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Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions

(liver/intercellular communication/fura-2)

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ABSTRACT **Hepatocytes are well coupled by gap junctions, which allow the diffusion of small molecules between cells. Although gap junctions in many tissues are permeable to molecules larger than cAMP and in several preparations gap junctions pass cAMP itself, little direct evidence supports permeation by other second-messenger species. Ca^{2+}, perhaps the smallest second messenger, would be expected to cross gap junctions, but the issue is complicated because gap-junction channels are closed when intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, is elevated to micromolar levels or above. Inositol 1,4,5-trisphosphate ($InsP_3$), a second messenger that can evoke Ca^{2+} release, might also reduce junctional permeability by this mechanism. We report here evidence for transjunctional flux of Ca^{2+} and $InsP_3$ in freshly isolated pairs or small clusters of rat hepatocytes. The Ca^{2+} indicator fura-2 was used to monitor transjunctional diffusion of Ca^{2+} directly or to detect passage of $InsP_3$ by localized Ca^{2+} release. Fura-2 injected as the free acid passed between cells. Injection of $InsP_3$ or $CaCl_2$ immediately increased $[Ca^{2+}]_i$ in the injected cell (peak values < 1 μM), and $[Ca^{2+}]_i$ increased rapidly in contacting cells (within seconds). The initial rise in $[Ca^{2+}]_i$ induced by $InsP_3$ was greater at discrete regions in the cytoplasm of both injected and uninjected cells and was inconsistent with simple diffusion of Ca^{2+}. In the coupled cells the regions of greatest increase were not necessarily near the contact zone. In contrast, the rise induced in $[Ca^{2+}]_i$ by $CaCl_2$ injection when cells were bathed in normal Ca^{2+} was always more diffuse than with $InsP_3$ injection, and in cells coupled to a cell injected with $CaCl_2$ the earliest and maximal increases occurred at the region of cell contact. This difference in distribution indicates that injected $InsP_3$ (or an active metabolite, but not Ca^{2+}) diffused between cells to cause localized release of Ca^{2+} from intracellular stores. Ca^{2+} injection induced a rise in $[Ca^{2+}]_i$ in coupled cells even when cells were maintained in Ca^{2+}-free saline, suggesting that changes in $[Ca^{2+}]_i$ seen in adjacent cells were due to transjunctional diffusion from the injected cell and not to uptake from the extracellular solution. However, in Ca^{2+}-free saline, $[Ca^{2+}]_i$ distribution was nonuniform, indicating that Ca^{2+}-releasing mechanisms contribute to the observed changes. No increase in $[Ca^{2+}]_i$ was seen in adjacent cells when Ca^{2+} was injected after treatment with the uncoupling agent octanol (500 μM), which itself did not change $[Ca^{2+}]_i$. These data provide evidence that the second messengers Ca^{2+} and $InsP_3$ can be transmitted from cell to cell through gap junctions, a process that may have an important role in tissue function.**

Gap junctions are permeable to small ions and molecules that are major cytoplasmic constituents, including nucleotides (1), sugars (2), glycolytic substrates (3), amino acids (4), small peptides (5), and cAMP (6–9). Gap junctions are also permeable to small fluorescent molecules (10, 11) and quaternary ammonium ions of a range of sizes (12). Junctional permeability to some of these molecules has been quantified (12–17).

Diffusion of molecules through gap junctions is driven by the electrochemical gradient across them. Permeant molecules may have a variety of signaling functions and may affect junctional permeability either through effects on gating (18–21), apparently mediated by phosphorylation of the channel protein (19), or through alteration of gene expression (22–26).

Ca^{2+} ions are recognized as second messengers within individual cells but have been largely overlooked in intercellular signaling functions because elevated intracellular Ca^{2+} level ($[Ca^{2+}]_i$) has been shown to reduce gap junctional conductance in a number of systems (27–30). However, the concentration of Ca^{2+} required to block gap junctions appears to be well above 1 μM (31), far higher than normal resting $[Ca^{2+}]_i$. From the size limit determined from permeability studies, Ca^{2+} at lower concentrations would be expected to permeate gap junctions. Indeed, another divalent cation, Co^{2+}, permeates gap junctions of the crayfish septate axon when injected at low concentrations but blocks permeation and electrical coupling at high (but unmeasured) concentrations (32).

