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Coordination of Hormone-Induced Calcium Signals in Isolated Rat Hepatocyte Couplets: Demonstration With Confocal Microscopy

Michael H. Nathanson and Angela D. Burgstahler

Departments of Medicine and Cell Biology and the Liver Center, Yale University School of Medicine, New Haven, Connecticut 06510

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Excitable cells often display rapid coordination of hormone-induced intracellular calcium signals. Calcium elevations that begin in a single epithelial cell also may spread to adjacent cells, but coordination of hormone-induced signals among epithelial cells has not been described. We report the use of confocal microscopy to determine the inter- and intracellular distribution of cytosolic calcium in isolated rat hepatocyte couplets, an isolated epithelial cell system in which functional polarity is maintained. Both vasopressin and phenylephrine evoked sequential coordinated calcium signals in the couplets, even during cytosolic calcium oscillations. The coupling was abolished by closure of intercellular gap junction channels by treatment with octanol. These observations demonstrate that hormone-induced intracellular calcium signals are coordinated among hepatocytes and suggest that gap junction channels mediate this intercellular integration of tissue responsiveness.

INTRODUCTION

Agonist-induced Ca^{2+} signals propagate through adjacent cells in excitable cell types ranging from astrocytes (Cornell-Bell *et al.*, 1990) to cardiac myocytes (Spray and Burt, 1990). This intercellular coordination appears to represent long-range signaling (Cornell-Bell *et al.*, 1990) and may be responsible for the synchronized and integrative behavior exhibited by cells comprising neuronal (Cornell-Bell *et al.*, 1990) or cardiac (Spray and Burt, 1990) tissue. In contrast, individual epithelial cells such as hepatocytes have been characterized by a lack of uniform Ca^{2+} signals in response to agonist stimulation (Monck *et al.*, 1988).

Hepatocytes are coupled via gap junctions that admit second messengers such as Ca^{2+} and inositol trisphosphate (IP_3) (Saez *et al.*, 1989). This may be of functional significance because microinjection of Ca^{2+} into one cell of an hepatocyte triplet (with a final Ca_i^{2+} concentration of 10 μM in that cell) leads to a Ca^{2+}-mediated event in the other two hepatocytes, i.e., contraction of the canalicular space they enclose (Watanabe *et al.*, 1985). These observations suggest that Ca^{2+} signals may be coupled in hepatocytes. In pancreatic acinar cells, another type of epithelium, disruption of gap junctions alters agonist-induced amylase secretion (Meda *et al.*, 1987). It has been difficult to adequately define hormone-induced intercellular Ca_i^{2+} signals in these types of cells because Ca_i^{2+} waves spread across isolated hepatocytes at 20–25 μm/s (Rooney *et al.*, 1990) and release of caged IP_3 results in localized Ca_i^{2+} increases within 50 ms (Parker and Ivorra, 1990), so that Ca_i^{2+} transients may occur at or exceed the limits of detection by digital epifluorescence imaging. In addition, epithelial cell preparations in which polarity has been maintained are often too thick or dense for detailed subcellular resolution of Ca_i^{2+} dyes by digital epifluorescence imaging. To better define the spatial and temporal profile of IP_3-mediated Ca_i^{2+} signals in epithelial cells, isolated rat hepatocyte couplets (Boyer *et al.*, 1988; Graf and Boyer, 1990) loaded with the Ca^{2+}-sensitive dye fluo3 (Kao *et al.*, 1989) were examined using confocal line scanning microscopy.

MATERIALS AND METHODS

Animals and Materials

Male Sprague-Dawley rats (180–250 g; Camm Research Lab Animals, Wayne, NJ) were maintained on Purina (Pittsburgh, PA) rodent chow under a constant light cycle and used for all experiments. Arg^8-vasopressin, phenylephrine, and octanol were obtained from Sigma Chemical Company (St. Louis, MO) and fluo3/AM was obtained from Molecular Probes (Pitchford, OR). All other chemicals were of the highest quality commercially available.

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Preparation of Isolated Hepatocytes and Hepatocyte Couplets

Isolated rat hepatocytes and hepatocyte couplets were prepared in the Hepatocyte Isolation Core Facility of the Yale Liver Center as described previously (Boyer *et al.*, 1988; Graf and Boyer, 1990). Briefly, rat livers were perfused with Hanks A then Hanks B medium containing 0.05% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 0.8 U trypsin inhibitor (Sigma Chemical)/U tryptic activity. Livers were then excised, minced, passed through serial nylon mesh filters, and the resultant cells washed. Cells were then suspended at a concentration of 5×10^5 cells/ml in Liebovitz L-15 medium (GIBCO, Grand Island, NY) containing 50 U penicillin and 50 μg streptomycin/ml and plated onto glass coverslips. Cell viability by trypan blue exclusion exceeded 85%.

Confocal Microscopic Measurements of Cytosolic Calcium

Isolated rat hepatocytes and hepatocyte couplets were prepared and plated onto glass coverslips as described above, incubated at 37°C for 2–6 h, then loaded with the Ca^{2+}-sensitive fluorescent dye fluo3/AM (6 μM) for 20 min at 37°C in L-15 medium containing 10% fetal calf serum. The coverslips containing these cells were transferred to a chamber on the stage of a Zeiss (Thornwood, NY) Axiovert microscope and the cells were perfused at 37°C with a *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered solution and observed using a Bio-Rad (Richmond, CA) MRC-600 confocal imaging system. An argon laser was used to excite the dye at 488 nm and emission signals above 515 nm were collected. Optical sections between 0.5 and 1.0 μm in thickness were obtained in the hepatocytes; in short-term culture these cells are spherical with a diameter of 20–25 μm. Neither autofluorescence nor other background signals were detectable at the machine settings (i.e., aperture, gain, and black level) that were used. There also was no change in size, shape, or location of cells or couplets during the experiments. Single cells and couplets were stimulated under one of the conditions described below and the resulting Ca_i^{2+} signals were detected by confocal line scanning microscopy. In the line scanning mode of confocal microscopy, fluorescence is determined at each point along a single line across the image, rather than at each point across the entire image (Figure 1). The advantage of this mode of confocal imaging is the speed at which images can be obtained; a single line can be scanned as frequently as every 4 ms, yet the spatial resolution of fluo3 fluorescence at each point along that scan line is the same as along any line in a full-screen confocal image (which can take up to 4 s to obtain). Velocities of intra- and intercellular Ca_i^{2+} waves were determined from the rate at which initial fluorescence increases moved along the scan line. This approach may overestimate the speed of Ca_i^{2+} waves that originate far from the scan line, but in hepatocyte couplets the estimated wave speed should be no more than 17% greater than the actual speed, on average (Appendix 1).

