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Coordinated intercellular calcium waves induced by noradrenaline in rat hepatocytes: dual control by gap junction permeability and agonist

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Calcium-mobilizing agonists induce intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes thought to trigger cellular responses. In connected cells, rises in $[Ca^{2+}]_i$ can propagate from cell to cell as intercellular Ca^{2+} waves, the mechanisms of which are not elucidated. Using fura2-loaded rat hepatocytes, we studied the mechanisms controlling coordination and intercellular propagation of noradrenaline-induced Ca^{2+} signals. Gap junction blockade with 18 α-glycyrrhetinic acid resulted in a loss of coordination between connected cells. We found that second messengers and $[Ca^{2+}]_i$ rises in one hepatocyte cannot trigger Ca^{2+} responses in connected cells, suggesting that diffusion across gap junctions, while required for coordination, is not sufficient by itself for the propagation of intercellular Ca^{2+} waves. In addition, our experiments revealed functional differences between noradrenaline-induced Ca^{2+} signals in connected hepatocytes. These results demonstrate that intercellular Ca^{2+} signals in multicellular systems of rat hepatocytes are propagated and highly organized through complex mechanisms involving at least three factors. First, gap junction coupling ensures coordination of $[Ca^{2+}]_i$ oscillations between the different cells; second, the presence of hormone at each hepatocyte is required for cell–cell Ca^{2+} signal propagation; and third, functional differences between adjacent connected hepatocytes could allow a 'pacemaker-like' intercellular spread of Ca^{2+} waves.

Keywords: Ca^{2+} waves/gap junction/hepatocytes/intercellular

Introduction

Hormone-induced intracellular increases in Ca^{2+} concentration have important roles in the physiology of most cell types (Berridge, 1993; Petersen *et al.*, 1994; Pozzan *et al.*, 1994; Thomas *et al.*, 1996). Isolated hepatocytes, as well as many other cells, exhibit cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) oscillations upon stimulation with low concentrations of inositol 1,4,5-trisphosphate ($InsP_3$)-dependent agonists (Woods *et al.*, 1986; Rooney *et al.*, 1989). Intracellular wave-like propagation of $[Ca^{2+}]_i$ oscillations across single cells is driven mainly by a regenerative process of Ca^{2+} release from the endoplasmic reticulum, mediated by $InsP_3$ receptors (Kasai and Petersen, 1994; Thomas *et al.*, 1996). In multicellular systems, intercellular waves can propagate from cell to cell by mechanisms which are not understood in all cell types (for review, see Sanderson *et al.*, 1994). Intercellular communication between adjacent connected cells can take different routes, including both extracellular (paracrine) and intracellular (cell coupling by gap junctions) pathways (Bruzzone *et al.*, 1996; Meda, 1996). Both these routes are involved in the propagation of intercellular Ca^{2+} waves in various cell types (for review, see Sanderson *et al.*, 1994). For example, ATP release into the extracellular medium has been reported to signal to distant cells and to generate an intercellular Ca^{2+} wave in rat basophilic leukaemia cells (Osipchuk and Cahalan, 1992), in neuroepithelioma cells (Palmer *et al.*, 1996) and in rat hepatocytes (Schlosser *et al.*, 1996). Nevertheless, diffusion of second messengers through gap junctions from one cell to the other appears to be responsible for the intercellular spread of Ca^{2+} waves in tracheal ciliated cells (Sanderson *et al.*, 1990; Sneyd *et al.*, 1995), glial cells (Charles *et al.*, 1992), pancreatic acinar cells (Loessberg-Stauffer *et al.*, 1993; Yule *et al.*, 1996) and many other cell types (Sanderson *et al.*, 1994).

In the liver, hepatocytes are tightly coupled by gap junctions (Nicholson *et al.*, 1987). Thus, intercellular communication is thought to occur mainly by spreading of second messengers or other molecules through these extensive connections, as in other epithelial cells (Bruzzone *et al.*, 1996; Meda, 1996). Hepatocyte gap junctions are freely permeable to both microinjected Ca^{2+} and $InsP_3$ (Saez *et al.*, 1989). However, there is no direct evidence for actual diffusion of Ca^{2+} or $InsP_3$ from one hepatocyte to the other during hormone-induced Ca^{2+} transient. In rat hepatocyte couplets, gap junction blockade results in the loss of Ca^{2+} signal synchronization between cells (Nathanson and Burgsthaler, 1992). In more complex multicellular systems of rat hepatocytes, we have demonstrated highly coordinated hormone-induced Ca^{2+} signals, with reproducible sequences of $[Ca^{2+}]_i$ rises in the different connected cells (Combettes *et al.*, 1994). Similarly, in the intact liver, highly coordinated $[Ca^{2+}]i$ signals in response to agonists have been described (Nathanson *et al.*, 1995; Robbgaspers and Thomas, 1995). However, the factors coordinating individual $[Ca^{2+}]_i$ oscillations between connected cells and propagating intercellular Ca^{2+} waves are not precisely known.

We studied multicellular systems of rat hepatocytes, and confirm that hormone-induced intracellular Ca^{2+} signals are coordinated between cells through the involvement of gap junctions. Surprisingly, neither intracellular rises of second messengers nor Ca^{2+} changes in one cell can entirely account for the intercellular propagation of hormone-induced Ca^{2+} waves. We show that coordinated intercellular Ca^{2+} waves in multicellular systems of rat

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hepatocytes require the additional presence of the agonist at the cell surface of each hepatocyte. Finally, we report functional differences between adjacent connected hepatocytes, which may be the basis of a 'pacemaker-like' mechanism for intercellular propagation of Ca^{2+} waves.

Results

The frequency of $[Ca^{2+}]_i$ oscillations elicited by a given agonist concentration differs between individual liver cells (Kawanishi *et al.*, 1989; Rooney *et al.*, 1989). However, in multicellular systems of rat hepatocytes (Nathanson and Burgstahler, 1992; Combettes *et al.*, 1994) and even in the intact liver (Nathanson *et al.*, 1995; Robbgaspers and Thomas, 1995), $[Ca^{2+}]_i$ oscillations are synchronized and highly coordinated. In our experiment, the behaviour of single cells and connected hepatocytes was very different. Noradrenaline treatment induced oscillating $[Ca^{2+}]_i$ rises of variable frequencies in single cells, whereas the $[Ca^{2+}]_i$ oscillations were tightly coordinated in hepatocyte triplets in the same microscope field (e.g. Figure 2A and data not shown).