Intercellular diffusion of Ca^{2+} or a Ca^{2+}-releasing second messenger has been demonstrated in the activation of photophores by electrically excitable supporting cells in the hydroid *Obelia* (33). Transjunctional Ca^{2+} fluxes could be the basis for propagated changes in smooth muscle tone in blood vessels (34) and could lead to increased secretion by coupled endocrine cells (35).

Inositol 1,4,5-trisphosphate ($InsP_3$) is a second messenger in hepatocytes generated through the phosphatidylinositol pathway. Stimulation of hepatocytes with vasopressin, angiotensin II, or noradrenaline induces hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce $InsP_3$ and diacylglycerol (36–38). $InsP_3$ has been shown to mobilize intracellular Ca^{2+} in hepatocytes (36, 39–41), and Ca^{2+} is the intracellular signal that links the cell-surface receptors for the three hormones cited above to induction of glucose formation from glycogen (42). In hormone-stimulated hepatocytes $[Ca^{2+}]_i$ shows repetitive transient rises to peak concentrations of $\approx$600 nM (43), but $InsP_3$ injection would not be expected to produce such oscillations (44).

Despite the possible relevance of transjunctional diffusion of $InsP_3$ and Ca^{2+} to physiological functions of liver and other tissues, whether $InsP_3$ or Ca^{2+} at physiological concentrations can permeate gap junctions has not been shown. The availability of fluorescent calcium indicators sensitive to physiological cytoplasmic Ca^{2+} concentrations and digital analysis of microscopic fluorescence images (45–47) allowed study of this question. We found that both Ca^{2+} and $InsP_3$ (or

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular free calcium concentration.

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a product of its action other than Ca^{2+}) permeate the gap junctions between adult rat hepatocytes. Preliminary findings of this study have been presented.[§]

METHODS

Preparation of Partially Dissociated Hepatocytes. Cells were dissociated from livers of adult Sprague–Dawley female rats (200–250 g) by a collagenase perfusion technique (48). Cells were suspended in Waymouth's medium supplemented with 10% fetal calf serum (GIBCO) and plated (10^4 cells per 23-mm culture dish) on polylysine-coated coverslips (18-mm diameter) for at least 1 hr at 37°C in humidified 5% CO_2/95% air. Coverslips were coated by immersion overnight in poly(L-lysine) solution at 20 μg/ml (Sigma) and rinsed twice with sterile water before use. All experiments were performed at 30°C.

Analysis of Intracellular Free Calcium Concentration. For most experiments the fluorescent calcium indicator, fura-2, was loaded into hepatocytes by incubation in medium containing the membrane-permeable fura-2 tetrakis(acetoxymethyl) ester (fura-2 AM) (45–47, 49). Plated cells were exposed for 20 min to a suspension of fura-2 AM that had been dissolved in dimethyl sulfoxide (DMSO) and mixed with Leibowitz's medium (GIBCO) at a nominal concentration of 5 μM fura-2 AM and 0.3% DMSO. Cells were rinsed before use with Krebs' saline or Ca^{2+}- and PO_4^{-3}-free Krebs' saline containing 0.1 mM EGTA. In other experiments fura-2 in the ionic form (5 mM in the pipette) was injected iontophoretically into hepatocytes through a 20–50 MΩ glass pipette. Fura-2 concentration was monitored with 360-nm excitation, at which fura-2 fluorescence is largely $[Ca^{2+}]_i$-independent. Intrinsic fluorescence of the cells measured at 360-nm excitation was <10% of that seen in cells after loading with the indicator.

Spatially resolved calcium measurements were made by acquiring two fluorescent images of the same microscope field with excitation at different wavelengths (340 and 380 nm) and detection through a 480-nm long-pass filter (47–50). After correcting for background fluorescence and camera dark current, a ratio image was calculated (340:380). Data acquisition for each ratio image required 0.9 sec. Ratio images were determined at either 2-sec or 5-sec intervals as noted in the text. Ratio images were converted to calcium concentration according to the following equation:

$$K_d \times \left(\frac{R - R_{min}}{R_{max} - R}\right) \times \left(\frac{F_o}{F_s}\right),$$

where K_d is the equilibrium dissociation constant of fura-2 for Ca^{2+} (≈220 nM at 30°C), the R terms are ratios of intensity of emission from 340- and 380-nm excitation (i.e., $R = F_{340}/F_{380}$) recorded for the particular pixel, R_{max} is the ratio for saturating levels of Ca^{2+}, R_{min} is the ratio for no added calcium and 5 mM EGTA, and F_0/F_s is the fluorescence ratio obtained at low and saturating Ca^{2+} levels with 380-nm excitation (13.5 in these studies, see ref. 45).