Experiment Design

Single cells and couplets were stimulated with either vasopressin (10 nM), vasopressin + octanol (250 μM), or phenylephrine (0.1–10 μM). Cells treated with both octanol and vasopressin were exposed to octanol for 15 s immediately before stimulation with vasopressin. Both vasopressin and phenylephrine result in a rapid IP_3-mediated increase in Ca_i^{2+} in hepatocytes (Monck *et al.*, 1988; Kawanish *et al.*, 1989; Rooney *et al.*, 1990), and phenylephrine often induces Ca_i^{2+} oscillations (Kawanish *et al.*, 1989; Rooney *et al.*, 1990). Octanol induces a selective and reversible inhibition of hepatocyte gap junction conductance (Spray *et al.*, 1986; Saez *et al.*, 1989). This combination of stimuli was chosen to compare patterns of IP_3-mediated rises in Ca_i^{2+} in single hepatocytes to patterns in hepatocyte couplets and to better define the role of gap junctions in the latter. Fluo3 fluorescence in response to each of these stimuli was recorded and the change in fluorescence over time at each point along the scan line was determined from the recorded image using an Itex Series 151 (Imaging Technology, Woburn, MA) image processor.

Microperfusion Studies

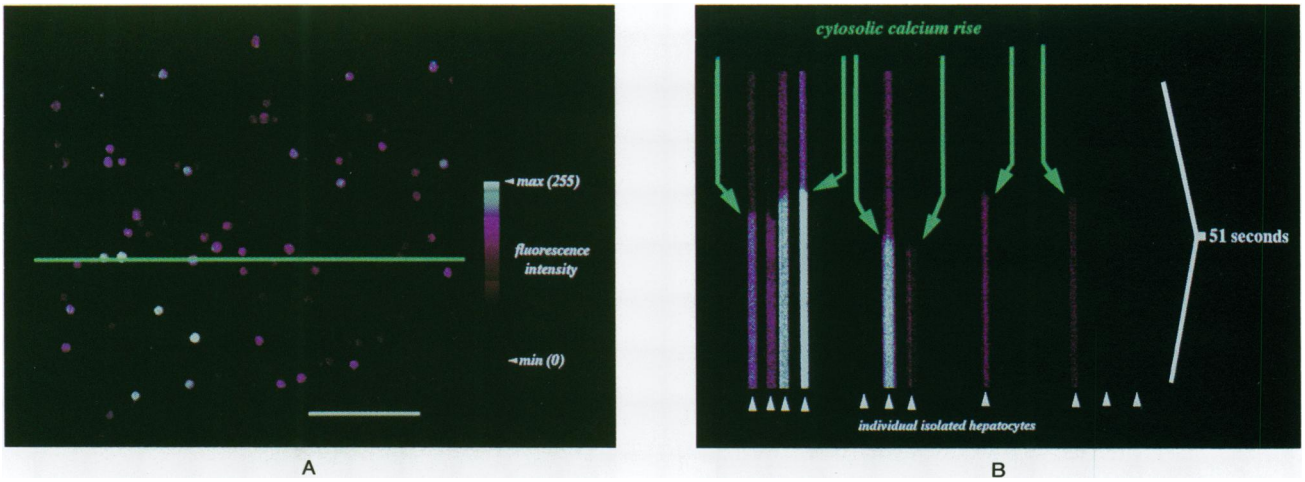
Individual hepatocytes were also selectively stimulated with vasopressin using an Eppendorf 5242 Microinjector (Eppendorf, Fremont, CA). Micropipettes were loaded with 1 μM vasopressin and had an orifice diameter of 2 μm and a constant positive pressure of 25 hPa, so that the calculated flow rate out of the tip was 0.14 μl/s. Tips were advanced toward a couplet (n = 16) or a pair of single cells (n = 11 pairs, average distance between each cell in a pair = 6.4 ± 5.3 μm) until a Ca^{2+} rise was detected (by line scanning) in the cell nearest to the pipette tip. In these studies, two-channel recordings of the confocal (fluorescent) and nonconfocal (transmission or light-microscopic) images were displayed simultaneously. With this approach, advancement of the pipette tip and resulting changes in the fluorescent signal in the hepatocyte(s) could be monitored at the same time (on the transmission and confocal channels, respectively). To minimize diffusion of vasopressin to nearby cells, tips were always advanced in the direction opposite to the direction of flow in the chamber; this flow rate was ~1.1 ml/min.

RESULTS

Stimulation of isolated hepatocytes with vasopressin caused a rise in Ca_i^{2+} that was preceded by a ~10-s latency period, as described previously (Monck *et al.*, 1988). The standard deviation of the latency period was used as an index of the cell-to-cell variation in this latency period and was measured using confocal line scanning of isolated hepatocytes (Figure 1). Seventy cells were studied (9 separate preparations, 6–11 cells per preparation), and the standard deviation of the latency period was 1.32 ± 1.07 s (mean ± SD). There was no correlation between latency period and either initial fluorescence intensity or position in the perfusion chamber.