Is a paracrine route involved in coordination and propagation of intercellular Ca^{2+} waves in multicellular systems of rat hepatocytes?

Single rat hepatocytes can signal to each other by releasing ATP into the extracellular medium following mechanical stimulation (Schlosser *et al.*, 1996). Thus, we tested for this type of intercellular communication in our experimental conditions, even though the incubation medium was renewed continuously by concurrent superfusion and aspiration (see Materials and methods). When superfusion of cells was performed according to our standard experimental conditions (i.e. at a rate of 1.5–2 ml/min), mechanical stimulation of fura2-loaded single cells elicited a $[Ca^{2+}]_i$ rise in the stimulated cell but was not followed by any Ca^{2+} response in the non-connected neighbouring cells (Figure 1B, left panel). In contrast, in the absence of continuous washing of the medium, mechanical stimulation of the same hepatocyte induced a $[Ca^{2+}]_i$ rise both in the stimulated cell and in non-connected neighbouring cells (Figure 1B, right panel). In these conditions, as reported recently (Schlosser *et al.*, 1996), the delay between the $[Ca^{2+}]_i$ rise in the stimulated cell and the Ca^{2+} response in non-connected cells increased with increasing distance between the stimulated hepatocyte and the other cells (cells 2– 4, Figure 1A and B, right panel). Finally, cells distant from the stimulated cell did not respond (e.g. cell 5, Figure 1A and B, right panel).

These results suggest that, in our conditions of continuous superfusion, paracrine mechanisms of intercellular communication cannot account for the propagation and coordination of Ca^{2+} signals in multicellular systems of rat hepatocytes.

Implication of the intercellular connection in the propagation and coordination of intercellular Ca^{2+} waves

To investigate the role of intercellular connection in coordination of noradrenaline-induced $[Ca^{2+}]_i$ oscillations, we compared Ca^{2+} signals elicited in hepatocyte triplets before (Figure 2A) and after (Figure 2B) excision of the intermediate cell (see Materials and methods). The two remaining cells were completely isolated one from the other but were not damaged, as indicated by the unchanged resting $[Ca^{2+}]_i$, respectively 115 ± 7 nM and 122 ± 10 nM before and after excision (n = 3 triplets). In these conditions, noradrenaline elicited $[Ca^{2+}]_i$ oscillations in both cells without coordination. One of the cells (cell 1) retained nearly the same pattern of $[Ca^{2+}]_i$ oscillations, whereas the other (cell 3) displayed a slower frequency and responded to noradrenaline after a longer delay. These observations were confirmed by the analysis of three triplets in three separate experiments (Table I).

These results suggest that intercellular connection is required for the coordination of hormone-induced $[Ca^{2+}]_i$ oscillations in multicellular systems of rat hepatocytes. Futhermore, one cell in the multiplet seems to impose its frequency and delay on the other connected cells.

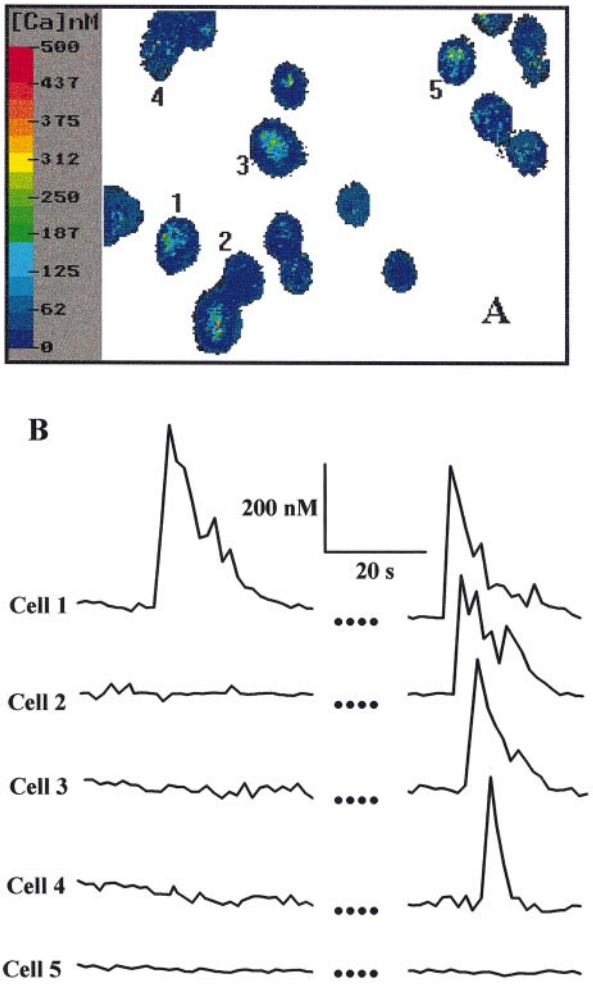

Fig. 1. Paracrine $[Ca^{2+}]_i$ signalling in isolated rat hepatocytes. (**A**) Pseudo-colour image of isolated hepatocytes loaded with fura2. The stimulated cell is referred to as cell 1. (**B**) Left: cell 1 was mechanically stimulated, while the preparation was continuously superfused (1.5–2 ml/min); a $[Ca^{2+}]_i$ increase was observed only in cell 1. Right: in the absence of continuous washing of the medium, mechanical stimulation of cell 1 induced a $[Ca^{2+}]_i$ rise in non-connected neighbouring cells. An increase in $[Ca^{2+}]_i$ was observed first in the mechanically stimulated cell (tracing 1), then in nearby cells, and finally in more distant cells (tracings 2–4). Tracings have been shifted arbitrarily along the *y*-axis for clarity. Recording of the traces was interrupted for 1 min between the experiments shown on the left and on the right.

