in the presence or absence of high agonist concentrations. This technique has the advantage over other methods to be relatively

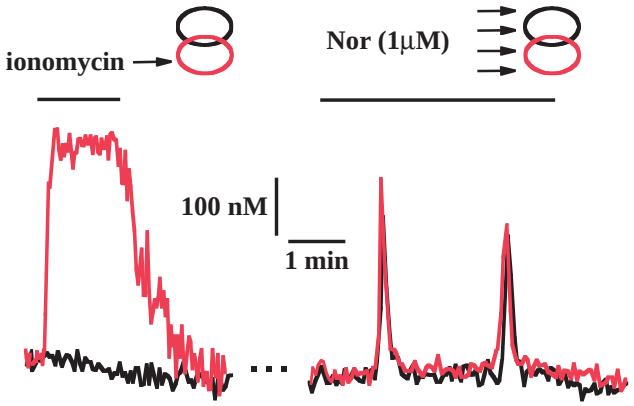

Fig. 1. Focal application of ionomycin to connected rat hepatocytes. Hepatocytes were injected with fura2. The two parts of the figure show successive measures of $[Ca^{2+}]_i$ in the same hepatocyte doublet and are representative of those obtained using 4 doublets in 3 independent experiments. The first part of the figure shows the Ca^{2+} response when one cell within the doublet was focally microperfused with ionomycin (500 nM in the micropipette) for the time shown by the upper horizontal bars. In these conditions, only the stimulated cell (indicated by arrow) within the doublet responded. Following ionomycin wash out, global superfusion of the doublet with noradrenaline (1 μM) induced tightly coordinated $[Ca^{2+}]_i$ oscillations in both cells. For technical convenience, tracings were interrupted (the gap represents 3 minutes).

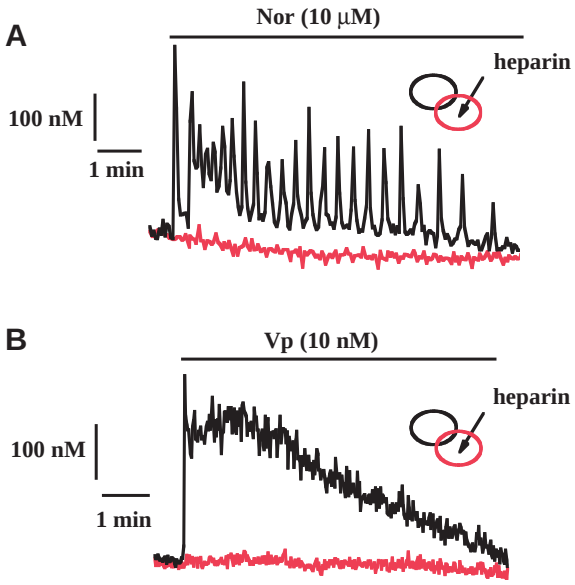

Fig. 2. Absence of apparent Ca^{2+} diffusion between connected rat hepatocytes. Hepatocyte doublets were injected with fura2 and heparin (10 mg/ml in the pipette) as described in methods section. After injection, perfusion (for the time shown by the horizontal bars) of high concentration of noradrenaline (Nor; 10 μM, A) or vasopressin (Vp; 10 nM, B), induced a rapid and strong increase in $[Ca^{2+}]_i$ in the noninjected cell only. Although this $[Ca^{2+}]_i$ increase was maintained for more than 3 minutes in the responding cell, no diffusion of Ca^{2+} was observed in the connected cell injected with heparin. These results are representative of those obtained using 10 doublets in four independent experiments.

non-invasive and quantitative. Indeed, when cells are loaded with a low molecular weight fluorescent probe (≤1 kDa), the recovery of fluorescence in the bleached target cell will reflect the influx of dye from the connected unbleached cells (Wade et al., 1986; Meyvis et al., 1999). Then, the rate of refill is a measure of gap-junction permeability. In the present study, hepatocytes were loaded with calcein, a low molecular weight (623 Da) fluorescent dye that has been previously reported to be an effective marker for gap-junction communication (Tomasetto et al., 1993; Jacobi et al., 1998) and Ca^{2+}-orange as an intracellular Ca^{2+} indicator. A representative confocal image of an hepatocyte doublet loaded with calcein and Ca^{2+}-orange is shown in Fig. 3C.