In addition, the Ca_i^{2+} rise was measured in eight hepatocyte couplets stimulated with vasopressin (Figure 2). The rise was initiated by a Ca^{2+} wave that spread across the cells at a rate of 109 ± 76 μm/s (range 36–207 μm/s). There was an average difference of 172 ± 77 ms (range 0–330 ms) between the time that the Ca_i^{2+} signal began to rise in the first and second cell of each couplet, although Ca^{2+} waves did not pause at cell boundaries. The probability that each half of a couplet would initiate a rise in Ca_i^{2+} independently within 330 ms in eight consecutive couplets is 1.7×10^{-7} (Appendix 2), indicating that the vasopressin-induced rise in Ca_i^{2+} in one cell of an hepatocyte couplet is not independent of the rise in the other cell. Because the time delay between the Ca_i^{2+} rise in the first and second cell of each couplet is the square root of the second moment about their mean latency period, this difference is the same statistic as the standard deviation of the latency period in populations of single cells, and the two statistics are directly comparable (Figure 2G).

To ascertain whether gap junctions are necessary for this coordinated rise in Ca_i^{2+}, couplets were pretreated



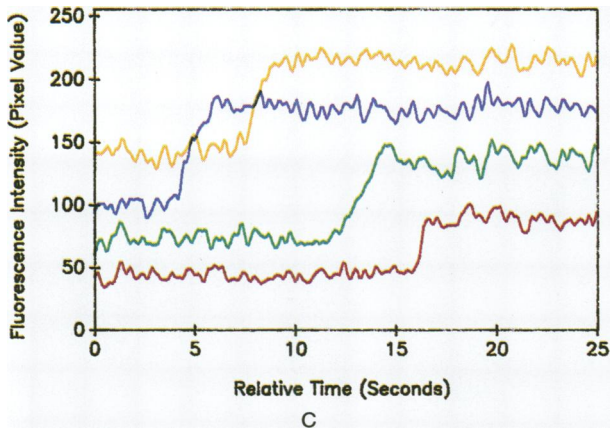
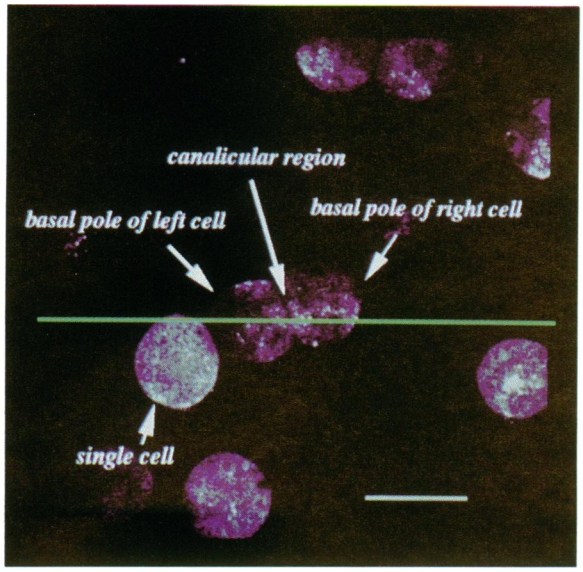


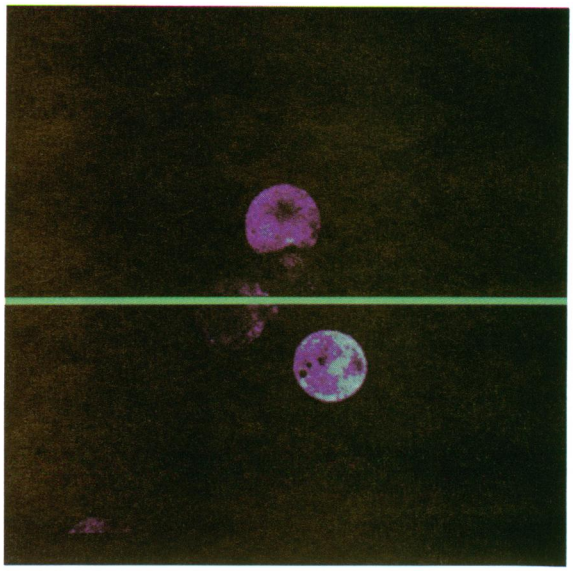
Figure 1. Variability in onset of cytosolic Ca^{2+} signals in isolated hepatocytes stimulated with vasopressin (10 nM). (A) Low-magnification confocal image of isolated rat hepatocytes loaded with the Ca^{2+}-sensitive dye fluo3. Fluorescence intensity in this and subsequent confocal images and line scans is represented by the pseudocolor scale shown on the right. Scale bar (lower right) is 250 μm. (B) Repetitive confocal scan ("line scan") along the line indicated in A. Each horizontal line in this figure corresponds to a scan along the line in A, so that each vertical stripe in this figure demonstrates fluorescence over time in an individual hepatocyte (bottom arrows). Abrupt increase in fluorescence (green arrows) denotes vasopressin-induced rise in cytosolic Ca^{2+}; note cell-to-cell variability in time of onset of the Ca^{2+} increase. Lines in this scan were acquired every 100 ms for a total of 51.2 s (top to bottom). (C) Graphical representation of fluorescence increase in four of the cells in A and B. Note uniformly rapid (<300 ms) rise time of Ca^{2+} signal among cells despite relatively large variability in time of onset from cell to cell. The rough correlation between initial fluorescence and latency period of the response was not a consistent finding (see results). Cytosolic Ca^{2+} is represented by intensity of fluo3 fluorescence scaled from 0 to 255.