Gap junction involvement in the propagation and coordination of intercellular Ca^{2+} waves

The long-chain alcohol octanol reduces gap junction permeability (Chanson *et al.*, 1989), and these properties have been described in hepatocytes (Spray *et al.*, 1986; Saez *et al.*, 1989). However, as described in other cell types (Deutsch *et al.*, 1995; Charles *et al.*, 1996; Young and Hession, 1996), octanol strongly diminished or even abolished hormone-induced $[Ca^{2+}]_i$ oscillations in single hepatocytes (data not shown). Thus, in addition to cell uncoupling, octanol may interfere with cellular processes involved in second messenger metabolism, impairing generation and spread of intra- and intercellular Ca^{2+} waves. We therefore tested the effects of a more specific and non-toxic agent, 18 α-glycyrrhetinic acid (AGA), which inhibits gap junction coupling in many cell types (Davidson *et al.*, 1986; Davidson and Baumgarten., 1988; Goldberg *et al.*, 1996) including rat liver epithelial cell lines (Guan *et al.*, 1996). Hepatocyte triplets were injected with fura2 (5 mM in the pipette) in control conditions or after incubation with 20 μM AGA for 10 min. In control conditions, Figure 3A shows that, after fura2 injection in the intermediate cell of a triplet, the dye began to be detectable in the two adjacent cells within ~10 s, so that these cells became fluorescent. In most cases, a steady-state level of fluorescence was reached in the non-injected cells after ~7 min (7 ± 1.2 min from four triplets; Figure 3A). It should be noted that intercellular fura2 transfer occurred in all doublets and triplets with dilated bile canaliculi, indicating that junctional cell coupling was efficient in these cases. Fura2 did not appear in non-injected cells as long as AGA (20 μM) was present in the medium (Figure 3B). When AGA was removed by washing the cells with a saline solution, fluorescence increased progressively in the non-injected cells (Figure 3B), confirming, as previously reported in other cell types (Davidson *et al.*, 1986), that the effect of AGA on gap junction cell coupling was reversible.

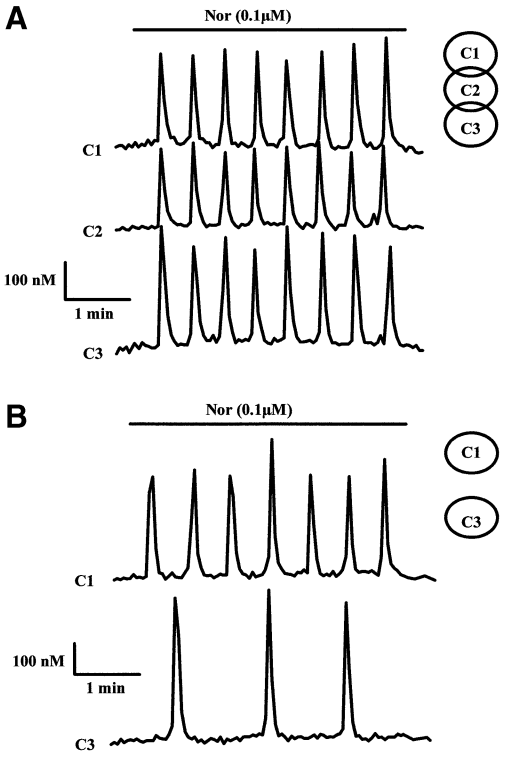

Fig. 2. Effect of intermediate cell excision on noradrenaline-induced $[Ca^{2+}]_i$ oscillations in rat hepatocyte triplets. Hepatocytes loaded with fura2 were challenged with noradrenaline (Nor, 0.1 μM) for the time shown by the horizontal bars. (**A**) Tightly coordinated $[Ca^{2+}]_i$ oscillations in the three connected cells under hormonal stimulation. (**B**) The intermediate cell was excised to eliminate physical contact between the two remaining cells, which were then perfused for at least 10 min and challenged with the agonist. This trace is representative of those obtained for seven triplets in four independent experiments. Tracings have been shifted arbitrarily along the *y*-axis for clarity.

We then tested the effects of AGA on cell coupling during noradrenaline-induced Ca^{2+} signals. Hepatocyte triplets were perfused with noradrenaline (0.1 μM) in the presence of AGA (20 μM) (Figure 4). Well-coordinated noradrenaline-induced $[Ca^{2+}]_i$ oscillations became highly uncoordinated. After removing AGA, $[Ca^{2+}]_i$ oscillations progressively recovered their synchronized pattern (Figure 4). Note that AGA, unlike octanol, did not significantly change the frequency of noradrenaline-induced $[Ca^{2+}]_i$ oscillations in single cells (data not shown). This result confirms that gap junctions are indeed involved in the coordination of $[Ca^{2+}]_i$ oscillations in the different hepatocytes of multicellular systems.

Is the noradrenaline-induced Ca^{2+} transient of one cell sufficient to trigger an intercellular Ca^{2+} wave in a multicellular system?

Our findings suggest that a signal diffuses from cell to cell, thereby coordinating and propagating intercellular Ca^{2+} waves. Although microinjected Ca^{2+} or $InsP_3$ in one cell of a doublet increases $[Ca^{2+}]_i$ in the connected cell (Saez *et al.*, 1989), little is known about the extent of such communication during physiological stimulation.

Microperfusion studies. We used a focal microperfusion procedure to investigate this question. One of two fura2-injected connected cells was focally microperfused with noradrenaline together with fluorescein to monitor the

Table I. Kinetics of noradrenaline-induced $[Ca^{2+}]_i$ oscillations before and after excision of intermediate cells in rat hepatocyte triplets

	Cell 1		Cell 3	
	Before excision	After excision	Before excision	After excision
Frequency (osc/min)	1.80 ± 0.3	1.77 ± 0.14	1.80 ± 0.3	0.85 ± 0.2
Delay (s)	22 ± 4	30 ± 8	25 ± 6	85 ± 23

Experimental conditions were as described for Figure 2. The frequency and delay of $[Ca^{2+}]_i$ oscillations were determined in hepatocyte triplets treated with noradrenaline (0.1 μM) for at least 5 min. Cell 2 was excised as described in Materials and methods. Data are the mean ± SEM of three hepatocyte triplets exhibiting the same range of frequencies and delays.

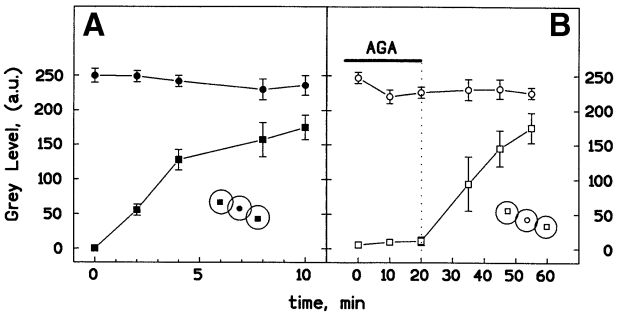

Fig. 3. Inhibition of intercellular dye transfer by AGA in rat hepatocyte triplets. (**A**) Fura2 was microinjected at time 0 into the intermediate cell of hepatocyte triplets. Emitted fluorescence at λ_{ex} = 360 nm was recorded and plotted as a function of time, both for the intermediate cell (●, n = 5) and for the two connected cells (■, n = 10). Results are shown as means ± SEM for five triplets in two independent experiments. (**B**) Hepatocyte triplets were pre-incubated with AGA (20 μM) for 10 min. After injection of fura2 into the intermediate cell (○, n = 3), appearance of fura2 fluorescence in connected cells was totally inhibited (□, n = 6) until AGA was washed out. Results are shown as the means ± SEM for three triplets in two independent experiments.