First, the baseline fluorescence of a doublet was obtained simultaneously for each dye by confocal microscopy (see Materials and Methods). After about 30 seconds, a maximal concentration of agonist was perfused. As revealed by the fluorescence increase of Ca^{2+}-orange, perfusion of vasopressin (10 nM) induced, as usual, a sustained $[Ca^{2+}]_i$ increase in the two cells (Fig. 3A). Then, as described in Materials and Methods, the fluorescence emitted by calcein was bleached in one cell (indicated as cell 2 in Fig. 3) by selectively exposing the latter cell to the full laser power at 488 nm. This resulted in the nearly complete ablation of the cell's calcein fluorescence (Fig. 3C,b; Fig. 3B, trace 2). Note that, in accordance with its excitation wavelength characteristic, Ca^{2+}-orange was not greatly affected by this strong illumination at 488 nm (Fig. 3C; Fig. 3A, trace 2). Subsequently, the microscope settings were returned to the recording configuration, and the refill was monitored (Fig. 3B). The fluorescence recovery was then plotted and fitted to an exponential function (Fig. 4), yielding two characteristic values: the degree (%) and the rate (seconds) of recovery. As shown in Fig. 4 and summarized in Table 1, hepatocyte doublets from control experiments were extensively interconnected by gap junctions, as they showed a rapid and high degree of recovery. As expected for a gap-junction-dependent effect, octanol (0.5 mM) nearly totally blocked this refill (Fig. 4; Table 1). At this concentration of octanol, the effect of gap-junction cell coupling was reversible as octanol could be washed out and coupling restored (data not shown). By contrast, in vasopressin (10 nM)- or noradrenaline (10 μM)-treated hepatocytes, we found that coupling was similar to control experiments, suggesting that stimulation with these maximal concentrations of agonists had no effect on gap-junction permeability (Table 1). Thus, it can be concluded that the passage of small signaling molecules is possible under conditions of physiological stimulation.

Are small amounts of Ca^{2+} sufficient to coordinate Ca^{2+} oscillations?

Together, these data suggest that, although Ca^{2+} can in principle flow through the gap junctions of connected hepatocytes, significant amounts of this messenger do not pass from one cell to the other. However, it has been proposed that a relatively small amount of Ca^{2+}, although undetectable by an increase in fluorescence, could be sufficient to account for the synchronization of Ca^{2+} oscillations via a Ca^{2+}-induced Ca^{2+} release mechanism (Ngezahayo and Kolb, 1993; Höfer, 1999). In an attempt to confirm or invalidate such a hypothesis in hepatocytes, we tried to abolish any Ca^{2+} movements in the intermediate cell of a triplet by injecting BAPTA in this cell to

