Ca²⁺-mobilizing hormones induce sequentially ordered Ca²⁺ signals in multicellular systems of rat hepatocytes

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The development of hormone-mediated Ca²⁺ signals was analysed in polarized doublets, triplets and quadruplets of rat hepatocytes by video imaging of fura2 fluorescence. These multicellular models showed dilated bile canaliculi, and gap junctions were observed by using an anti-connexin-32 antibody. They also showed highly organized Ca²⁺ signals in response to vasopressin or noradrenaline. Surprisingly, the primary rises in intracellular Ca²⁺ concentration ([Ca²⁺]_i) did not start randomly from any cell of the multiplet. It originated invariably in the same hepatocyte (first-responding cell), and then was propagated in a sequential manner to the nearest connected cells (cell 2, then 3, in triplets; cell 2, 3, then 4 in quadruplets). The sequential activation of the cells appeared to be an intrinsic property of multiplets of rat hepatocytes. (1) In the continued presence of

hormones, the same sequential order was observed up to six times, i.e. at each train of oscillations occurring between the cells. (2) The order of [Ca²⁺]_i responses was modified neither by the repeated addition of hormones nor by the hormonal dose. (3) The mechanical disruption of an intermediate cell slowed down the speed of the propagation, suggesting a role of gap junctions in the rapidity of the sequential activation of cells. (4) The same multiplet could have a different first-responding cell for vasopressin or noradrenaline, suggesting a role of the hormonal receptors in the sequentiality of cell responses. It is postulated that a functional heterogeneity of hormonal receptors, and the presence of functional gap junctions, are involved in the existence of sequentially ordered hormone-mediated [Ca²⁺]_i rises in the multiplets of rat hepatocytes.

INTRODUCTION

In situ, the hepatocyte is a complex polarized epithelial cell. Its plasma membrane displays three morphologically and functionally different regions. The sinusoidal domain is in contact with the plasma and contains the hormonal receptors. The lateral domain is involved in cell–cell interaction and segregates gap and tight junctions. Finally, the canalicular domain is involved in secretion of bile acids and formation of native bile (for references, see Schachter, 1988; Nathanson and Boyer, 1991; Evans et al., 1992; Arias et al., 1993). During preparation of isolated hepatocytes, the resulting single cells lose their polarity, and all the proteins originally segregated into the different domains redistribute randomly throughout the whole plasma membrane (Maurice et al., 1985; Groothuis et al., 1981; Zeitlin and Hubbard, 1982). However, occasionally the hepatocyte suspensions contain doublets (or pairs of cells) which can express, under certain conditions, properties of epithelial cells (Gautam et al., 1987). These doublets display morphologically and functionally distinct domains in their plasma membrane (Maurice et al., 1985; Nathanson and Boyer, 1991; Arias et al., 1993). For instance, the sinusoidal domain, which contains the hormonal receptors, segregates PtdIns(4,5)P₂ (Tran et al., 1993). The lateral domain of the plasma membrane of the doublet contains tight junctions and gap junctions (Graf et al., 1984; Spray et al., 1986; Gautam et al., 1987; Saez et al., 1989), and the canalicular domain segregates the specific proteins involved in the secretion of native bile (Nathanson and Boyer, 1991; Arias et al., 1993).

Although they have lost their polarity, single cells have been widely used to study hormone-mediated Ca²⁺ signals (for refer-

ences, see Jacob, 1990; Claret et al., 1991; Menniti et al., 1992; Berridge, 1993). In single hepatocytes, the InsP₃-dependent hormones generate Ca²⁺ oscillations, the frequency of which is tightly dependent on the concentration of agonists (Woods et al., 1986; Cobbold et al., 1989; Rooney et al., 1990; Thomas et al., 1991; Capiod et al., 1991). In 24 h cultures of rat hepatocytes, video imaging has shown that the intracellular Ca²⁺ wave arises from a specific sub-plasma-membrane locus, and propagates across the hepatocyte at a constant speed of 20–25 μm/s, independent of the hormone concentration (Rooney et al., 1990; Thomas et al., 1991). In polarized doublets of rat liver cells, Nathanson and Burgstahler (1992a) and Nathanson et al. (1993) have reported that InsP₃-linked agonists, namely vasopressin and the α₁-adrenergic agonist phenylephrine, induce different types of [Ca²⁺]_i rises with equal frequency: either homogeneous increases of [Ca²⁺]_i throughout the cytoplasm, or local [Ca²⁺]_i rises at the sinusoidal domain of the plasma membrane that spread at a constant rate to the canaliculus (basal to apical), or Ca²⁺ waves initiated from the canalicular domain (apical to basal Ca²⁺ wave). In any case, the Ca²⁺ wave then spreads across the adjacent cell of the hepatocyte doublet, in a constant wave-like fashion. Other interesting studies have demonstrated the importance of epithelial polarity for localized or propagated variations of [Ca²⁺]_i in acinar cells (Nathanson et al., 1992; Kasai et al., 1993; Thorn et al., 1993; Loessberg Stauffer et al., 1993) and many other cells (Sanderson et al., 1994).

We report an analysis of the hormone-mediated Ca²⁺ signals, by video imaging of fura2 fluorescence, in multicellular models which retain expression of polarized functions (doublets, triplets and quadruplets of rat hepatocytes). Our results show that the

Abbreviations used: [Ca²⁺]_i, concentration of Ca²⁺ in the cytosol; InsP₃, inositol 1,4,5-trisphosphate.

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Ca^{2+} signals do not arise randomly from any cell constituting the multiplet, but originate in a particular cell presumably more sensitive to hormones than its associated cells.