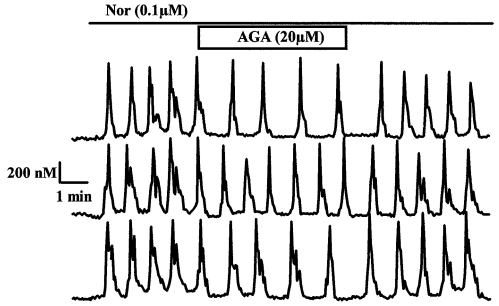

Fig. 4. Effects of AGA treatment on noradrenaline-induced $[Ca^{2+}]_i$ oscillations in rat hepatocyte triplets. Hepatocytes loaded or injected with fura2 were challenged with noradrenaline (Nor, 0.1 μM) for the time shown by the horizontal bar. AGA (20 μM) was applied as indicated by the open box. Tracings represent $[Ca^{2+}]_i$ in three connected cells, and have been shifted arbitrarily along the *y*-axis for clarity. Following addition of AGA to the bath together with the agonist, noradrenaline induced non-coordinated $[Ca^{2+}]_i$ oscillations in the three cells, each cell responding with its own frequency. Following AGA washout, the three cells rapidly recovered synchronized $[Ca^{2+}]_i$ oscillations. These results are representative of those obtained using five triplets in two independent experiments.

area perfused (see Materials and methods and Figure 8). The noradrenaline-treated cell exhibited $[Ca^{2+}]_i$ oscillations and the non-perfused cell did not (Figure 5, left part). Similarly, when the focal noradrenaline perfusion was restricted to the other cell, $[Ca^{2+}]_i$ oscillations were limited to that hepatocyte and did not show up in the connected cell (Figure 5, middle part). At the end of this experiment, the doublet was globally superfused with noradrenaline (0.1 μM), and both cells then exhibited $[Ca^{2+}]_i$ oscillations which were tightly coordinated (Figure 5, right part). It should be noted that, when stimulated independently by microperfusion, the two connected cells did not exhibit the same delays and $[Ca^{2+}]_i$ oscillation frequencies, whereas they were tightly coordinated when globally superfused.

These results show that intracellular rises in $InsP_3$, Ca^{2+} or other second messengers elicited by an $InsP_3$-dependent hormone, although required for coordination, are not sufficient to trigger an intercellular Ca^{2+} wave. As in previous experiments (see Figure 2), these results also suggest that one cell in the multiplet is able to impose its frequency and delay on the other connected cells.

Sudden noradrenaline removal during agonist-induced Ca^{2+} response. We then used an alternative technical approach to verify the observation reported above. Fura2-loaded hepatocyte multiplets were stimulated with noradrenaline until the first cell(s) had fully reached its peak(s) $[Ca^{2+}]_i$ rise, then the hormone was removed rapidly (see Materials and methods). The triplet displayed coordinated $[Ca^{2+}]_i$ oscillations before noradrenaline removal, but one of the three connected cells no longer responded when the hormone was suddenly removed after the first and the second cells had responded (Figure 6). This confirms that agonist-induced Ca^{2+} mobilization in one hepatocyte is not sufficient to trigger an intercellular Ca^{2+} wave in connected cells. The presence of noradrenaline appears to be required at each hepatocyte for the response of the whole multicellular system.

Heparin microinjection. We finally studied the role of putative $InsP_3$ intercellular diffusion in synchronization of $[Ca^{2+}]_i$ oscillations by microinjecting heparin in the intermediate cell of a triplet. Heparin inhibits both $InsP_3$ binding and the resulting $InsP_3$-induced Ca^{2+} release (Worley *et al.*, 1987; Cullen *et al.*, 1988) as well as α_1-adrenoreceptor–G protein coupling (Dasso and Taylor, 1991). Triplets were loaded with fura2 and treated with noradrenaline (0.1 μM), eliciting trains of coordinated $[Ca^{2+}]_i$ oscillations in the three connected cells (Figure 7, left panel). After washing out noradrenaline, the intermediate cell of the triplet was microinjected with heparin. The renewed superfusion of noradrenaline (0.1 μM) elicited $[Ca^{2+}]_i$ oscillations only in the non-injected cells, whereas $[Ca^{2+}]_i$ remained at a low basal level in the heparin-injected cell (Figure 7, bottom panel). $[Ca^{2+}]_i$ oscillations in the two remaining responding cells were coordinated for the first minute, and exhibited an increasing difference in their frequencies. To ensure that the intermediate cell had not been damaged during the heparin microinjection process, the triplet was treated with taurolithocholate sulfate (200 μM), a Ca^{2+}-mobilizing agent which does not involve $InsP_3$-dependent mechanisms (Capiod *et al.*, 1991). This elicited a $[Ca^{2+}]_i$ rise in the three cells of the triplet (Figure 7, right panel). When heparin was injected together with fura2 in the intermediate cell of triplets, similar results were observed in the two remaining responding cells (data not shown).

These results confirm that Ca^{2+} does not diffuse from cell to cell in detectable amounts during noradrenaline stimulation and that intercellular transfer of $InsP_3$, probably insufficient towards cell 3 in these experiments, may be involved in the synchronization of agonist-induced $[Ca^{2+}]_i$ oscillations.

Discussion

Although molecular mechanisms underlying intracellular Ca^{2+} signalling have received considerable attention (Berridge, 1993; Pozzan *et al.*, 1994; Thomas *et al.*, 1996), intercellular propagation of Ca^{2+} signals, which constitutes