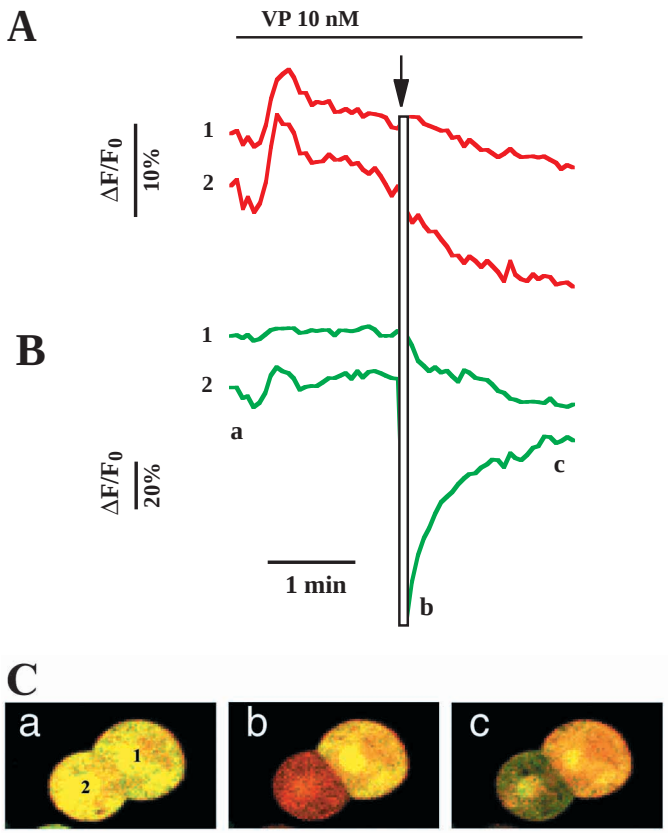

Fig. 3. Simultaneous determination of gap-junction permeability and $[Ca^{2+}]_i$ increase in hepatocyte doublets. Hepatocytes loaded with calcein and Ca^{2+}-orange were imaged by confocal microscopy (as described in Materials and Methods) and appear brightly fluorescent (C). Panel A shows the time course of vasopressin (Vp)-induced $[Ca^{2+}]_i$ increase in a doublet of hepatocytes observed at 580 nm using Ca^{2+}-orange excited at 546 nm. Simultaneous determination at 520 nm of calcein fluorescence excited at 488 nm is shown in B (note that traces have been shifted for clarity). In contrast to Ca^{2+}-orange, the small molecular weight of calcein allows it to diffuse freely across gap junctions so that gap-junction permeability can be evaluated by fluorescence recovery after photobleaching (FRAP). Basal fluorescence level of the two dyes was measured for 30 seconds. Then perfusion of Vp (10 nM) induced a rapid and maintained $[Ca^{2+}]_i$ increase in both cells of the doublet (A). After about 1 minute the cell indicated as cell 2 was bleached by focused 100% intensity laser excitation at 488 nm for 15 seconds (arrows and open box). Subsequently, the cell's fluorescence recovered by diffusion of unbleached dye from the adjacent cell through the gap junctions (B, traces 1 and 2; see C). Note that fluorescence of Ca^{2+}-orange was slightly affected by photobleaching, as indicated by the small decrease in fluorescence intensity observed at 580 nm (panel A, trace 2) and the fact that the photobleached cell appeared greener after recovery (C, c). Panel C shows a merged image of a doublet of hepatocytes loaded with calcein and Ca^{2+}-orange before (a), immediately (b) and about 2 minutes after photobleach (c). Cell 2 was photobleached as described above.

see if coordination of Ca^{2+} signals was affected. However, as expected from its low molecular weight, BAPTA was able to spread from the injected cell to the other connected cells (data not shown) and the concentration of BAPTA-Dextran (10 kDa) that could reach the cell after injection (100-200 μM) was not

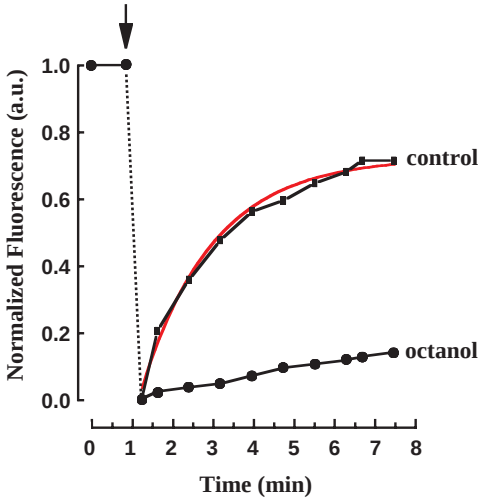

Fig. 4. Hepatocyte gap junctions remain open during InsP_3-dependant agonists stimulation. Typical calcein fluorescence recovery analysis. Each curve was normalized and then fitted to an exponential function (red line) as described in Materials and Methods. Analysis allowed us to obtain two values: the degree of recovery (%) and the rate of recovery (τ). As shown here, recovery was large (>60%) and rapid (τ≈100 seconds) in the control experiment. By contrast, octanol reduced almost totally the fluorescence recovery after photobleach.