Injection of InsP_3 or $CaCl_2$. InsP_3 (2.5 mM in 150 mM lithium chloride or 150 mM potassium glutamate) was pressure-injected through glass pipettes. Injection of either saline alone had no effect (n = 1 for each). Ca^{2+} was injected by pressure or by iontophoresis using short depolarizing pulses through pipettes containing 1 mM $CaCl_2$.

[§]Sáez, J. C., Connor, J. A., Spray, D. C. & Bennett, M. V. L., Fourth International Congress on Cell Biology, Aug. 14–19, 1988, Montreal, p. 243 (abstr.).

RESULTS

Gap Junctions Are Permeable to Fura-2. Fura-2 injected iontophoretically into one cell of a small group of hepatocytes spread to other cells. In the example shown in Fig. 1, the middle cell of a triplet seen by endogenous fluorescence at high sensitivity (Fig. 1*A*) was rapidly injected with fura-2 and seen using 360-nm excitation. Immediately after injection, fluorescence of the injected cell was intense so that, at the reduced sensitivity used, the background autofluorescence in the adjacent cells was almost undetectable (Fig. 1*B*). Three minutes later fura-2 had spread throughout the lower adjacent cell and was detectable in the upper cell (Fig. 1*C*). The level of fluorescence was greater in the nuclei than in the cytoplasm. Additional dye spread had occurred after 6 min (Fig. 1*D*). Similar results were obtained in three additional cases.

Gap Junctions Are Permeable to InsP_3. In cells loaded with fura-2 applied as the membrane-permeant derivative (fura-2 AM), $[Ca^{2+}]_i$ showed no significant changes over 3–4 hr. The average was 228.5 ± 41.5 nM (mean ± SEM; n = 28).

No significant increase in $[Ca^{2+}]_i$ was seen in hepatocytes during or after application of current to one cell sufficient to depolarize or hyperpolarize it by ±40 mV (n = 2; data not shown).

Injection of InsP_3 caused immediate localized increases in $[Ca^{2+}]_i$ in the injected cell and later localized increases in $[Ca^{2+}]_i$ in adjacent cells (Fig. 2). The left-hand cell of the fura-2 AM-loaded triplet shown in Fig. 2 *A* (phase) and *B*

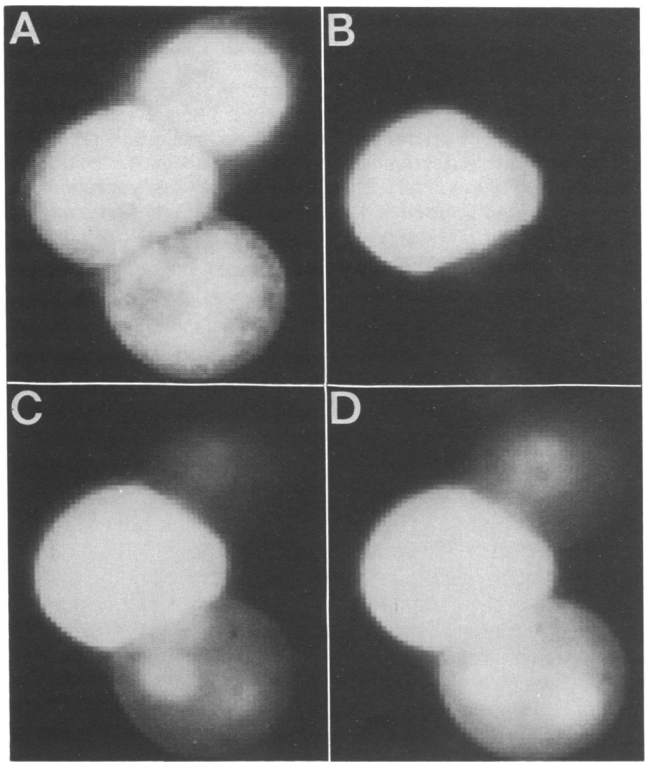

FIG. 1. Liver gap junctions are permeable to fura-2. (*A*) Background emission with 360-nm excitation, sensitivity about 25 times higher than in *B–D*, where background emission is too faint to be apparent. The nucleus is darker than the cytoplasm, probably reflecting the distribution of NADH. (*B–D*) Fluorescent images with 360-nm excitation. (*B*) The middle cell of the triplet was pressure-injected with fura-2. (*C*) Three minutes later transfer to both neighboring cells of fura-2 had occurred, with more extensive transfer to the lower cell. Fura-2 was more concentrated in the nuclei (which may have been due to binding to nuclear components) and possibly other subcellular organelles, although the cytoplasm of each cell shows diffuse staining. (*D*) Six minutes later fluorescence intensities were increased in the uninjected cells.