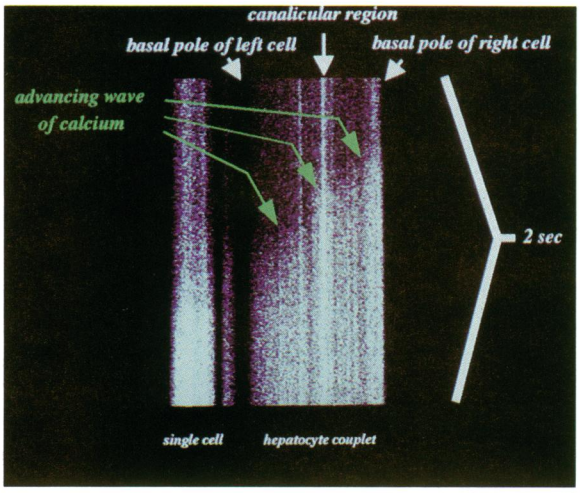
for 15 s with octanol. There was a 1.37 ± 0.56 s (n = 5) delay between the time the first and second cell of each of these couplets exhibited a Ca_i^{2+} rise, which was eight times longer than the delay seen in couplets not pretreated with octanol ($p < 0.005$) but not significantly different from the standard deviation of the latency period in vasopressin-stimulated isolated hepatocytes with (1.21 ± 0.61 s, n = 38 cells in 5 preparations) or without (1.32 ± 1.07 s) octanol pretreatment (Figure 2G). Thus, couplets pretreated with octanol responded to hormonal stimulation in the same manner as isolated hepatocytes rather than as couplets.

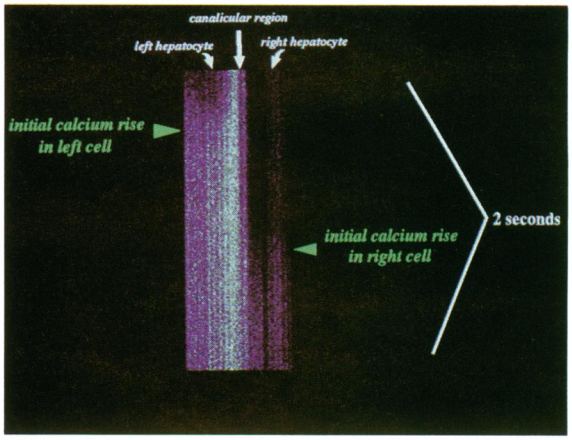
Isolated hepatocytes and hepatocyte couplets were also stimulated with 0.1, 1, or 10 μM phenylephrine to induce Ca_i^{2+} oscillations (Rooney *et al.*, 1990) (Figure 3). Oscillations were observed in 79% (15 of 19) of cells stimulated with 0.1 μM, 81% (17 of 21) of cells stimulated with 1 μM, and 55% (92 of 166) of cells stimulated with 10 μM phenylephrine; the period of these oscillations was 35.0 ± 18.2, 25.0 ± 8.5, and 22.3 ± 13.7 s, respectively. Oscillations were synchronized in couplets, even though couplets did not oscillate in synchrony with single cells nearby (Figure 3). The period of the oscillations was 34.0 ± 14.0 s in couplets stimulated with 0.1 μM (n = 4), 24.4 ± 10.7 s in couplets stimulated with 1 μM (n = 4), and 20.3 ± 2.8 s in couplets stimulated with 10 μM phenylephrine (n = 16); none of these periods differ significantly from those seen in single cells exposed to the same concentration of phenylephrine. These results show that Ca_i^{2+} oscillations are also coordinated in hepatocyte couplets.

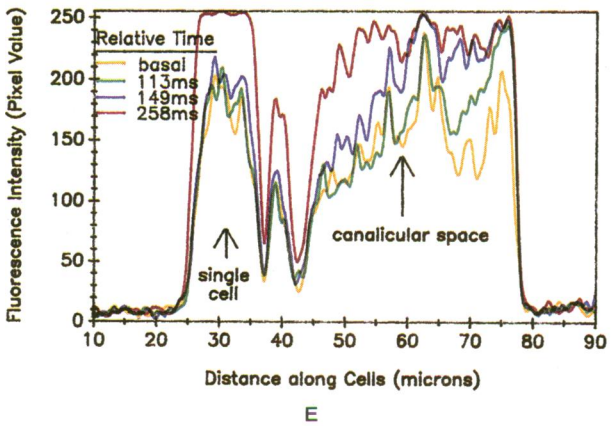
Using a micropipette, individual hepatocytes were selectively stimulated with vasopressin (Figure 4). Although it was difficult to reproducibly expose only one cell in a field to vasopressin, there was a measurable time lag between stimulation of the cell nearest the micropipette tip and the other cell(s) in the field. The time lag between cells in a couplet (n = 16 couplets) was 7.4 times shorter ($p < 0.005$) than the time lag between paired single (control) cells 6.4 ± 5.3 μm apart (both of the single cells were stimulated in 7 of 11 pairs, whereas only the cell closer to the pipette tip was stimulated in the remaining 4 pairs). To account for the different distance vasopressin would have to travel between cells


canalicular region
basal pole of left cell
basal pole of right cell
single cell




canalicular region
basal pole of left cell
basal pole of right cell
advancing wave of calcium
2 sec
single cell
hepatocyte couplet


canalicular region
left hepatocyte
right hepatocyte
initial calcium rise in left cell
initial calcium rise in right cell
2 seconds


Relative Time
basal
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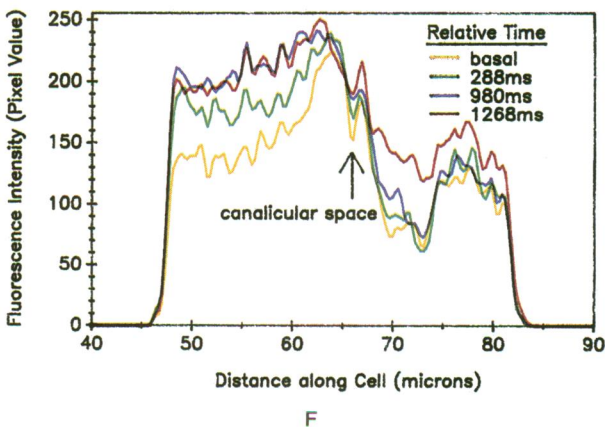
149ms
258ms
Fluorescence Intensity (Pixel Value)
Distance along Cells (microns)
single cell
canalicular space

E


Relative Time
basal
288ms
980ms
1268ms
Fluorescence Intensity (Pixel Value)
Distance along Cell (microns)
canalicular space