Table 1. Assessment of hepatocyte gap-junction permeability

	Recovery %	$t_{\frac{1}{2}}$ seconds
Control *n*=49	64±2	105±6
Vasopressin (10 nM) *n*=14	56±3	84±11
Noradrenaline (10 μM) *n*=8	76±10	81±14
8Bromo-cAMP (10 μM) *n*=16	64±4	100±12
Octanol (500 μM) *n*=3	11±3	ND

In contrast with octanol, neither noradrenaline nor vasopressin at maximal concentrations affects significantly the permeability of the gap junctions to the calcein. Data are the mean±s.e.m. ND, not done.

sufficient to completely block vasopressin or noradrenaline-induced Ca^{2+} increase in those cells (data not shown). Thus, it was not possible to directly estimate in the latter conditions whether Ca^{2+} is involved in the coordination of Ca^{2+} oscillations.

Nevertheless, it should be remembered that coordination could extend across an intermediate cell in which the release of intracellular Ca^{2+} was inhibited by heparin, at least for the first few spikes following stimulation (Tordjmann et al., 1997). This suggests that an increase in Ca^{2+} is not crucial for the coordination of Ca^{2+} oscillations.

Investigation of the possible role of InsP_3 as a coordinating messenger for Ca^{2+} spiking among connected hepatocytes

Ca^{2+} signals in response to increasing agonist concentrations

The other likely candidate that could be responsible for the coordination of Ca^{2+} spiking among connected hepatocytes is InsP_3. If junctional InsP_3 diffusion is involved, it can be

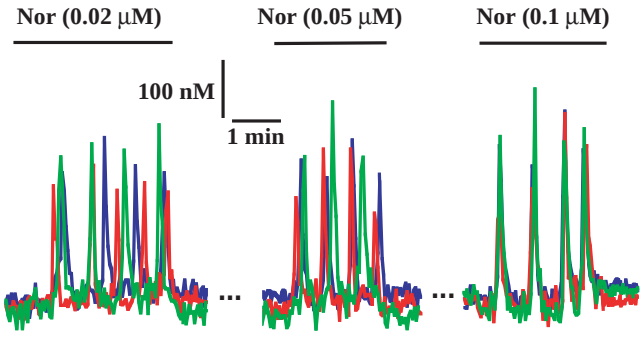

Fig. 5. Coordination of Ca^{2+} oscillations increase with the increase of agonist concentration in connected hepatocytes. Hepatocytes were loaded or injected with fura2. The figures show successive measures of $[Ca^{2+}]_i$ in the same hepatocyte triplet. Cells were sequentially stimulated with increasing concentrations of noradrenaline (0.02, 0.05 and 0.1 μM) for the time shown by the horizontal bars. Addition of the lowest dose of noradrenaline to the bath was followed by oscillations in the three cells that were not coordinated (left). After washing, addition of a higher concentration of noradrenaline (0.05 μM) induced coordinated Ca^{2+} oscillations (middle part). Finally, very well coordinated oscillations were observed in the presence of 0.1 μM noradrenaline (right). The same results were obtained in hepatocyte doublets and are representative of those obtained using 12 triplets and 21 doublets in five independent experiments. Recording of the traces was interrupted during the washing process (5 minutes).