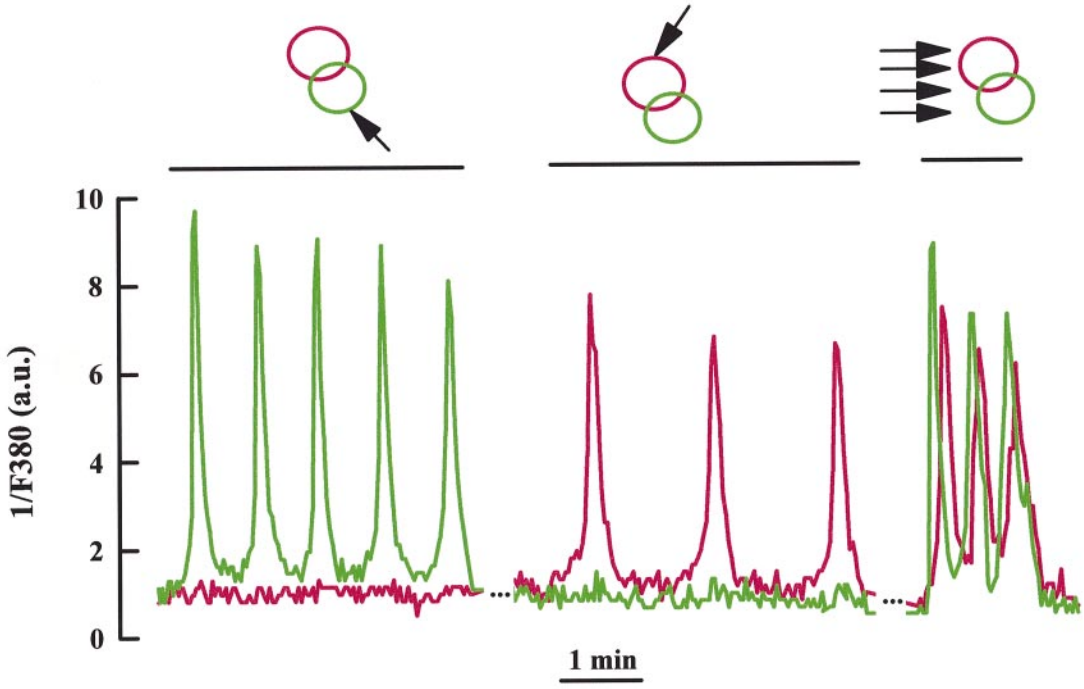

Fig. 5. Focal stimulation of connected rat hepatocytes. Hepatocytes were loaded or injected with fura2. The left, middle and right parts of the figure show successive measurements of $[Ca^{2+}]_i$ in the same hepatocyte doublet. The left and middle parts show the Ca^{2+} response, when one cell within the doublet was focally microperfused with noradrenaline (10 μM in the micropipette) for the time shown by the upper horizontal bars. In these conditions, only the stimulated cell (indicated by an arrow) within the doublet responded. In the right part, at the end of the experiment, the doublet was globally superfused with noradrenaline (0.1 μM): both cells of the doublet exhibited tightly coordinated $[Ca^{2+}]_i$ oscillations. The cell preparation was alternately illuminated at 450 nm to gauge the extent of the microperfused area, and at 380 nm to measure noradrenaline-induced $[Ca^{2+}]_i$ increases. Thus, tracings are the inverted graphical representation of the Ca^{2+}-associated fluorescence decrease observed at λ_{ex}= 380 nm, λ_{em} = 510 nm. For technical convenience, tracings were interrupted (the gap represents 3 min). These results are representative of those obtained using four doublets in three independent experiments.

a potentially important mechanism of cell–cell communication, is poorly understood. Coordination and propagation of agonist-induced intercellular Ca^{2+} signals in connected hepatocytes have been reported previously but the mechanisms have not been clarified (Nathanson and Burgstahler, 1992; Combettes *et al.*, 1994; Nathanson *et al.*, 1995; Robbgaspers and Thomas, 1995).

Paracrine communication does not contribute to intercellular Ca^{2+} wave coordination and propagation

In several cell types, including isolated rat hepatocytes, mechanical stimulation induces the release of molecules into the extracellular medium, inducing a $[Ca^{2+}]_i$ increase in adjacent cells (Osipchuk and Cahalan, 1992; Grierson and Meldolesi, 1995; Palmer *et al.*, 1996; Schlosser *et al.*, 1996). It was not known whether such paracrine secretion co-exists with direct gap junctional communication during hormone stimulation in hepatocytes. We demonstrated that paracrine mechanisms cannot account for the highly coordinated intercellular Ca^{2+} waves observed under high perfusion flow rates (see Figure 1). Moreover, no coordination of intercellular Ca^{2+} signals was observed between neighbouring non-connected hepatocytes (data not shown), as would have been expected if there was paracrine secretion. Finally, as we have reported previously, the direction of wave propagation was not related to the direction in which the cells were superfused (Combettes *et al.*, 1994; and data not shown).

Gap junctional cell coupling contributes to coordinate intercellular Ca^{2+} signals

Thus, intercellular connections are presumably responsible for intercellular coordination and propagation of Ca^{2+} signals. Freshly isolated multicellular systems of rat hepatocytes are composed of connected cells with different types of intercellular junctions, especially gap junctions and components of tight junctions (Gautam *et al.*, 1987). This role of gap junctions had been reported previously in octanol-treated hepatocyte couplets (Nathanson and Burgstahler, 1992). We confirmed this conclusion by using the more specific gap junction blocker AGA. In our experiments, noradrenaline-induced $[Ca^{2+}]_i$ oscillations were not synchronized in the presence of AGA, indicating that communication via gap junctions indeed controls the synchronization of Ca^{2+} signals between connected cells. Finally, intermediate cell excision from a triplet confirmed that coordinated changes in $[Ca^{2+}]_i$ in connected cells were not simply the result of independent responses of individual cells showing the same $[Ca^{2+}]_i$ oscillation frequency. Note that intermediate cell excision had larger effects on noradrenaline-induced Ca^{2+} signalling in the remaining cells than gap junction inhibition alone, suggesting an additional role for tight junctions or adhesion molecules (Dedhar and Hannigan., 1996).

Diffusion through gap junctions is insufficient to induce intercellular Ca^{2+} waves

Cell–cell propagation of Ca^{2+} signals has been observed in cultured tracheal epithelial cells and in glial cells (for