MATERIALS AND METHODS

Materials

Anti-connexin-32, a purified antibody directed against a synthetic peptide of 17 amino acids corresponding to residues 266–282 of the C-terminus of the rat liver connexin-32, was previously raised in chicken, and purified and characterized (Mazet et al., 1992), and was generously given by Professor D. Gros. It was used at a concentration of 3 $\mu\text{g}/\text{ml}$. Fluorescein-conjugated second antibody, rabbit anti-chicken IgG, was purchased from Jackson ImmunoResearch Laboratories. Fura2/AM was obtained from Molecular Probes. Collagenase was from Worthington or Boehringer Mannheim. Williams' Medium E was from GIBCO. All other chemicals used were from Sigma.

Preparation of primary cultures of single cells, doublets, triplets and quadruplets of rat hepatocytes

Multicellular and single hepatocytes were prepared by collagenase perfusion of liver from fed female Wistar rats as previously described (Combettes et al., 1986; Mauger et al., 1989). Rat hepatocytes were maintained (2×10^6 cells/ml) in Williams' medium E supplemented with 10% foetal-calf serum, penicillin (200 000 units/ml), streptomycin (100 mg/ml), and gassed with O_2/CO_2 (19:1) (pH 7.4) at 22 °C. Cell viability, estimated by Trypan Blue exclusion, was consistently above 96% and remained stable for 4–5 h.

Loading of hepatocytes with fura2

Small portions of the suspended liver cells (5×10^6 cells) were diluted in 2 ml of Williams' medium E supplemented with 10% foetal calf serum, penicillin (200 000 units/ml), streptomycin (100 mg/ml) and 2–3 μM fura/AM, then plated on to glass dish coverslips coated with collagen and incubated for 60 min at 37 °C under an atmosphere containing 5% CO_2 (Gryniewicz et al., 1985). The dish coverslips were made by cutting a hole (12 mm diameter) in small plastic Petri dishes (35 mm diameter) and attaching a glass coverslip to the bottom of each dish with adhesive (Selgard) to cover the hole.

Polarized doublets and triplets were distinguished from aggregates of non-connected cells or from doublets and triplets which have lost their polarity by screening dilated bile canaliculi, taken as a marker of polarity (Gautam et al., 1987). These authors have shown that most of the canalicular spaces in freshly isolated doublets of rat hepatocytes are collapsed, because the high permeability of tight junctions (freely permeable to Ruthenium Red) prevents them from swelling as bile is secreted. The canalicular domain of the plasma membrane is devoid of the two markers of the canalicular membrane, Mg^{2+} -ATPase and thiamin pyrophosphatase. During monolayer culture, hepatocyte doublets reorganize their canalicular membrane (reorganization appreciable at 1 h, complete within 2.5–4.5 h). The canalicular domain segregates Mg^{2+} -ATPase and thiamin pyrophosphatase, and the canalicular space, which excludes Ruthenium Red, expands its resealed space as bile is secreted. Interestingly, all the doublets with dilated canalicular spaces are polarized (impermeable to Ruthenium Red and segregation of canalicular markers). In the present study, the yield of our polarized preparations with swollen canaliculi was 20–30%. These preparations segregated adequately the usual markers poly-B1 (baso-

lateral), mono-B1 (lateral) and mono-ZO1 (tight junctions) in the right domains of the plasma membrane, and $\text{PtdIns}(4,5)\text{P}_2$ in the sinusoidal domain of the plasma membrane (Tran et al., 1993; and results not shown). Finally, the present results show that the lateral domain of the plasma membrane segregates connexin-32, and that multiplets respond to agonists in exactly the same way (three types of Ca^{2+} waves) as do doublets settled for 4 h (Nathanson and Burgstahler, 1992a; Nathanson et al., 1993).

$[\text{Ca}^{2+}]_i$ imaging

After the fura2 loading period, coverslips were washed twice with modified Eagle's medium (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 , 20 mM Hepes, 1g/l glucose, pH 7.4). Dish coverslips were put into a thermostatically controlled holder on the stage of a Zeiss Axiovert 35 microscope set up for epifluorescence microscopy, and immediately superfused with modified Eagle's medium. The medium incubating the cells was continuously renewed at a flow rate of 1.5 ml/min and the volume maintained constant at 150 μl , so that the time constant of the medium exchange was 6 s. The solution was supplied by several inlet tubes containing control and hormone solutions converging on the chamber. The temperature was kept at 34 °C, because, in agreement with the findings of Kawanishi et al. (1989), intracellular fura2 fluorescence decreased rapidly at 37 °C due to the dye being extruded from liver cells. The excitation light was supplied by a high pressure xenon arc lamp (75 W), and the excitation wavelengths were selected by 350 nm and 380 nm filters (10 nm bandwidth) mounted in a processor-controlled rotating filter between the u.v. lamp and the microscope. Photons emitted by the fura2 within the cells were collected by the objective (Zeiss Neofluar; $\times 40$).

Ca^{2+} imaging, developed by A. Trautmann in collaboration with the IMSTAR Co. (Paris), was essentially as described by Tan et al. (1992) and Donnadiou et al. (1992). Briefly, Ca^{2+} images were acquired by using an intensified charge-coupled device camera with a sensitivity of 10^{-5} lx (Lhesa, France). At low concentrations of hormones which elicited low-frequency $[\text{Ca}^{2+}]_i$ oscillation, $[\text{Ca}^{2+}]_i$ was calculated from 350 nm and 380 nm frame pairs captured at low frequency (every 3 s) to measure a true ratioed image for a prolonged period (10–20 min). In contrast, at high hormone concentrations, which generated fast Ca^{2+} signals lasting several seconds from their initiation to their full development, the frames of the digital video imaging were captured at high frequency to provide an image every 110 ms. A reference image was acquired at 360 nm and the filter was changed to 380 nm a few seconds before hormone application. Up to 100 images were gathered at 380 nm, and the Ca^{2+} images were computed off-line by using the 360 nm reference.