expected that the degree of coupling increases with the level of InsP_3, and thus also with the concentration of the agonist. To test this hypothesis, hepatocyte multiplets were perfused with increasing concentrations of InsP_3-dependent agonists (Fig. 5). Note that the proportion of responsive cells in these conditions was low (30-50%). Nevertheless, application of very low concentrations of noradrenaline (0.02 μM), to doublet or triplet of hepatocytes sometimes induced Ca^{2+} oscillations (Fig. 5 and data not shown). However, these oscillations were not coordinated, as if the cells were not functionally coupled by gap junctions (each cell having its own oscillation frequency) even though gap junctions efficiently connected these cells as they were loaded by cell to cell diffusion of microinjected fura2. Raising the concentration of noradrenaline (0.05 μM) not only increased the frequency of the Ca^{2+} oscillations as expected (see blue and green traces in Fig. 5 for example), but also led to coordinated oscillations (Fig. 5, middle panel). Finally, perfusion of a higher concentration of noradrenaline (0.1 μM) induced a good coordination of the Ca^{2+} oscillations among the connected hepatocytes. Because, in contrast with the amplitude of Ca^{2+} oscillations, the InsP_3 level increases with increasing concentrations of agonists (Thomas et al., 1991; Thomas et al., 1996), these results suggest that InsP_3, rather than Ca^{2+}, is responsible for the coordination of Ca^{2+} oscillations between connected hepatocytes. At low stimulation, production of InsP_3 is indeed too small to allow for an efficient junctional InsP_3 diffusion, and the cells thus oscillate at their own intrinsic frequencies; raising the level of InsP_3, via the increase of agonist concentrations, allows the diffusion of significant amounts of InsP_3 through gap junctions, a phenomenon that can account for the observed coordination in Ca^{2+} oscillations (Dupont et al., 2000).

Coordination of Ca^{2+} signals after sensitization of the InsP_3Rs

We used an other approach to validate the latter mechanism of coordination. Our approach was to act on the affinity of the InsP_3 receptors (InsP_3R) for InsP_3. This was achieved with the use of thimerosal and cAMP, as both are known to increase the affinity of the InsP_3R to InsP_3. Indeed, in many cell types and notably in hepatocytes, numerous studies have shown that the affinity of the InsP_3R for InsP_3 increased in the presence of cAMP (Burgess et al., 1991; Bird et al., 1993; Joseph and Ryan, 1993; Hajnoczky et al., 1993). This effect results from the ability of the cAMP-dependent protein kinase to phosphorylate the three types of InsP_3 receptors (Wojcikiewicz and Luo, 1998).

As described previously, InsP_3-dependent agonists at low concentration induced uncoordinated Ca^{2+} oscillations among connected hepatocytes (Fig. 6, left panels). However, when cells were incubated with 8Bromo-cAMP (10 μM) for 5 minutes we observed that, in 70% of all the multiplets analyzed (15 doublets and 8 triplets), re-addition of the same low concentration of vasopressin or noradrenaline, induced well-coordinated $[Ca^{2+}]_i$ oscillations (Fig. 6A and data not shown). This suggest that in these conditions, the small quantity of InsP_3 diffusing through gap junctions was sufficient to coordinate Ca^{2+} oscillations. Alternatively, it has been shown that cAMP can increase the permeability of gap junctions (Loewenstein, 1985; Burghardt, 1995), an effect that could account for coordination of Ca^{2+} signals. Although the concentration of 8Bromo-cAMP required to increase gap-junction permeability is usually higher (0.1-1 mM) than that used in this study (10 μM), this hypothesis has been checked. Gap-junction permeability was quantified using FRAP in dying hepatocytes doublets incubated for 5 minutes with or without 8Bromo-cAMP (10 μM). Results are summarized in Table 1 and show that gap-junction permeability was not significantly affected by 8Bromo-cAMP. Thus, the cAMP-induced increase in the degree of coordination of Ca^{2+} oscillations observed at low levels of stimulation might result from the shift in the sensitivity of the InsP_3R.

These results were confirmed with the use of the thiol reagent thimerosal. This compound is known to increase the sensitivity of the InsP_3R to InsP_3, especially in hepatocytes (Missiaen et al., 1992; Bird et al., 1993; Hilly et al., 1993). In agreement with previous observations (Green et al., 1999), application of 1 μM thimerosal to fura2-loaded hepatocytes did not elevate Ca^{2+} by itself but induced a slight rise in the frequency of noradrenaline-induced Ca^{2+} oscillations (Fig. 6B). More interestingly, in the majority of multiplets (5/8 doublets and 3/5 triplets), noradrenaline-induced Ca^{2+} oscillations that were uncoordinated in the absence of thimerosal, became well coordinated after co-application of thimerosal (Fig. 6B). In most cases, coordination was maintained during the first few peaks (Fig. 6B, upper traces), but in some doublets coordination remained for much longer periods of time (Fig. 6B, lower traces).