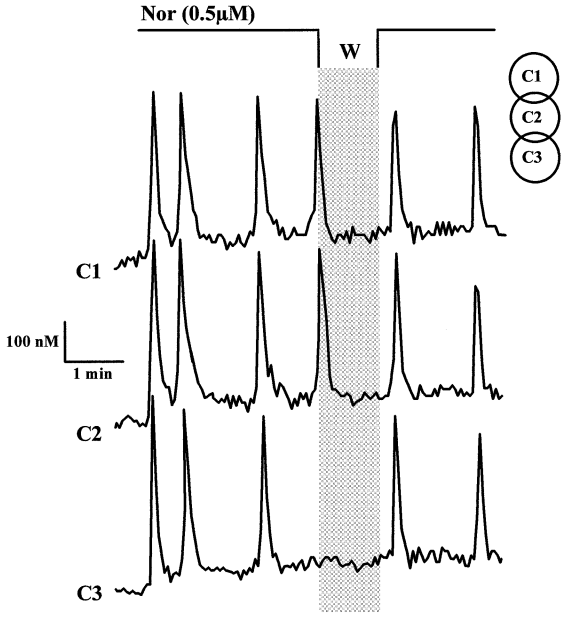

Fig. 6. Effect of sudden noradrenaline removal during coordinated $[Ca^{2+}]_i$ oscillations in multicellular systems of rat hepatocytes. Hepatocytes loaded with fura2 were challenged with noradrenaline (Nor, 0.5 µM) for the time shown by the horizontal bar. The medium was washed out rapidly (W) as indicated by the step (and dashed area). Tracings have been shifted arbitrarily along the *y*-axis for clarity. Following noradrenaline addition to the bath, intercellular Ca^{2+} waves initiated in cell 1 (determined by examination of a video recording) propagate to cells 2 and 3. Following washout of noradrenaline at the peak of the $[Ca^{2+}]_i$ increase in cell 2 (see third oscillation), there was no further propagation to cell 3. These results are representative of those obtained using eight triplets in four independent experiments.

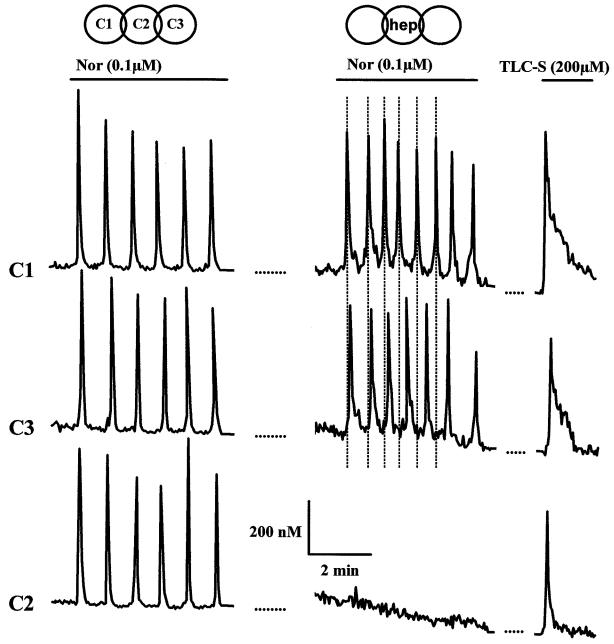

Fig. 7. Effect of heparin microinjection on noradrenaline-induced $[Ca^{2+}]_i$ oscillations in multicellular systems of rat hepatocytes. Hepatocyte triplets loaded or injected with fura2 were challenged with noradrenaline (Nor, 0.1 µM) for the time shown by the horizontal bars. Tracings, representing $[Ca^{2+}]_i$ in the three connected cells, have been shifted arbitrarily along the *y*-axis for clarity. The left, middle and right parts of the figure show successive measurements 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 and intercellular Ca^{2+} waves, initiated in cell 1 (determined by examination of a video recording) and propagating to cells 2 and 3. The intermediate cell of the triplet was then injected with heparin (10 mg/ml in the pipette) as described in Materials and methods. After injection (middle part), noradrenaline elicited $[Ca^{2+}]_i$ oscillations in cell 1 and cell 3 but not in the injected cell (cell 2), in which $[Ca^{2+}]_i$ stayed at a low basal level. Note that cell 3 rapidly developed a different oscillation frequency. Dotted lines have been added to facilitate the comparison between cell 1 and cell 3 frequencies. At the end of the experiment, the same triplet was challenged with taurolithocholate sulfate (TLC-S, 200 µM), which elicited a Ca^{2+} response in the three connected cells (right part). These results are representative of those obtained using five triplets in four independent experiments.

review, see Sanderson *et al.*, 1994), as well as in pancreatic acinar and β cells (Loessberg-Stauffer *et al.*, 1993; Meda, 1996; Yule *et al.*, 1996). In these different cell types, intercellular Ca^{2+} waves are thought to be mediated by passive $InsP_3$ diffusion through gap junctions from one stimulated cell to the connected cells (Boitano *et al.*, 1992; Demer *et al.*, 1993; Sneyd *et al.*, 1995; Yule *et al.*, 1996). To study this putative diffusion of $InsP_3$, heparin was injected into the intermediate cell within a hepatocyte triplet. After heparin injection, $[Ca^{2+}]_i$ did not increase in the injected cell, and noradrenaline-induced $[Ca^{2+}]_i$ oscillations in the two remaining responding cells rapidly became unsynchronized. This confirms that cell–cell diffusion of Ca^{2+} is not detectable during hormone stimulation and suggests that, alternatively, intercellular transfer of $InsP_3$ could be implicated in the synchronization of agonist-induced $[Ca^{2+}]_i$ oscillations. Yet, diffusion of $InsP_3$ is not sufficient by itself to propagate intercellular Ca^{2+} waves. Using different technical approaches, we found that stimulation of only one hepatocyte in a multiplet is not sufficient by itself to trigger a $[Ca^{2+}]_i$ rise in adjacent connected cells. Thus, passive diffusion to connected cells of the messengers produced in the stimulated cell cannot alone drive intercellular Ca^{2+} waves in multicellular systems of rat hepatocytes. This is clearly demonstrated by focal microperfusion experiments (Figure 5), and confirmed by the sudden removal of agonist (Figure 6). The apparent discrepancy with previous observations on hepatocyte couplets could have resulted from the use of either $InsP_3$ injection (Saez *et al.*, 1989) or addition of a maximal concentration of vasopressin (Nathanson and Burgstahler, 1992), the most efficient agonist for increasing intracellular $InsP_3$ concentration in hepatocytes (Hansen *et al.*, 1986; Combettes *et al.*, 1988). Both types of cell stimulation probably induce stronger $InsP_3$ intracellular increases than perfusion of low agonist concentrations, leading to sufficient $InsP_3$ intercellular diffusion. It should be noted that in the intact liver, intercellular Ca^{2+} waves can be elicited by $InsP_3$-dependent agonists in a range of concentrations similar to that used in this study (Nathanson *et al.*, 1995; Robbgaspers and Thomas, 1995).