Calibration of fura2 fluorescence to measure $[\text{Ca}^{2+}]_i$ was performed in cells and calculated from the ratio of 350 nm/380 nm fluorescence values as described by Gryniewicz et al. (1985). The results were fitted by using the equation:

$$[\text{Ca}^{2+}] = K_i[(R - R_{\min.})/(R_{\max.} - R)]$$

where R is the ratio between the 350 nm and 380 nm fluorescence values, $R_{\min.}$ and $R_{\max.}$ are the minimum and maximum values of this ratio, and K_i is a proportionality constant. To determine $R_{\max.}$, at the end of an experiment ionomycin (1 μM) was added to the fura2-loaded cells to increase the intracellular Ca^{2+} concentration to high levels, resulting in maximal Ca^{2+} -dependent fura2 fluorescence (Williams and Fay, 1990). To calculate $R_{\min.}$, 3 mM EGTA was then added to the bathing solution.

Localization of gap junctions by immunofluorescence

Single hepatocytes and multicellular systems (hepatocyte doublets, triplets and quadruplets) were plated on to collagen-coated coverslips exactly as described for Ca²⁺ imaging, i.e. in Williams' medium E at concentration of 10⁶ cells/ml for 1 h. Cells were washed and fixed with 2% formaldehyde for 30 min in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4). Hepatocytes were washed with PBS and non-specific binding was eliminated by a 15 min incubation in PBS supplemented with 0.5% ovalbumin. Finally, cells were equilibrated overnight at 4°C with the purified anti-connexin-32 antibody (3 µg/ml). Specificity of the immunofluorescent reaction was verified by omission of the primary antibody. Cells were washed with PBS and incubated with fluorescein-conjugated rabbit anti-chicken IgG at 1/100 dilution for 30 min. Finally, cells were mounted in Citifluor and observed under either an epifluorescence photomicroscope (Axioskop Zeiss) or a laser scanning confocal microscope. Series of optical sections (average of 10–15) of cells were collected with a Bio-Rad MRC-600 laser scanning confocal microscope in mono-label mode.

RESULTS

Doublets and triplets contain bile canaliculi and gap junctions

True polarized doublets or triplets were distinguished from aggregates of non-connected cells, occasionally present, by screening dilated bile canaliculi (Gautam et al., 1987) by conventional light microscopy (see the Materials and methods section). This procedure does not damage the cells, and can thus be used before recording Ca²⁺ signals in fluorescence microscopy. Generally, these canaliculi appeared as depressions of the two adjacent plasma membranes (arrows in Figure 1a). The degree of swelling of the bile canaliculi was variable, which is believed to indicate the existence of a basal secretion of native bile: secretion is low in the absence of stimulatory bile acids (Gautam et al., 1987; Nathanson and Boyer, 1991; Arias et al., 1993).

Another marker of cell polarity is the presence of gap junctions between the connected cells (Malewicz et al., 1990; Evans et al., 1992). In the hepatocyte doublets, gap junctions have been characterized functionally (Graf et al., 1984; Saez et al., 1989), and morphologically by freeze-fracture and transmission electron microscopy in the hepatocyte doublets (Spray et al., 1986). In the present work, they were immunolocalized by using a specific antibody and confocal microscopy. This anti-gap-junction antibody directed against residues 266–282 of the rat connexin-32 has recently been characterized and used to immunolocalize gap junctions in the whole rat liver (Mazet et al., 1992). The antibody labelled local aggregates in the lateral domain of contiguous hepatocytes of the triplet (arrowheads in Figure 1b), a distribution observed in the whole liver (Evans et al., 1992; Mazet et al., 1992). The staining was not observed with control antisera (results not shown). Eight cell doublets, four triplets and one quadruplet were examined by confocal microscopy, and all displayed this segregated distribution of gap junctions.

Spatio-temporal development of sequential hormone-mediated Ca²⁺ signals in multiplets of rat hepatocytes

The hormone-mediated Ca²⁺ signals were examined in multiplets of rat hepatocytes at concentrations ranging from 0.1 to 10 nM vasopressin and from 0.1 to 1 µM noradrenaline. Except for the dose–response relationship shown in Figure 7, only the experiments performed with concentrations close to the EC₅₀ for each

Figure 1 Localization of bile canaliculi by conventional microscopy (a) and of gap junctions by indirect immunofluorescence and confocal microscopy (b) in the different domains of the plasma membrane of doublets and triplets of rat hepatocytes

Primary cultures of hepatocytes, containing single cells, doublets and triplets of hepatocytes, were spread on microscope dish coverslips and equilibrated with Williams' medium E as indicated in the Materials and methods section. (a) One triplet and one doublet observed by conventional microscopy show bile canaliculi at the secretory pole of the plasma membrane (arrows). Magnification $\times 190$. (b) Cells were isolated and fixed with formaldehyde as described in the Materials and methods section. Gap junctions were localized by indirect immunofluorescence after incubation with the anti-connexin-32 antibody (3 µg/ml), an antibody directed against the rat liver connexin-32 C-terminus. The panel shows one triplet of rat hepatocytes observed by scanning laser confocal microscopy. Labelling by the antibody mainly appears as localized patches in the lateral domains of the apposing plasma membranes (arrowheads). One bile canaliculus is also visible (arrow). Magnification $\times 600$.

hormone (1 nM vasopressin and 0.5 µM noradrenaline) are illustrated. The temporal analysis of the subcellular [Ca²⁺]_i variations was done from the digitized images collected either at high frequency (every 100–300 ms for analysis of fast phases of Ca²⁺ signals), or at low frequency (every 2.5–3.0 s for slow Ca²⁺ signals).