Thus, both cAMP and thimerosal significantly increased the level of coordination of Ca^{2+} spiking between connected hepatocytes at low agonist concentrations. Our interpretation of the latter results is that a shift in the affinity of the InsP_3Rs for InsP_3 potentiates the coordinating effect of the small amounts of InsP_3 flowing through gap junctions. It should be

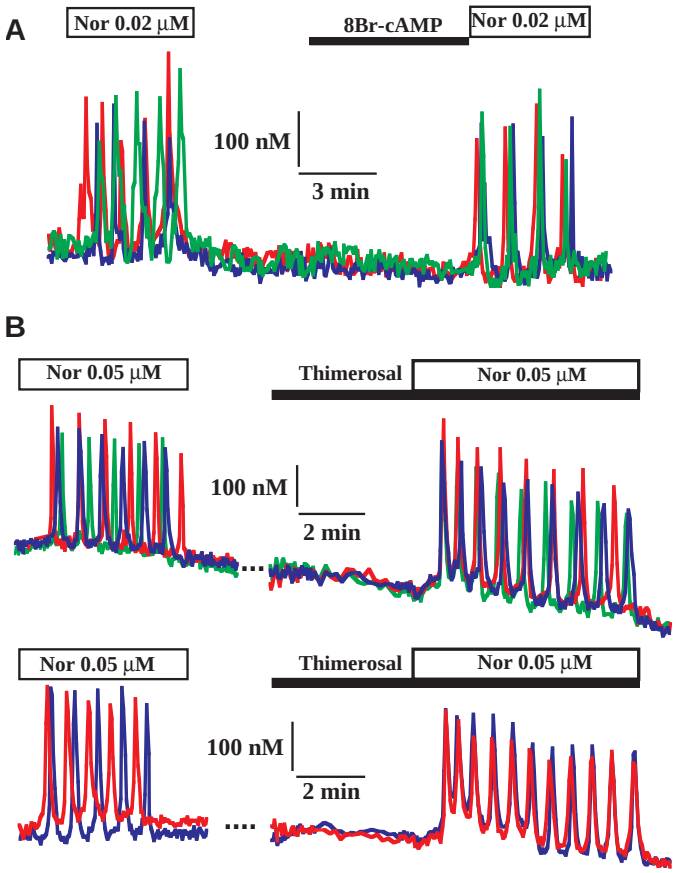

Fig. 6. Increase in InsP_3 receptor sensitivity induces coordination of Ca^{2+} oscillations in connected hepatocytes. Hepatocyte multiplets were loaded with fura2. Addition of a low concentration of noradrenaline (0.02 μM, A; 0.05 μM, B) for the time shown by the open box induced uncoordinated Ca^{2+} oscillations. However, when the same cells were incubated in the presence of 8Bromo-cAMP (10 μM, for the time shown by the black box; A), or when noradrenaline was perfused in the presence of thimerosal (10 μM, for the time shown by the black box; B), the same dose of noradrenaline elicited coordinated Ca^{2+} oscillations. The same results were obtained in hepatocyte doublets and are representative of those obtained using 7 triplets and 12 doublets in four independent experiments. For technical reasons, recording of the traces was interrupted during the washing process (3 minutes) and frame pairs were captured every 10 seconds during a part of thimerosal perfusion.