F

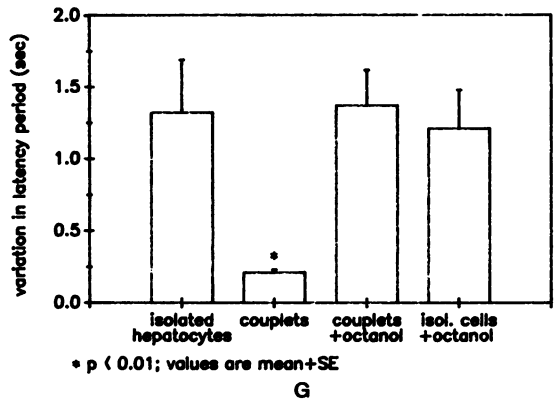


Figure 2. Spatial and temporal distribution of the cytosolic Ca^{2+} signal in isolated rat hepatocyte couplets stimulated with vasopressin (10 nM). (A and B) Confocal images of hepatocyte couplets loaded with fluo3. Scale bar in A is 25 μm, and scale in A is 1.2 times the scale in B. (C) Repetitive confocal scan along the line indicated in A, obtained while the couplet was stimulated with vasopressin. Increase in fluorescence (green arrows) indicates rise in Ca_i^{2+} in response to perfusion with vasopressin. Note that the Ca_i^{2+} rise begins at the basolateral pole of the right hepatocyte and spreads in a continuous wave-like fashion (at ~160 μm/s) to the pericanalicular region of both cells, then to the basolateral pole of the left hepatocyte. In contrast to the line scan in 1B, this line was scanned every 4 ms for a total of 2 s (from top to bottom). (D) Repetitive confocal scan along the line indicated in B; couplet was pretreated with octanol (250 μM) for 15 s, then stimulated with vasopressin. Ca_i^{2+} rise in the left hepatocyte precedes the rise in the right cell by ~1 s (green arrows); time scale here is the same as in 2C. (E and F) Graphical representation of fluorescence intensity as a function of distance along the hepatocyte couplets in C and D, respectively, at four selected times during the 2 s of observation. E illustrates the rapid continuous progression of the vasopressin-induced Ca_i^{2+} wave observed in an hepatocyte couplet under normal conditions; Ca_i^{2+} initially rises only in the basal region of the cell on the right (green tracing), and this spreads to the pericanalicular region of both cells within 36 ms (blue tracing) and to the basal region of the left cell after an additional 109 ms (red tracing). In contrast, F demonstrates dissociation between the vasopressin-induced Ca^{2+} signals in the two cells of the octanol-treated couplet; the initial Ca_i^{2+} rise in the cell on the left (green tracing) precedes the rise in the cell on the right (red tracing) by 980 ms. Note that the rates at which Ca_i^{2+} waves travel across cells (as shown here) are different from the rate at which Ca_i^{2+} increases at single points within cells (as shown in Figure 1C). (G) Variability in initiation of Ca^{2+} rise in hepatocytes stimulated with vasopressin under different circumstances. In isolated hepatocyte preparations with (n = 5) and without (n = 9) octanol, this variability is the standard deviation of the latency period. In hepatocyte couplets with (n = 5) and without (n = 8) octanol, this variability is the mean time difference between initiation of the Ca^{2+} rise in both halves of each couplet. The variability in onset of Ca^{2+} rise is decreased sixfold in vasopressin-stimulated couplets with intact gap junctions ($p < 0.01$), relative to each of the three other groups. Values in this graph are means ± SEM.

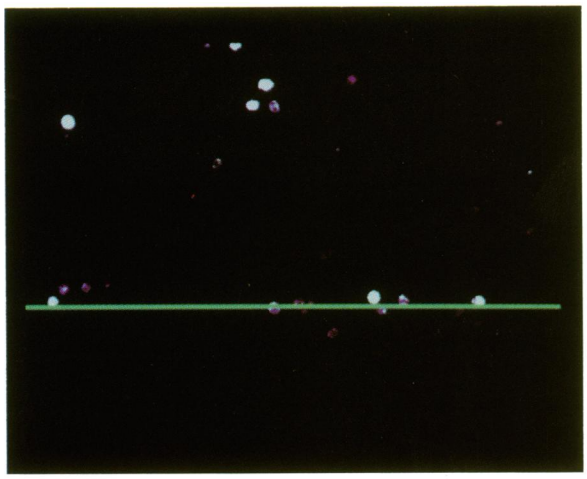
in couplets and paired single cells, the time interval between the initial Ca_i^{2+} rises (in both cells of a couplet or in paired single cells) was divided by the difference in the shortest distance between each of the two cells and the pipette tip. This "apparent velocity" with which vasopressin would have to travel between cells to account for the time lag was over six times greater in couplets (13.9 ± 17.0 vs. 2.1 ± 1.3 μm/s; $p < 0.005$), providing complementary evidence for coordination of Ca_i^{2+} signals in hepatocyte couplets.