Vasopressin and α_1 -adrenergic agonists induced either basal-to-apical or apical-to-basal Ca²⁺ waves, or a uniform increase in [Ca²⁺]_i throughout the cytoplasm (Nathanson and Burgstahler, 1992a; Nathanson et al., 1993). Then, [Ca²⁺]_i increased in a sequential manner in the adjacent cells. Figure 2(A) illustrates a homogeneous [Ca²⁺]_i rise followed by a sequential activation of hepatocytes in a triplet treated with vasopressin. The basal [Ca²⁺]_i (time 61.3 s) was low, although not totally uniform, throughout the cytoplasm of the three cells of the triplet A. The hormone elicited a relatively uniform increase in [Ca²⁺]_i throughout the cytoplasm of the first-responding cell at times 65.8, 66.0 and 66.8 s (Figure 2A, hormone addition at 46 s). Then, after a short pause of a few seconds (time 69 s), [Ca²⁺]_i rose in the

Figure 2 Spatio-temporal development of $[Ca^{2+}]_i$ rises in the different cells of a triplet stimulated by $InsP_3$ -linked hormones (fast kinetics)

Primary cultures of hepatocytes containing fura2-loaded associated hepatocytes were spread on microscope dish coverslips and equilibrated in modified Eagle's medium (34 °C) as indicated in the Materials and methods section. Ca^{2+} images were acquired by using an intensified charge-coupled device camera. The 350 nm and 380 nm frames were captured at high frequency to provide an image every 110 ms. (A) Imaging of the development of $[Ca^{2+}]_i$ rises in the three cells of a triplet of rat hepatocytes. Vasopressin (1 nM) was added at time 46 s. In this particular triplet, the hormone initiates a progressive homogeneous increase in $[Ca^{2+}]_i$ throughout the cytoplasm of the first-responding cell (cell 1, times 65.8, 66.0 and 66.8 s), then sequential increases in the connected cells 2 (time 69.0 s) and then 3 (times 72.2 s). (B) Time course of the sequential responses of $[Ca^{2+}]_i$ observed in the three cells of the triplet shown in (A). Each point of the three curves is the average of the total $[Ca^{2+}]_i$ captured every 110 ms throughout cells 1, 2 and 3. The hormone triggers a train of $[Ca^{2+}]_i$ rises in the three cells with rise times of 1.3, 1.2 and 1.0 s, respectively.

nearest connected cell 2, and finally, after a new delay of a few seconds (72.2 s), in the nearest cell 3. At that time, $[Ca^{2+}]_i$ was high, with a few variations, in the three cells of the triplet A. The time courses of the averaged $[Ca^{2+}]_i$ captured every 110 ms in cells 1, 2 and 3 (Figure 2B) show in another way the sequential pattern of $[Ca^{2+}]_i$ responses observed in the three cells of the triplet A. Rise times, taken as the time between the points where the line through the maximal rate of $[Ca^{2+}]_i$ rise intersected the basal and maximal levels of Ca^{2+} signals, were not very different between the three connected cells (1.3, 1.2 and 1.0 s respectively).

Vasopressin and noradrenaline could also elicit $[Ca^{2+}]_i$ signals which originated from the sinusoidal domain (basal-to-apical

direction). A typical example of this behaviour is illustrated in Figure 3 with noradrenaline (0.5 μ M). The agonist added at time 16 s initiated a localized increase in $[Ca^{2+}]_i$ in the sinusoidal domain of the plasma membrane (time 28.8 s, Figure 3A). Then, the cytoplasmic $[Ca^{2+}]_i$ rapidly became high throughout cell 1 (time 31.1 s). After a delay, $[Ca^{2+}]_i$ increased in the connected cell 2 (time 34.5 s). Analysis of the time courses of the hormone-mediated $[Ca^{2+}]_i$ transients in cells 1 and 2 (Figure 3B) indicated that rise times were 2.6 and 2.1 s respectively. Finally, the two agonists generated $[Ca^{2+}]_i$ rises localized at the canalicular domain (apical-to-basal direction; results not shown). Similar results were obtained with other multicellular systems treated with

Figure 3 Localized increase of $[Ca^{2+}]_i$ induced by $InsP_3$ -linked hormones in the first-responding cell of a doublet of rat hepatocytes (fast kinetics)

The frames of the digital video imaging of fura2 fluorescence of primary cultures of hepatocytes were captured every 260 ms. (A) Spatio-temporal development of $[Ca^{2+}]_i$ rises in the cells of a doublet of rat hepatocytes. Noradrenaline ($0.5 \mu M$) was added at time 16 s. In this doublet, the hormone initiates a localized increase in $[Ca^{2+}]_i$ in the sinusoidal domain of the plasma membrane (time 28.8 s). Then the cytoplasmic $[Ca^{2+}]_i$ becomes high throughout cell 1 (time 31.1 s). After a delay, $[Ca^{2+}]_i$ increases in the connected cell 2 (time 34.5 s). Similar heterogeneous increases were also observed with vasopressin. (B) Time course of the sequential responses of $[Ca^{2+}]_i$ observed in cells 1 and 2 of the doublet shown in (A). The hormone triggers a train of $[Ca^{2+}]_i$ transients, with rise times of 2.6 and 2.1 s respectively.

vasopressin or noradrenaline (48 doublets, 28 triplets, 5 quadruplets and 1 quintuplet).