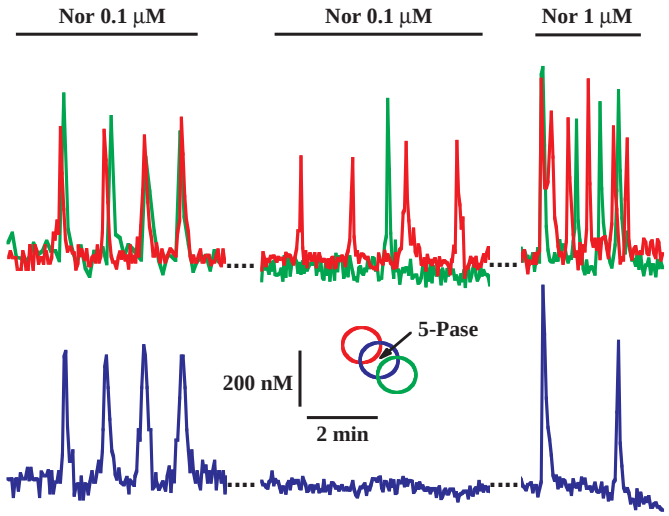

Fig. 7. InsP_3 5-phosphatase prevents coordination of Ca^{2+} oscillations induced by InsP_3-dependent agonists in connected hepatocytes. Hepatocyte triplets loaded or injected with fura2 were challenged with noradrenaline (0.1 μM and 1 μM) for the time shown by horizontal bars. Tracings, representing $[Ca^{2+}]_i$ in the three connected cells, have been shifted arbitrarily along the y-axis for clarity. For technical convenience, tracings were interrupted but left, middle and right parts of the figure show successive measurement of $[Ca^{2+}]_i$ in the same hepatocyte triplet. In the left part, noradrenaline addition to the bath was followed by coordinated $[Ca^{2+}]_i$ oscillations. The intermediate cell of the triplet was then injected with InsP_3 5-phosphatase, as described in Materials and Methods. After injection (middle), application of noradrenaline did not elevate $[Ca^{2+}]_i$ in the injected cell. By contrast, noradrenaline still elicited Ca^{2+} oscillations, but these oscillations were not coordinated at all. Finally, at the end of the experiment, the same triplet was challenged with a higher concentration of noradrenaline (1 μM), which elicited a Ca^{2+} response in the three connected cells (right). Note the oscillatory pattern for the InsP_3 5-phosphatase injected cell. These results are representative of those obtained using 9 triplets in three independent experiments.

emphasized that the increase in coordination by cAMP and thimerosal would not be predicted by a model based on Ca^{2+} diffusion through gap junctions, as the amplitude of Ca^{2+} oscillations does not depend on the presence of cAMP or thimerosal.

Effect of the increase of InsP_3 5-phosphatase activity in hepatocytes

Finally, we have studied the role of a putative InsP_3 intercellular diffusion in synchronization of Ca^{2+} oscillations by microinjection of type I InsP_3 5-phosphatase in the intermediate cell of a triplet. Type I InsP_3 5-phosphatase is the most widespread InsP_3 5-phosphatase and efficiently metabolizes InsP_3 to produce inositol 1,4-bisphosphate, which does not mobilize Ca^{2+} (Putney et al., 1989; Erneux et al., 1989; Verjans et al., 1994). It has been shown recently that over-expression of this enzyme in CHO cells deeply affected the pattern of Ca^{2+} oscillations; in some cases it even abolished the stimulus-induced Ca^{2+} signal (De Smedt et al., 1997). In the present study, we thus used microinjected InsP_3 5-phosphatase to specifically decrease the level of InsP_3 in a particular hepatocyte. Triplets were first loaded with fura2 and treated with noradrenaline (0.1 μM), which elicited trains of coordinated Ca^{2+} oscillations in the three connected cells (Fig. 7, left panel). After washing out noradrenaline, the intermediate cells of the triplets were microinjected with InsP_3 5-phosphatase, as described in Materials and Methods. In the vast majority of injected multiplets (9/13), the renewed superfusion of noradrenaline (0.1 μM) elicited Ca^{2+} oscillations in the non-injected cells only, whereas Ca^{2+} remained at a low basal level in the InsP_3 5-phosphatase-injected cell. In the remaining injected cells (4 out of 13), the frequency of oscillations was strongly reduced (data not shown). In all cases, Ca^{2+} oscillations in the two remaining responding cells appear uncoordinated (n=13 triplets). It is worth noting that InsP_3 5-phosphatase did not inhibit gap-junction permeability because, when fura2 was co-injected

with 5-phosphatase into the intermediate cells of the triplets, the dye diffused into the two adjacent connected cells and again, no coordination was observed in the two remaining responding cells (n=3, data not shown).