DISCUSSION

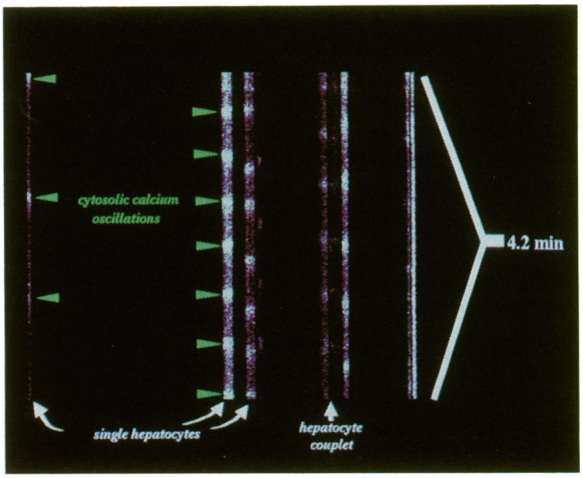
Stimulation of vasopressin V_1 receptors on the basolateral membrane of the hepatocyte results in an IP_3-mediated rise in Ca_i^{2+} that is preceded by a brief latency period (Monck *et al.*, 1988; Rooney *et al.*, 1990). The cell-to-cell variation in this latency period was measured in isolated hepatocytes using confocal line scanning microscopy, and the lack of correlation between latency period and either initial fluorescence intensity or position in the perfusion chamber suggests that this variability reflects intrinsic differences among cells rather than buffering of Ca_i^{2+} by fluo3 or delays in response due to transit time of vasopressin along the perfusion chamber. This cell-to-cell variation was reduced by an order of magnitude in hepatocyte couplets, which indicates that the vasopressin-induced rise in Ca_i^{2+} in one cell of an hepatocyte couplet is linked to the rise in the other cell. This highly coordinated rise in Ca_i^{2+} between cells was abolished by octanol, which suggests that the organization of vasopressin-induced Ca_i^{2+} signals in hepatocyte couplets depends on preserved function of gap junctions (Spray *et al.*, 1986). Similarly, phenylephrine-induced Ca_i^{2+} oscillations were synchronized in couplets, even though couplets did not oscillate in synchrony with single cells nearby. These results further demonstrate that Ca_i^{2+} signals are coordinated in hepatocyte couplets.

As an alternative way to examine Ca_i^{2+} signals in the couplets, a micropipette was used to selectively stimulate individual hepatocytes with vasopressin. Although it was difficult to reproducibly expose only one cell in a field to vasopressin, the sequential rise in Ca_i^{2+} observed in each couplet was too rapid to be explained by independent stimulation of both cells of the couplet. This observation provides complementary evidence for coordination of Ca_i^{2+} signals in hepatocyte couplets.

The Ca^{2+} dye fluo3 appears to distribute nonuniformly in some hepatocytes (Figure 2, A and B), and its punctate fluorescence distribution is suggestive of compartmentalization of some of the dye (Kawanish *et al.*, 1989). When encountered during digital epifluorescence imaging (Kawanish *et al.*, 1989), this phenomenon may introduce errors in Ca_i^{2+} measurements due to fluorescence contributions from noncytosolic compartments. With confocal microscopy, in contrast, fluorescence signals from within the cytosol can clearly be distinguished from compartmentalized regions of dye (Figure 2), whether these compartments are within or beyond the plane of focus. Thus, both the progression of Ca_i^{2+} waves across the cytosol and changes in fluo3 fluores-



A



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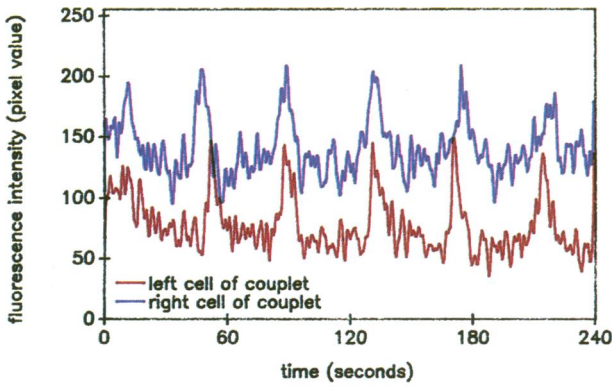
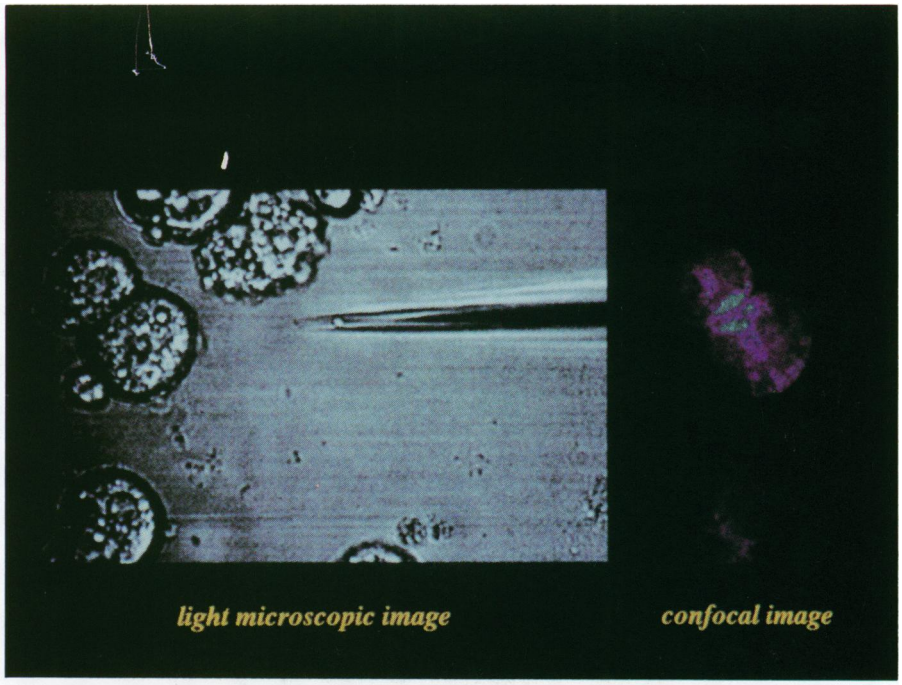