Sequential $[Ca^{2+}]_i$ responses and intercellular delay

The intercellular delay between the different cells of the same multiplet was taken as the times between the successive rapid rising phases. In the experiment illustrated in Figure 4(a), noradrenaline ($0.5 \mu M$, added at 5 s) elicited a sequential activation from the first-responding cell 1 (shaded cell) to cells 2 and 3, with rise times of 1.7, 1.7 and 2.3 s, and intercellular delays of 2.2 and 1.8 s, respectively. Since the mean distance between cells 1 and 3 of the triplet was little different from $50 \mu m$, the

sequential activation of the three cells took place with a speed of approx. $13 \mu m/s$.

To investigate whether suppression of the intermediate cell altered the responsiveness of the other cells to the hormone, cell 2 was mechanically disrupted by use of a micropipette, and the resulting $[Ca^{2+}]_i$ traces were recorded from the subsequently independent cells 1 and 3 (Figure 4b). Cells 1 and 3 displayed low $[Ca^{2+}]_i$, were able to respond individually to noradrenaline and therefore to express functional hormonal receptors. Interestingly, the sequential order between cells 1 and 3 was retained. However, the intercellular delay between the separated cells 1 and 3 was longer (19 s) than the intercellular delay (2.2 plus 1.8 s) when they had been associated in a triplet. Six other experiments with triplets confirmed the view that disruption of the intermediate

Figure 4 Effect of disruption of the intermediate hepatocyte on the hormonal responsiveness of the other cells (fast and slow kinetics)

(a) The hormone ($0.5 \mu\text{M}$ noradrenaline), added at time 5 s, triggers a train of $[Ca^{2+}]_i$ rises in the three connected cells, with rise times of 1.7, 1.7 and 2.3 s respectively. $[Ca^{2+}]_i$ starts in the peripheral cell 1 (shaded cell), then rises in cell 2 with an intercellular delay of 2.2 s and in cell 3 with a delay of 1.8 s. The final $[Ca^{2+}]_i$ was high throughout the triplets, for at least 10 s. Frame ratioing: 295 ms. (b) Disruption of the intermediate cell 2 of the triplet shown in (a) prolongs the intercellular delay between cell 1 and cell 3 (19 s). The hormone was added at 13 s. Frame ratioing: 2.5 s.

cell did not prevent cells 1 and 3 from responding individually with the same order to the hormones. The only clear effect of disruption of the intermediate cell was to lengthen the intercellular delay between cells 1 and 3.

The primary $[Ca^{2+}]_i$ rise originates from the same cell at each oscillation during a prolonged application of hormones

Low and intermediate concentrations of vasopressin ($0.1\text{--}1 \text{ nM}$) and α_1 -adrenergic agonists ($0.1\text{--}0.5 \mu\text{M}$) applied during long periods elicited co-ordinated $[Ca^{2+}]_i$ oscillations of low frequencies ($0.5\text{--}1$ spike/min) between the associated cells (Nathanson and Burgstahler, 1992b). In the triplet treated with $0.5 \mu\text{M}$ noradrenaline for 9 min (added at time 26.5 s; Figure 5), the three connected cells appeared to oscillate at exactly the same frequency. Further examination of the successive spikes revealed that the primary $[Ca^{2+}]_i$ rise did not start randomly from any cell of the triplet. It arose six times from the same cell located in a central position within the triplet (shaded cell 1, Figure 5). Ca^{2+}

Figure 5 A long application of noradrenaline initiates $[Ca^{2+}]_i$ oscillations six times from the same central cell of a triplet

The triplet represented above the trace was incubated with $0.5 \mu\text{M}$ noradrenaline, added at time 26.5 s. The first $[Ca^{2+}]_i$ arises six times from the same cell 1 (shaded cell, black squares) located in a central position, and then, after intercellular Ca^{2+} delays from 6.6 to 9.6 s, $[Ca^{2+}]_i$ increases at the same time in the peripheral cells 2 (white diamonds) and 3 (white squares). Frame ratioing: 3 s.

never originated in either cells 2 or 3, although they were in permanent contact with noradrenaline for at least 9 min. It is noteworthy that, since the first-responding cell 1 was located in a central position, $[Ca^{2+}]_i$ rose exactly at the same time in the connected cells 2 and 3, after pauses ranging from 6.6 to 9.6 s. Oppositely directed sequential activations were therefore observed. Similar results were obtained in other experiments carried out with prolonged addition of vasopressin or noradrenaline. Thus 14 multiplets (10 doublets, 3 triplets and 1 quadruplet) were observed in which vasopressin or noradrenaline promoted repeated $[Ca^{2+}]_i$ rises in the same cell, then delayed activations of the same connected cells.

The localization of the first-responding cell does not depend on the site of application of the hormone

The fact that $[Ca^{2+}]_i$ rise originated from a particular cell during long periods of superfusion with vasopressin and noradrenaline did not result from non-homogeneous applications of hormones. First, the time constant of washing of the dish coverslip was 6 s (see the Materials and methods section), i.e. much shorter than the period of time ($8\text{--}10$ min) during which each hepatocyte maintained its function of being either the first, or the second or the third responding cell. Second, oppositely oriented $[Ca^{2+}]_i$ propagations were observed in doublets, triplets and quadruplets settled in close and parallel positions on the dish coverslips. In other terms, first-responding cells were therefore located at opposite ends of the pair of parallel multiplets. If the localization of the two cells had resulted from the site of hormone application, the hormone flow should have triggered $[Ca^{2+}]_i$ rises in the two most closely located cells, and thus elicit parallel propagations of

Figure 6 Repeated application of vasopressin does not alter the position of the first-responding cell in a quadruplet of rat hepatocytes

First (a), second (b) and third (c) application of vasopressin (1 nM) was added three times at zero time after extensive washings between each trace. The shaded peripheral cell initiates a succession of similar $[Ca^{2+}]_i$ rises in the four connected cells of the quadruplet. Frame ratioing: 110 ms.