Agonist–receptor interactions at each cell are required to generate intercellular Ca^{2+} waves

When connected by gap junctions, hepatocytes, like other epithelial cells, are considered to be like a syncytial system, throughout which information acquired by one cell can be transferred freely to all other cells (Bruzzone *et al.*, 1996; Meda, 1996). Nevertheless, as shown above,

a simple syncytial transmission of messengers cannot be the sole basis for the propagation of intercellular Ca^{2+} waves in multicellular systems of rat hepatocytes. The presence of the hormone is required by each cell of the hepatocyte multiplet for the generation of an intercellular Ca^{2+} wave. These stringent conditions for propagation of coordinated Ca^{2+} signals in epithelial cells have not been described in any other multicellular system. This liver-specific type of intercellular Ca^{2+} wave propagation may result from the morphological organization of the liver cell plate, a one cell thick cord entirely bathed in blood-borne factors including hormones through sinusoids (Gumuccio *et al.*, 1994). More generally, our results might indicate that the concept of an excitable medium—previously shown to allow characterization of Ca^{2+} waves in large cells like oocytes (Lechleiter and Clapham, 1992; Berridge, 1997)—can usefully be applied to the assembly of much smaller cells (like hepatocytes) communicating via intercellular junctions. To allow the Ca^{2+} signal to propagate from cell to cell, diffusion is required (presumably $InsP_3$ diffusion through gap junctions), but each cell must also have reached a certain level of excitability, ensured by the presence of noradrenaline. Only under such conditions will diffusion from cell to cell of a small additional stimulus be sufficient to synchronize the otherwise uncoordinated oscillations of the various cells. Heparin perfusion into the intermediate cell of a triplet, or sudden removal of the agonist, will drive this cell into a refractory state which will prevent propagation of the wave.

Evidence for a 'pacemaker-like' organization in multicelllar systems of rat hepatocytes

The requirement for the multicellular system to have all of its individual cells in a state of sufficient excitability for wave propagation to occur provides the explanation for the fact that upon coupling of these individual oscillating units, the cell with the shortest delay and the highest frequency of oscillation will act as a pacemaker for the other cells. One hepatocyte in a multiplet can thus impose its delay and frequency of $[Ca^{2+}]_i$ oscillations on other connected cells. When completely disconnected, previously coupled hepatocytes recover an individual pattern of Ca^{2+} signalling, one cell exhibiting a shorter delay and a faster $[Ca^{2+}]_i$ oscillation frequency than the other (Figure 2). Similarly, when individually stimulated, two adjacent connected hepatocytes differ with regard to these two Ca^{2+} signalling variables, whereas global stimulation of the cell pair results in equal $[Ca^{2+}]_i$ oscillation frequencies with an intercellular time lag of a few seconds (Figure 5). These results suggest that agonist-induced intercellular Ca^{2+} waves are initiated by an $InsP_3$ increase in the most sensitive cell (Combettes *et al.*, 1994). Intercellular diffusion of $InsP_3$, although insufficient by itself to produce a $[Ca^{2+}]_i$ increase in connected cells, may synchronize $[Ca^{2+}]_i$ oscillations in coupled hepatocytes. Such an intercellular organization of signalling has not been reported previously in non-excitable and non-automatic epithelial cells and can be compared with cell–cell triggering in cardiac pacemaker cells (Irisawa *et al.*, 1993). We previously have suggested that this Ca^{2+} signalling pattern could result from an *in situ* gradient of cellular sensitivity to agonists in hepatocyte plates (Tordjmann *et al.*, 1996), a possibility which needs to be investigated further.

In this study on multicellular systems of rat hepatocytes, we show that noradrenaline-induced $[Ca^{2+}]_i$ oscillations are synchronized at a multicellular level by diffusion of a messenger through gap junctions, and that intercellular Ca^{2+} waves do not propagate through a mechanism only involving passive cell–cell diffusion. We identified three factors which are required simultaneously to trigger coordinated intercellular Ca^{2+} waves. First, gap junction coupling allows diffusion of an intercellular messenger; second, the presence of the agonist is required to ensure excitability of each cell in the multicellular system; and third, functional cellular differences between adjacent hepatocytes may be the basis of sequentially ordered patterns of Ca^{2+} signalling through a 'pacemaker-like' mechanism.

Materials and methods

Materials

Fura2-PE3/AM and fura2 were obtained from Teflab and Molecular Probes Inc. respectively, 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

Single hepatocytes and multicellular systems were prepared from fed female Wistar rats by limited collagenase digestion of rat liver, as previously described (Combettes *et al.*, 1994). After isolation, rat hepatocytes were maintained (2×10^6 cells/ml) at 4°C in Williams' medium E supplemented with 10% fetal calf serum, penicillin (200 000 U/ml) and streptomycin (100 mg/ml). Cell viability, assessed by trypan blue exclusion, remained >96%, during 4–5 h.

Loading of hepatocytes with fura2

Hepatocytes were loaded with fura2 either by injection (see below) or by incubation with the dye. As fura2 may be partly compartmentalized (Chiavaroli *et al.*, 1994; Combettes *et al.*, 1996), hepatocytes were loaded with the new fura2 analogue, fura2-PE3, which has the same spectroscopic characteristics as fura2 but which is less susceptible to compartmentalization (Vorndran *et al.*, 1995). 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 glass dish coverslips coated with collagen I, and incubated for 60 min 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-PE3/AM. The hepatocytes were incubated for 30 min at 37°C under an atmosphere containing 5% CO_2. The coverslips were then washed twice with a saline solution (10 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).

As previously described (Combettes *et al.*, 1994), freshly isolated doublets and triplets 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).

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

Dish coverslips were put onto a thermostatted holder (34°C) on the stage of a Zeiss Axiovert 35 microscope set up for epifluorescence microscopy. The excitation light was supplied by a high pressure xenon arc lamp (75 W), and the excitation wavelengths were selected by 340 and 380 nm filters (10 nm bandwidth) mounted in a processor-controlled rotating filter wheel (Lhesa, France) between the UV lamp and the microscope.

Ca^{2+} imaging was as described by Combettes *et al.* (1994). Briefly, fluorescence images were collected by a low-light level ISIT camera (Lhesa, France), digitized and integrated in real time by an image processor (IMSTAR, France). $[Ca^{2+}]_i$ was determined in individual cells from the ratio of 340/380 nm fluorescence values as described by Grynkiewicz *et al.* (1985) using a K_d for Ca^{2+}–dye of 250 nM.