C

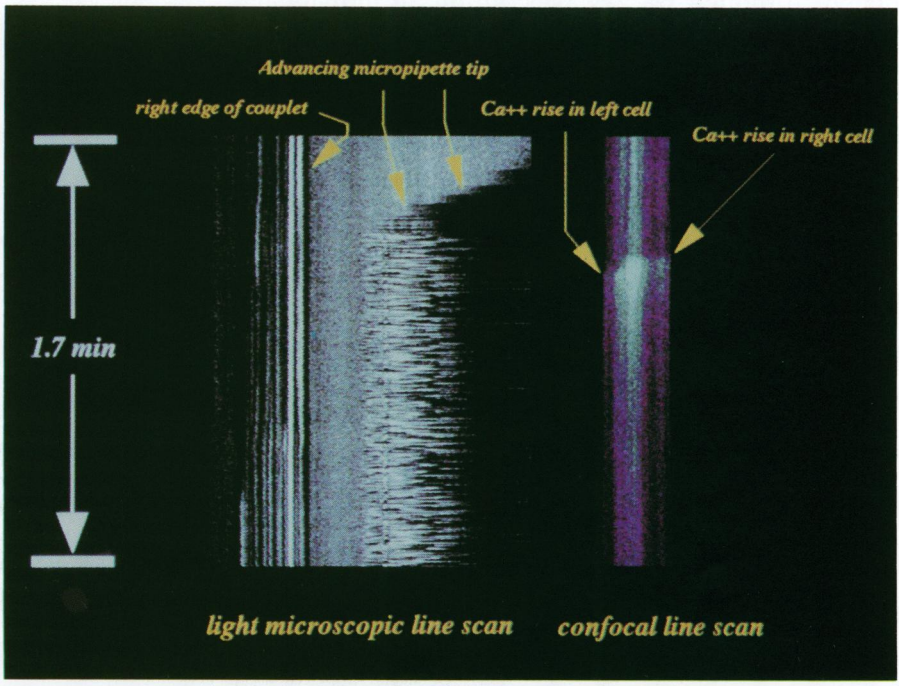
Figure 3. Oscillations in free cytosolic Ca^{2+} in isolated hepatocytes and hepatocyte couplets stimulated with phenylephrine (0.1 μM). (A) Low-magnification confocal image of isolated hepatocytes loaded with fluo3. Note that the line to be scanned intersects a couplet. (B) Repetitive confocal scan of the line indicated in A. Alternating increases (green arrows) and decreases in fluorescence indicate phenylephrine-induced Ca_i^{2+} oscillations. Note the variations in phase and frequency of the oscillations among cells. Line was scanned every 500 ms for a total of 256 s. (C) Graphical representation of fluorescence change over time in both cells of the hepatocyte couplet in B. Note that oscillations in fluorescence intensity are synchronous in the couplet, in contrast to the varied oscillatory patterns among individual isolated hepatocytes seen in B.

cence over time at specific locations within the cytosol can be followed accurately despite compartmentalization of the dye elsewhere in the cell.

Cytosolic Ca^{2+} waves, both intra- and intercellular, have been observed in a variety of epithelia (Jacob, 1990; Kasai and Augustine, 1990; Rooney *et al.*, 1990; Sanderson *et al.*, 1990). Wavefront speeds ranging from 15 to 50 μm/s have been reported within single cells (Jacob, 1990; Kasai and Augustine, 1990; Rooney *et al.*, 1990; Sanderson *et al.*, 1990), whereas intercellular Ca_i^{2+} waves appear to be slower (up to 25 μm/s) and to pause at cell boundaries (Sanderson *et al.*, 1990). On the basis of such observations, it has been estimated that the diffusion coefficient D for Ca_i^{2+} in hepatocytes is 330 $\mu m^2/s$ (Meyer, 1991), which is similar to the diffusion coefficient for Ca_i^{2+} in other cell types (Lechleiter *et al.*, 1991; Meyer, 1991). The present work indicates that Ca^{2+} signals that result from hormonal stimulation travel across hepatocyte couplets and triplets at average speeds > 100 μm/s (with speeds of over 200 μm/s in some couplets), almost an order of magnitude faster than speeds of Ca_i^{2+} waves reported in other epithelia. On the basis of these velocity measurements and the assumption that D is proportional to the square of the velocity (Meyer, 1991), estimates of D would be on the order of 8000 $\mu m^2/s$. This diffusion coefficient is over 20 times the value expected from simple diffusion of Ca_i^{2+} and over 200 times the value expected from diffusion of IP_3 (Lechleiter *et al.*, 1991). In previous studies of intercellular Ca_i^{2+} signaling, individual epithelial cells were stimulated either mechanically (Sanderson *et al.*, 1990) or by microinjection of Ca^{2+} or IP_3 (Saez *et al.*, 1989; Sanderson *et al.*, 1990), and the resultant Ca^{2+} wave was followed as it crossed into adjacent cells that had not been stimulated. In the current study, in contrast, both cells of each couplet were stimulated simultaneously. This led to a Ca^{2+} signal that appeared to begin at a single locus in one cell of the couplet, even though both cells had been stimulated. This Ca_i^{2+} signal then spread at an unexpectedly high speed across both of the cells, without pausing at cell boundaries. This finding suggests that hormonal stimulation induces a type of coordinated intercellular response that is different from and much faster than is seen by



A



B

Figure 4. Selective stimulation of individual hepatocytes by microperfusion with vasopressin. (A) Simultaneous light microscopic transmission (left) and confocal (right) image of fluo3-loaded hepatocytes. Micropipette tip is seen in the transmission image. (B) Simultaneous transmission (left) and confocal (right) line scan as the micropipette tip is advanced toward the hepatocyte couplet. Abrupt sequential increase in Ca^{2+} in the two halves of the couplet (right) occurs when the tip approaches the right cell of the couplet (left).

simple diffusional spread of a second messenger (such as Ca_i^{2+} or IP_3) among cells.