$[Ca^{2+}]_i$ rises. These opposite propagations were observed several times. Among the 74 associated cells examined (2 quintuplets, 6 quadruplets, 28 triplets and 38 doublets), 11 pairs were in strictly parallel positions: 5 pairs showed oppositely directed Ca^{2+} propagations and 6 parallel propagations. Thus the observation of sequential responses from a first-responding cell was not due to a limited or partial diffusion of the hormone.

The first-responding cell retains its property under repeated additions of hormones

We then investigated whether the first $[Ca^{2+}]_i$ rises started from the same cell when hormones were applied repeatedly. A quadruplet was successively bathed and washed with 1 nM vasopressin (at each time added at zero time). As expected from the above data, the hormone elicited a sequential activation from cell 1 to cells 2, 3 and 4 (Figure 6a). After washes, a second and then a third challenge with the same concentration of vasopressin elicited exactly the same pattern of cell responses (Figures 6b and 6c). Thus the cell retained its ability to be the first cell to respond to successive applications of the hormones. Twenty multiplets (13 doublets and 7 triplets) maintained the function of first-responding cell when they were treated with vasopressin or noradrenaline and then washed three times, and in two cases four times. Thus the fact of being able to recognize agonists first in the continued or interrupted presence of the hormones shows that

Figure 7 The position of the first-responding cell and the sequential order do not depend on the concentration of the circulating hormone

The hormone vasopressin (VP) was added, and then washed three times, each at zero time, and with a different concentration. This treatment does not alter the position of the first-responding cell (shaded cell), but decreases in a dose-dependent manner the intercellular delay between cells 1 and 2, and cells 2 and 3. Frame ratioing: 110 ms. (a) 0.1 nM vasopressin. First intercellular delay (cell 1 and 2): 7.3 s. Second intercellular delay (cell 2 and 3): 4.7 s. The rise times were 1.1, 0.8 and 1.3 s in cells 1, 2 and 3 respectively. (b) 1 nM vasopressin. First intercellular delay: 4.9 s. Second intercellular delay: 1.3 s. Rise times: 1.1, 1.0 and 1.2 s. (c) 10 nM vasopressin. First intercellular delay: 2 s. Second intercellular delay: 0.76 s. Rise times: 0.7, 0.7 and 0.8 s.

the first-responding cell differs functionally from its associated cells.

Effect of the concentration of hormones on the position of the first-responding cell

A question raised by these observations is whether the function of the cell depended on the concentrations of the stimulatory hormones. The position of the cell was not altered when the triplet was sequentially treated with 0.1 nM (Figure 7a), 1 nM (Figure 7b) and then 10 nM (Figure 7c) vasopressin, at each time added at zero time. As reported in the legend to Figure 7, the rise times of the Ca^{2+} transients were little different between the connected cells, and were slightly decreased during the treatment of the triplet with increasing concentrations of vasopressin (approx. 1.2 s at 0.1 nM, 1.1 s at 1 nM, and 0.75 s at 10 nM). In contrast, the intercellular delay between cells 1 and 2, and cells 2 and 3, diminished from approx. 6 s to 3.1 s, and then to 1.4 s, respectively. As a result, the speed of propagation of Ca^{2+} signals increased from approx. 4 to 8, and then to 18 $\mu\text{m/s}$. Thus, an

interesting observation was that, particularly at low agonist concentration (0.1 nM), the intercellular delays were longer than the rise times of individual cells. In other terms, $[Ca^{2+}]_i$ rose rapidly in the cytoplasm of three cell, but after marked pauses at the boundary between cells 1 and 2, and then cells 2 and 3. Such propagations of Ca^{2+} signals delayed at the distal cell boundaries have been observed in other epithelial cells (Sanderson et al., 1994). In contrast, at high concentration of vasopressin (10 nM), the diminution of the intercellular delays decreased this apparent pausing of $[Ca^{2+}]_i$ at the boundaries of connected cells.

Twelve doublets, eight triplets and one quadruplet were similarly treated with a series of concentrations of vasopressin and noradrenaline. In all cases, the position of the special cell was not affected by the concentration of the hormone. The only apparent effect of changing the hormone concentration was to accelerate the propagation of Ca^{2+} rises between cells.

Peripheral or central position of the first-responding cell

Examination of a number of hormone-mediated $[Ca^{2+}]_i$ traces from triplets and quadruplets clearly revealed that a peripheral position in the associated cells was not required to express the function of the first-responding cell: the primary $[Ca^{2+}]_i$ rises could originate from either a peripheral (see Figures 2, 4, 6 and 7) or a central (see Figure 5) position. Among 28 triplets and 4 quadruplets treated with vasopressin, the cell was peripheral 25 times and central 7 times. Similarly, in the 12 triplets and the 2 quadruplets treated with noradrenaline, the cell was peripheral 8 times and central 6 times.

Do vasopressin and noradrenaline use a common first-responding cell?

Two possibilities can explain the observation that the first-responding cell differs functionally from its associated cells. It could possess either the most numerous or highest-affinity surface receptors, or the most efficient intracellular coupling mechanism [PtdIns(4,5) P_2 , G-proteins, phospholipase C- β , protein kinase C, Ins P_3 receptor]. To distinguish between these two possibilities, we investigated whether a cell which was the first cell during vasopressin stimulation remained the first during subsequent stimulation with noradrenaline. Doublets and triplets were successively treated with vasopressin and then with noradrenaline, or vice versa. Experiments performed with 11 multiunits (7 doublets, 3 triplets and 1 quadruplet) showed that the first-responding cell was either the same (6 times) or different (5 times) for the two hormones. In addition, the initial position (either common or different) of the cell was not altered by repeated treatments with vasopressin and noradrenaline. Thus the potency of the cell to recognize hormones probably results from the possession of more efficient hormonal receptors.