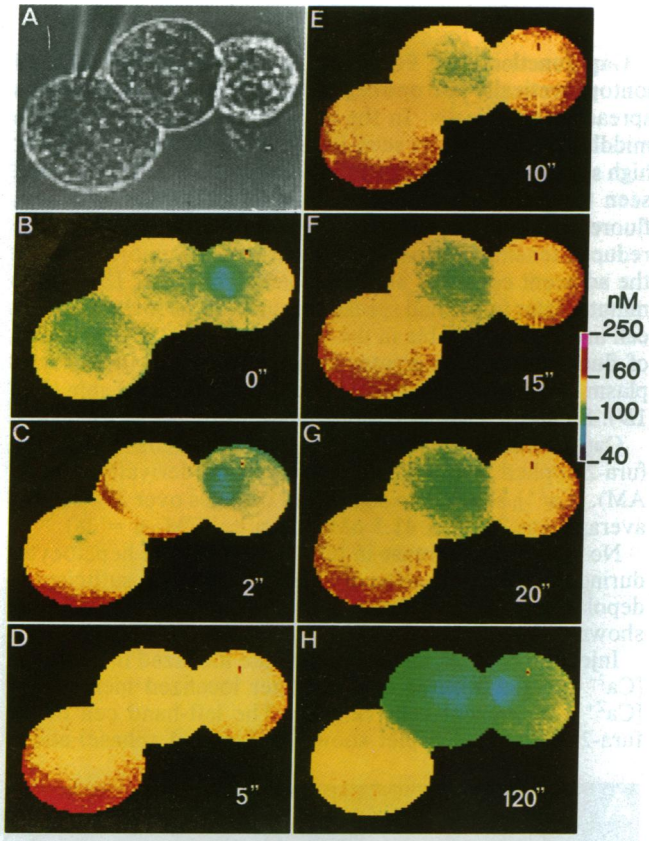

FIG. 2. Liver gap junctions are permeable to InsP_3. (*A*) Nomarski image of a triplet of hepatocytes plated 3 hr previously. (*B–H*) Ca^{2+} levels in cells determined from fluorescence ratios (see *Methods*); times after injection are indicated in seconds. (*B*) Before injection $[Ca^{2+}]_i$ was similar in all three cells. (*C*) InsP_3 injection into the leftmost cell caused a rapid (<2-sec latency) and transient increase in $[Ca^{2+}]_i$ at discrete regions of that cell and the adjacent one. (*D*) Five seconds later $[Ca^{2+}]_i$ was reduced in the middle cell and had increased in the righthand cell. (*E*) $[Ca^{2+}]_i$ had continued to increase in the far cell and had nearly recovered in the middle cell. (*F–H*) In the middle and far cells $[Ca^{2+}]_i$ decreased to values lower than the initial ones, whereas in the injected cell $[Ca^{2+}]_i$ progressively declined toward control values. The injection pipette remained in the left cell during the recording period, which could account for the slower and less complete recovery of its $[Ca^{2+}]_i$. Ca^{2+} levels are given in the color scale at the right.

(ratio image) was pressure-injected with InsP_3, leading to a rapid (<2-sec latency) increase in $[Ca^{2+}]_i$ from 120 nM to 180 nM in a discrete region of the injected cell (Fig. 2*C*, red region in lower left corner). $[Ca^{2+}]_i$ was also elevated in the middle cell at the area of contact with the injected cell. Approximately 5 sec later $[Ca^{2+}]_i$ had increased to at least 160 nM in the most distal region of the far cell (Fig. 2*D*, red region in this cell). After another 5 sec $[Ca^{2+}]_i$ had increased further in the far cell and decreased in the middle cell (Fig. 2*E*). $[Ca^{2+}]_i$ then declined in both far and middle cells to below the initial levels (Fig. 2 *F–H*).