The potential significance of coordination of intracellular Ca_i^{2+} signals in hepatocytes is that it permits integrative "organ-level" responses of these cells to hormonal stimuli. This work confirms that hormone-induced Ca_i^{2+} signals begin in a single intracellular locus in hepatocytes and demonstrates that such signals travel across hepatocyte couplets and triplets in a continuous wave-like fashion. These findings are not surprising because hepatocyte gap junctions admit Ca^{2+} and IP_3. Unexpectedly, these Ca_i^{2+} waves travel at speeds greater

than would be expected by simple intra- and intercellular diffusion of a second messenger. The mechanism for propagation of such Ca_i^{2+} waves is unclear though. Rapid propagation of ions across the cytosol is suggestive of an electrically mediated event, but hepatocytes lack voltage-dependent Ca^{2+} channels (Mauger and Claret, 1988). A positive-feedback mechanism, such as Ca^{2+}-induced Ca^{2+} release (CICR), could also be responsible for cytosolic events that occur more quickly than can be explained by diffusion. However, it is not clear why CICR would not also lead to rapid Ca_i^{2+} waves after microinjection of Ca^{2+} nor is it apparent why Ca_i^{2+} waves are slower (<25 μm/s) in hormone-stimulated isolated hepatocytes in longer-term culture (15–16 h, as opposed to 2–6 h in the current study) (Rooney *et al.*, 1990). An alternative explanation is that coupled hepatocytes are in near-identical physiological states relative to their neighbors, so that their response to hormonal stimulation appears to be synchronized. The functional significance of this high degree of coordination among hepatocytes is also unclear. In the hepatocyte, such pericanalicular functions as tight junction permeability (Lowe *et al.*, 1988), canalicular contraction (Watanabe *et al.*, 1985), and canalicular bile secretion (Nathanson *et al.*, 1992) are Ca^{2+}-mediated. Although Ca_i^{2+} waves within an individual pancreatic acinar cell coordinate basolateral and apical chloride transport (Kasai and Augustine, 1990), it is unknown whether Ca^{2+} waves are also important for regulating polarized cell functions in the hepatocyte. Further work will be needed to clarify the interrelationship between intra- and intercellular Ca^{2+} signaling and cell function in hepatocytes and other epithelia.

APPENDIX 1

To determine the relationship between the velocity (v) and the "apparent" or measured velocity (v_a) of a Ca_i^{2+} wave in an isolated hepatocyte, we make the following assumptions (Figure 5):

1) The hepatocyte is a sphere with radius (R) centered at (0, 0);
2) The Ca_i^{2+} wave emanates from a point (P) (x, y) at a constant velocity (v);
3) Line scanning measurements are performed along the y-axis of the cell.

Because a sphere is radially symmetric, without loss of generality we can consider the plane through the cell containing both the scan line and P. The apparent velocity along the scan line will be

$$v_a = v(R - y)/\{(x^2 + [R - y]^2)^{1/2} - x\}$$

To determine the mean value for v_a ($\bar{v}_a$), we must evaluate

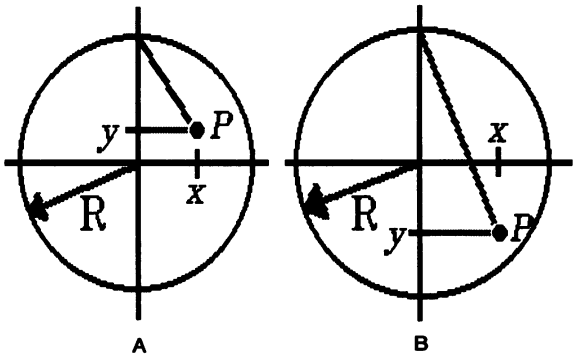


Figure 5. Relationship between the speed of a Ca_i^{2+} wave as measured by confocal line scanning microscopy and the actual speed. The hepatocyte is represented as a sphere of radius R, a Ca_i^{2+} wave emanates from a point P within the sphere, and line scans are collected along the y-axis. (A) The Ca_i^{2+} wave originates in the top half of the cell ($x > 0$). (B) The Ca_i^{2+} wave originates in the bottom half of the cell ($x < 0$).

$$\bar{v}_a = (v/\pi R^2) \int\int_S (R - y)/\{(x^2 + [R - y]^2)^{1/2} - x\}dxdy$$

where S denotes (integration over) the area within the circle. This integral can be evaluated most simply by evaluating separately the regions in which $y > 0$ (Figure 5A, region A) and $y < 0$ (Figure 5B, region B)

$$\text{A: } \bar{v}_a = (2v/\pi R^2) \int_0^R \int_{-(R^2-y^2)^{1/2}}^{+(R^2-y^2)^{1/2}} (R - y)/$$
$$\{(x^2 + [R - y]^2)^{1/2} - x\}dxdy = 1.47v$$

$$\text{B: } \bar{v}_a = (2v/\pi R^2) \int_{-R}^0 \int_{-(R^2-y^2)^{1/2}}^{+(R^2-y^2)^{1/2}} (R - y)/$$
$$\{(x^2 + [R - y]^2)^{1/2} - x\}dxdy = 1.07v$$

so that $\bar{v}_a = 1.27v$

and v is ~79% of v_a in single isolated hepatocytes, on average. By similar considerations the average speed of a Ca_i^{2+} wave across a couplet can be calculated. In particular, as the intracellular portion of the scan line increases relative to the distance between P and the scan line, the extent to which v_a overestimates v will decrease. Thus, in an hepatocyte couplet

$$\bar{v}_a < 1.17v$$

and v is over 85% of v_a on average.

APPENDIX 2

To test the hypothesis that Ca_i^{2+} signals begin independently in each half of a couplet, the probability that both cells would independently initiate a Ca_i^{2+} rise within 0.33 s is

$$P(|X_1 - X_2| < 0.33) = P(|z| < 0.33/(1.32\sqrt{2}))$$
$$= P(|z| < 0.18)$$
$$= \Phi(0.18) - \Phi(-0.18)$$
$$= 0.143$$

where X_1 and X_2 are random variables representing the time lag between vasopressin stimulation and increased Ca_i^{2+} in the two cells, $X_i \sim N(\mu, 1.32^2)$, $X_i - X_j \sim N(0, 2[1.32^2])$, $z \sim N(0, 1)$, and Φ is the cumulative distribution function for z. The probability that this event would occur eight consecutive times, as we experimentally observed, is 0.143^8 (or 1.7×10^{-7}), which indicates that the vasopressin-induced rise in Ca_i^{2+} in one cell of an hepatocyte couplet is not independent of the rise in the other cell.

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