Doublets and triplets are more responsive to hormones than single cells

It has been previously reported that hepatocyte doublets display a shorter hormonal lag, taken as the time between the addition of hormones and the onset of the rapid phase of the $[Ca^{2+}]_i$ rise, than single cells (Nathanson and Burgstahler, 1992b). We investigated whether there was also a difference in responsiveness to hormones between triplets of cells and single cells. $[Ca^{2+}]_i$ spikes and oscillations were recorded from single cells, doublets and triplets on the same dish coverslips. Cells were treated for 180 s with low concentrations of vasopressin (0.1 nM) or noradrenaline (0.1 μ M) which evoked $[Ca^{2+}]_i$ rises and oscillations in most of cells. The number of responsive cells was then counted

Table 1 Hepatocyte doublets and triplets are apparently more responsive than single cells to vasopressin and noradrenaline

Primary cultures of hepatocytes containing fura2-loaded single cells, doublets and triplets of hepatocytes, were spread on dish coverslips as indicated in the Materials and methods section. They were equilibrated with modified Eagle's medium in the presence of either 0.1 nM vasopressin or 0.1 μ M noradrenaline for 3 min. The responsive single cells, doublets and triplets present on the same dish coverslips were counted. Values are means $\pm$ S.E.M. Values in parentheses are the total numbers of single cells, doublets and triplets present on the dish coverslips. It should be noted that 90% of single cells responded to further additions of 10 nM vasopressin and 10 μ M noradrenaline.

Hormone	Triplets	Doublets	Single cells
Vasopressin (0.1 nM)	90 $\pm$ 6% (18)	90 $\pm$ 5% (38)	53 $\pm$ 7% (230)
Noradrenaline (0.1 μ M)	89 $\pm$ 7% (13)	88 $\pm$ 5% (29)	67 $\pm$ 9% (116)

(Table 1): 90% of the connected cells and only 53% of co-recorded single cells responded to 0.1 nM vasopressin. Similarly, 88% of the doublets, 89% of the triplets and 67% of the single cells responded to 0.1 μ M noradrenaline. It is noteworthy that the single cells resistant to low concentrations of hormones were sensitive to further additions of either 10 nM vasopressin or 10 μ M noradrenaline (see legend to Table 1). This indicates that the lower percentage of single cells sensitive to low concentrations of vasopressin (0.1 nM) and noradrenaline (0.1 μ M) did not result from a loss of agonist surface receptor or other cell function during isolation and short-term culture. These results are consistent with the view that multiunits are more sensitive to hormones than are single cells.

DISCUSSION

Different data reported in this study and the literature support the view that doublets, triplets and quadruplets of rat hepatocytes with dilated bile canaliculi retained the properties of polarized epithelial cells (see the Introduction and the Materials and methods sections). These multicellular systems appeared also to express functional gap junctions. In rat hepatocytes, gap junctions have been previously identified by freeze-fracture and transmission electron microscopy in the hepatocyte doublets (Spray et al., 1986), and shown to be freely permeable to ions and small molecules (Graf et al., 1984; Spray et al., 1986; Saez et al., 1989). Our results showed that they could be easily identified in the lateral domains of the plasma membrane by using an anti-connexin-32 antibody and confocal microscopy, not only in doublets, but also in triplets or quadruplets of rat hepatocytes. The immunolabelling appeared as highly localized microdomains, suggesting the presence of plaques of aggregated gap junctions, a distribution similar to that detected by anti-connexin-32 in the whole liver (Evans et al., 1992; Mazet et al., 1992), and more generally, in the other tissues (Malewicz et al., 1990).

At the intracellular level, the multiunits displayed homogeneous increases in $[Ca^{2+}]_i$, basal-to-apical and apical-to-basal Ca^{2+} waves in response to hormones (Nathanson and Burgstahler, 1992a; Nathanson et al., 1993). At a more integrated level, they showed also highly organized hormone-mediated Ca^{2+} signals. Notably, Ca^{2+} signals originated in a hepatocyte, then propagated in a sequentially manner to the nearest connected cells (cell 2, then 3, in triplets; cell 2, then 3, then 4, in quadruplets). This sequential activation of the cells from the first-responding cell appeared to be an intrinsic property of the multicellular systems of rat hepatocytes. First, during the pro-

longed periods with hormones producing trains of repeated [Ca²⁺]_i oscillations, [Ca²⁺]_i rose up to six occasions from the same cell. Second, the order of activation of cell responses was not modified during repeated applications of the hormones. Third, the cell sequence of the multicellular systems was not changed by the 100-fold increase in the concentrations of vasopressin and noradrenaline. Fourth, the intercellular delay, and thus the speed of activation of the successive cells, were markedly dependent on hormone doses. Fifth, suppression of the intermediate cell localized in the central part of multiplets did not alter the initial order of responses of the remaining cells. Finally, multiplets were more sensitive than single cells to hormones.