After $[Ca^{2+}]_i$ in the injected cell reached its maximum value (Fig. 2*E*), it decreased toward the initial concentration but remained somewhat elevated (Fig. 2 *F–H*). Although recovery was seen in all injected cells, it was generally incomplete and slower than in coupled cells, probably because of leakage of InsP_3 from the tip of the injecting pipette, which was maintained within the cell throughout the experiment.

The same pattern of changes in $[Ca^{2+}]_i$ was seen with InsP_3 dissolved in 150 mM potassium glutamate (n = 8) or 150 mM lithium chloride (n = 14), although Li^+ blocks degradation of InsP_3 (51). When initial $[Ca^{2+}]_i$ was higher in one or more cells, increase in Ca^{2+} was not seen in these cells (n = 3).

In most of the InsP_3 injection experiments the $[Ca^{2+}]_i$ increases occurred in more or less restricted regions, and there were intervening regions in which $[Ca^{2+}]_i$ changed little. In two instances an increase occurred in the third cell of the connected string, with little or no increase in the second cell. This observation suggests that InsP_3 and not Ca^{2+} was spreading between cells and causing local release of Ca^{2+} from intracellular stores, which were small or depleted in the intermediate cell. Alternatively there might be another diffusible substance than Ca^{2+}, the concentration of which is increased by InsP_3 and which also acts to release Ca^{2+}. The different appearance after $CaCl_2$ injection (see below) confirms that InsP_3 or a similar molecule is acting as a diffusible messenger.

Gap Junctions Are Permeable to Ca^{2+}. Injection of $CaCl_2$ by small positive current pulses or by pressure (electrode position indicated in Fig. 3) raised $[Ca^{2+}]_i$ throughout the cytoplasm of the injected cell and rapidly elevated $[Ca^{2+}]_i$ in adjacent cells (n = 4, Fig. 3 *A* and *B*). In three cases the $[Ca^{2+}]_i$ levels in the adjacent cell approached those in the injected cell (Fig. 3*B*). In one case a wave of elevated $[Ca^{2+}]_i$ was seen moving from the injected cell to the adjacent cell. In a fifth case no Ca^{2+} movement was observed, and the cells presumably were uncoupled. In the experiment illustrated in Fig. 3, a second injection of $CaCl_2$ caused an increase of $[Ca^{2+}]_i$ to 600 nM, but there was no further diffusion to the adjacent cell in which $[Ca^{2+}]_i$ recovered its initial value (Fig. 3 *C* and *D*). This observation might have been due to cell uncoupling as a direct or indirect consequence of $CaCl_2$ injection. Partial recovery of $[Ca^{2+}]_i$ in the injected cell occurred later. Partial recovery in both injected and uninjected cells was observed in another case, but was not examined in the remaining cases.

Spread of elevated $[Ca^{2+}]_i$ between cells after $CaCl_2$ injection was completely prevented by the addition of 500 μM 1-octanol (n = 3, data not shown), a concentration that completely blocks the junctional conductance between pairs of hepatocytes (unpublished observation). Although reversibility was not studied in a single pair, in two cases spread of Ca^{2+} from an injected to an adjacent cell was seen after washing out an uncoupling concentration of octanol.

When $CaCl_2$ was injected into cells maintained in Ca^{2+}-free Krebs' saline, intercellular diffusion of Ca^{2+} was also seen (n = 3; Fig. 3 *F–J*). The resting pattern of Ca^{2+} distribution in Ca^{2+}-free or Ca^{2+}- and PO_4^{-3}-free saline was different from that seen in cells maintained in Ca^{2+}-containing medium, in that levels of $[Ca^{2+}]_i$ were higher in the periphery of the cells (except in the contact regions, compare Fig. 3 *A* and *F*). Injection of $CaCl_2$ increased $[Ca^{2+}]_i$ throughout the cytoplasm of the injected cell (top cell, Fig. 3 *G–J*) and caused a progressive increase in $[Ca^{2+}]_i$ in the adjacent cell that was most pronounced in the periphery. Between the region closest to the junctions and the periphery there was always an intervening region in which $[Ca^{2+}]_i$ had not changed significantly when the increase in the periphery was first detected. In two of these experiments electrical coupling was demonstrated qualitatively during diffusion of Ca^{2+}.