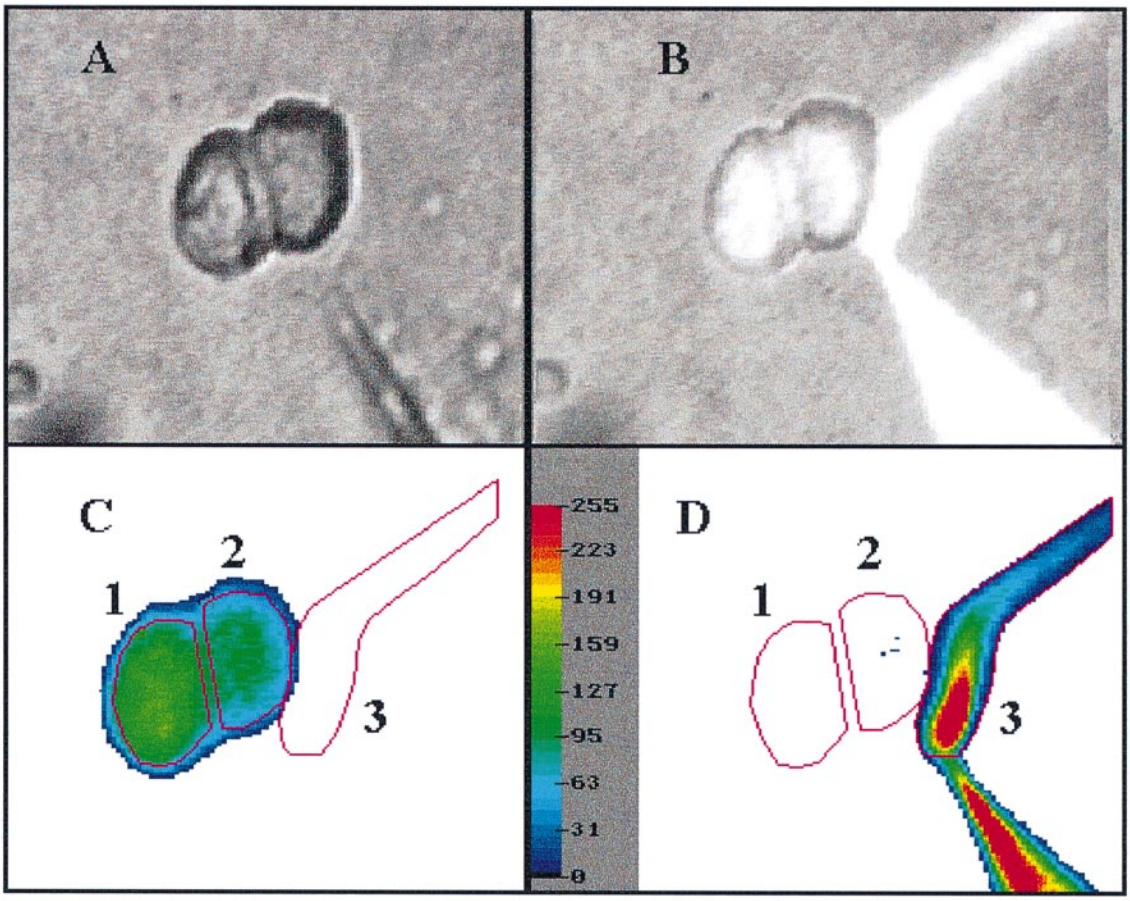

Fig. 8. Optical control of focal microperfusion. (**A**) A hepatocyte doublet was observed under phase-contrast light microscopy (×40) and a micropipette was brought carefully to the cell of interest. Noradrenaline was then applied focally (microperfusion), as described in Materials and methods, by positioning the micropipette close to the cell and applying a constant pressure (60 hpa), to deliver picolitre quantities of agonist-containing solution together with carboxyfluorescein (30 μM). (**B**) Carboxyfluorescein image superimposed on the phase-contrast image, demonstrating the absence of dye diffusion against the washing perfusion flow. (**C**) Cells loaded with fura2 were illuminated at 380 nm and fluoresced brightly. (**D**) When illuminated at 450 nm, cells are no longer observed, as expected. In contrast, the carboxyfluorescein included in the microperfused solution fluoresced very brightly and allowed the extent of the microperfused area to be gauged. Regions of interest (limits of the cells and of the microperfused solution) are outlined in red, showing that only one cell was perfused in these conditions. Images (C and D) have been pseudocoloured to represent fluorescence intensity. The pseudo-colour scale is displayed on the left of (D).

R_{max} and R_{min} were determined as described previously (Combettes *et al.*, 1994).

Superfusion, microinjection, mechanical stimulation and microperfusion of cells

Superfusion. Cells were superfused continuously with control or test solutions by six inlet tubes converging on the coverslip chamber. The perfusion rate was 1.5–2 ml/min and the chamber volume was ~0.2 ml. The medium was renewed continuously by aspiration. The agonist was removed rapidly during the Ca^{2+} response with this superfusion system, at a perfusion rate of 4 ml/min to improve the washing efficiency. Octanol-containing solutions were not added through the superfusion inlet tubes because of the affinity of this molecule for hydrophobic plastic surfaces. These solutions were transferred directly into the chamber with a glass pipette.

Microinjection. Microinjection was performed according to the method of Graessmann *et al.* (1980). Briefly, we used an Eppendorf 5242 Microinjector mounted on the stage of an Axiovert 35 microscope, positioned on a vibration isolated platform. 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 20 mM K_2HPO_4, 10 mM NaH_2PO_4, 30 mM KH_2PO_4 adjusted to pH 7.1. Solutions were filtered through a 0.22 μm filter. After microinjection, cells were allowed to recover for at least 10 min. Excision of intermediate cells of hepatocyte triplets was performed with this microinjection system, by impaling and carefully removing the cell with a micropipette so that no physical contact persisted between the two remaining cells. The success of both microinjection and cell excision 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 $[Ca^{2+}]_i$.

Microperfusion. Agents were applied locally by positioning a micropipette (Femtotips, Eppendorf) close to the cell of interest (Figure 8A) and applying a constant pressure (60 hpa) via the Eppendorf injector, delivering picolitre quantities of agonist-containing solution. In these experiments, carboxyfluorescein (30 μM) was included in the micropipette to gauge the extent of the microperfused area (see Figure 8B and D). As expected, the best focused cell treatment was ensured by concurrently applying the microperfused agent and a continuous retrograde washing perfusion at a rate of 4 ml/min (Figure 8B–D). In these experiments, the 340 nm excitation wavelength was changed to 450 nm to monitor the carboxyfluorescein image. Consequently, fura2 fluorescence change was only observed at 380 nm.

Mechanical stimulation. Single cells were mechanically stimulated by gradually deforming the hepatocyte membrane with a micropipette, as described by Schlosser *et al.* (1996).

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