The fact that the same multiplet could have different first-responding cells for vasopressin and for noradrenaline suggests that the extracellular step, and particularly surface hormonal receptors, plays a role more important in the function of the cell than do the intracellular steps of the signalling pathway. The first-responding cell is likely to be more sensitive to the hormones than the second, the third or the fourth cells, presumably because it possesses more numerous and/or higher-affinity hormonal receptors. Therefore, it should display the highest rate of InsP₃ generation and the quickest hormonal lag (Rooney et al., 1989; Miledi and Parker, 1989; Capiod et al., 1991; Lipinsky et al., 1993; Ogden et al., 1993). The sequential activation of cells from the first-responding cell could thus result from the heterogeneity of lag times in multiplets. Consistent with this view, the liver cell plate in the hepatic acinus displays heterogeneous expression of a number of biological functions (Jungermann and Katz, 1989). A few studies have suggested the existence of gradients of receptors to vasopressin (Ostrowski et al., 1993) and glucagon (Berthoud et al., 1992) along the hepatocyte cord. We are currently studying these putative gradients of receptors in the hepatic acinus.

The intercellular connection by gap junctions augmented the homogeneity of hormonal [Ca²⁺]_i signals, and led to tightly co-ordinated [Ca²⁺]_i oscillations between the different cells, which has been observed in hepatocyte doublets (Nathanson and Burgstahler, 1992b) and in other systems of connected cells (Loessberg Stauffer et al., 1993; Sanderson et al., 1994). This is not an unexpected result, since gap junctions equalize hormonal responses and homogenize the different cells constituting the multicellular systems (Graf et al., 1984; Spray et al., 1986; Saez et al., 1989; Nathanson and Burgstahler, 1992b). Since the destruction of the intermediate cell of the triplet shown in Figure 4 augmented the intercellular delay from 4 to 19 s, as chemical inhibition of gap junctions decreases the propagation of the intercellular Ca²⁺ wave in hepatocyte doublets (Nathanson and Burgstahler, 1992b) and in other cells (Sanderson et al., 1994), it may be reasonably concluded that intercellular communication by gap junctions is also involved in the rapidity of the sequential activation of hepatocytes. Since hepatic gap junctions are freely permeable to small molecules (Graf et al., 1984; Spray et al., 1986; Saez et al., 1989), a fraction of the relatively mobile InsP₃ (Albritton et al., 1992), produced at the sub-plasma-membrane level in the first-responding cell, could diffuse at the cell boundary and cross gap junctions (Loessberg Stauffer et al., 1993; Sanderson et al., 1994). This can be expected to accelerate the rate of InsP₃ generation and decrease the hormonal lag of the nearest cell. The existence of gap junctions would then allow the first-responding cell to sensitize its accompanying cells to the circulating hormone. This could explain why the triplets and the doublets appeared to be more sensitive than single cells to vasopressin and noradrenaline (Table 1), and exhibit a shorter hormonal lag than do single liver cells (Nathanson and Burgstahler, 1992b). Thus the sequential [Ca²⁺]_i responses from

the first-responding cell could be explained by the combination of at least two main mechanisms, which both remain hypothetical: first, a heterogeneous distribution of the hormonal receptors between hepatocytes of the hepatic acinus; second, an intercellular propagation of cytoplasmic messengers such as InsP₃ through the gap junction.

Most often, at low hormone concentration, the sequential activation of the different associated hepatocytes took place very slowly (2–4 μm/s). As the activation time of each cell (rise time) was shorter than the activation time between two cells (intercellular delay), [Ca²⁺]_i rose rapidly in the cytoplasm of cells with pauses at cell boundaries, as observed in other epithelial cells (Sanderson et al., 1994). At high hormone concentration, the diminution of the intercellular delay decreased this apparent pausing of [Ca²⁺]_i at the boundaries of connected cells, and the sequential activation of the hepatocytes took place rapidly (20–25 μm/s). In contrast, as already observed in single hepatocytes (Woods et al., 1986; Cobbold et al., 1989; Rooney et al., 1989; Thomas et al., 1991), rise times of the Ca²⁺ transients of each cell were only slightly dependent on the concentrations of hormones. In addition, the intracellular Ca²⁺ wave in rat liver cells propagates across the hepatocyte at a constant speed of 20–25 μm/s, independent of the hormone concentration (Rooney et al., 1990; Thomas et al., 1991). The fact that speeds of intercellular Ca²⁺ propagation and the intracellular Ca²⁺ wave are differently related to the agonist dose leads to the suggestion that distinct processes are occurring during their propagations. The intracellular Ca²⁺ wave might be more dependent on a regenerative release of Ca²⁺ pools, in which Ca²⁺ released by InsP₃ from a sub-plasma-membrane pool sensitizes deeper pools (Berridge and Irvine, 1989; Rooney et al., 1990; Berridge, 1993). At the multicellular level, the dose-dependent acceleration of the intercellular Ca²⁺ wave might be more dependent on gradients of hormonal receptors and on the permeability of gap junctions to second messengers, including InsP₃ (Loessberg Stauffer et al., 1993; Sanderson et al., 1994).

The whole rat liver (Graf et al., 1987) displays oscillations of Ca²⁺ releases in response to vasopressin, in a dose-dependent manner similar to that seen in single cells (Woods et al., 1986; Cobbold et al., 1989; Rooney et al., 1990; Thomas et al., 1991; Capiod et al., 1991), doublets (Nathanson and Burgstahler, 1992b), triplets and quadruplets of rat hepatocytes (the present work). Such behaviour requires co-ordination of the potential individual oscillators present in each cell constituting the organ. Several factors can contribute to the co-ordination of the Ca²⁺ signals in the whole liver (Graf et al., 1987). First, the network of hepatic sinusoids which bathes the basal membranes of each hepatocyte (Campora and Reynolds, 1988). This provides rapid access of circulating hormones to the entire network of hepatocytes. Second, the particular distribution of hormonal receptor density or affinity in the hepatic acinus. Third, the presence of gap junctions between the different cells of the network, transforming the hepatic tissue into a true syncytium (Graf et al., 1984; Spray et al., 1986; Saez et al., 1989).

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