Thus, it can be concluded that, if the level of InsP_3 is too low in the intermediate cell, coordination cannot be achieved between the two end cells of the triplet. It could be argued that such an absence of coordination is simply due to the fact that the Ca^{2+} increases cannot propagate through the intermediate cell in the absence of an effective InsP_3-sensitive Ca^{2+}-induced Ca^{2+} release; in other words, as diffusion of Ca^{2+} is very slow, the increase of Ca^{2+} occurring in the first cell of the triplet could not be detected by the last one in the absence of any regenerative process. That this is not the case is clearly visible in the last panel of Fig. 7, which shows Ca^{2+} oscillations in the three cells of the same triplet at 1 μM noradrenaline. There, owing to the high level of stimulation, the concentration of InsP_3 is clearly sufficient to activate the InsP_3Rs even in the 5-phosphatase-injected cell (Fig. 7, right panel). However, even in this case, the Ca^{2+} oscillations remained uncoordinated among the three cells of the triplet. Thus, the absence of coordination in the Ca^{2+} oscillations in the InsP_3 5-phosphatase-injected cells can be explained only by a difference in the levels of InsP_3 between the connected cells, which cannot be erased by the low permeability of the gap junctions. Moreover, the latter experiment indicates that the intermediate cell had not been damaged during the 5-phosphatase microinjection process. It also gives an idea of the efficiency of the InsP_3 5-phosphatase to metabolize InsP_3. Indeed, Ca^{2+} oscillations such as those seen in the intermediate cell are usually observed at 10 time lower agonist concentrations (see Fig. 7, left panel; Fig. 5; Fig. 6).

To summarize, there is no coordination of Ca^{2+} oscillations when the production of InsP_3 is too low, that is, in the presence of low concentrations of agonist or when InsP_3 is rapidly metabolized (by InsP_3 5-phosphatase). By contrast, coordination can be achieved either by increasing the InsP_3 concentration (via a rise in agonist concentration) or by increasing the affinity of the InsP_3 receptor for InsP_3, even at a very low levels of stimulation.

In conclusion, we have shown that in doublets of hepatocytes connected by gap junctions, a high and sustained increase in $[Ca^{2+}]_i$ does not induce any detectable $[Ca^{2+}]_i$ rise in the adjacent cells, although gap junctions are fully operable. Similar results have been obtained in other cell types (Demer et al., 1993; Yule et al., 1996; reviewed by Sanderson et al., 1994) and are in agreement with the poor ability of Ca^{2+} to diffuse over a long distance (Albritton et al., 1992). However, because Ca^{2+} is known to be the principal regulator of its own release through an increase in the effect of InsP_3 on its receptor (reviewed by Taylor, 1998), it has been suggested in other cell types, such as aortic endothelial cells, pancreatic acinar cells or articular chondrocytes, that the diffusion of small amounts of Ca^{2+} between adjacent cells could serve to sensitize InsP_3R in the neighboring cells and thus allow for the intercellular propagation of the signal (Yule et al., 1996; D'Andrea and Vittur, 1997; reviewed by Tordjmann et al., 2000).

Our results in hepatocytes cannot exclude any role for Ca^{2+} in the coordination of Ca^{2+} spiking in this cell type. It remains plausible that diffusion of Ca^{2+} might play a role when the Ca^{2+} sequestration mechanism is partly impaired (i.e. at high levels of InsP_3). In this case, intercellular diffusion of Ca^{2+} could smooth out the differences in Ca^{2+} signals among connected cells caused by possible heterogeneities in cell shape or in the diverse InsP_3-independent fluxes (Höfer, 1999). Our results show however, that even if such Ca^{2+}-activated Ca^{2+} release through InsP_3R takes place in hepatocytes (Taylor, 1998), it is not crucial for the coordination of Ca^{2+} oscillations induced by InsP_3-dependent agonists among connected cells. By contrast, coordination of Ca^{2+} oscillations among connected hepatocytes is fully dependent on the level of InsP_3 diffusing from cell to cell.

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