DISCUSSION

The levels of resting $[Ca^{2+}]_i$ (228.5 ± 41.5 nM) that we observed in adult rat hepatocytes are similar to those reported by others using different calcium-sensitive indicators, arsenazo III (52), quin-2 (53), and aequorin (43). We found that injections of InsP_3 increased $[Ca^{2+}]_i$ in adjacent cells. The spatial distribution of the increases indicated that Ca^{2+} was released locally from intracellular stores, presumably calciosomes (54), and it is likely that InsP_3 itself diffuses from cell to cell. Increases in $[Ca^{2+}]_i$ evoked by InsP_3 were never large, ≈100 nM, but we did not attempt to generate a dose–

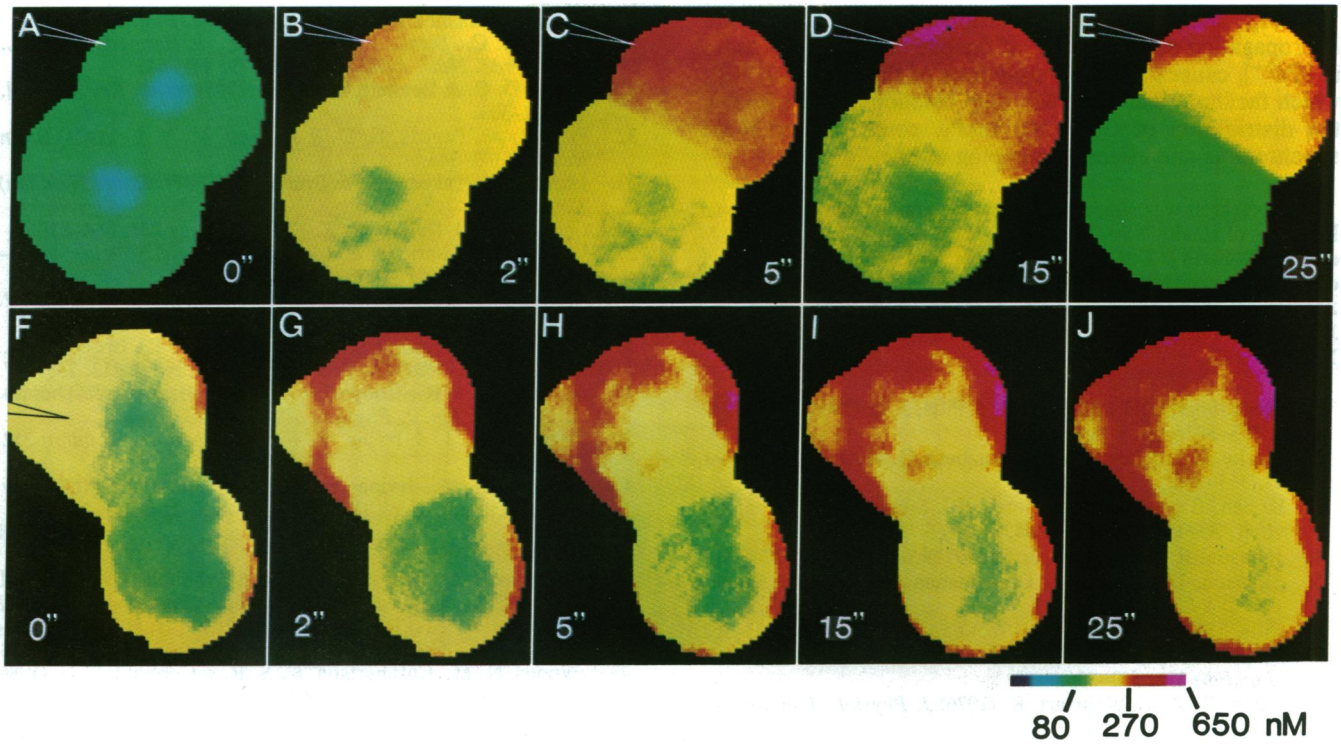

FIG. 3. Liver gap junctions are permeable to Ca^{2+} either in the presence (*A–E*) or the absence of extracellular Ca^{2+} (*F–J*). (*A*) $[Ca^{2+}]_i$ image of two cells in contact after 8-hr culture. (*B*) Pressure-injection of $CaCl_2$ (electrode position indicated as drawn) rapidly increased $[Ca^{2+}]_i$ from 120 nM to 250 nM in the injected cell and from 120 nM to 230 nM in the adjacent cell. (*C*) Ten seconds later $[Ca^{2+}]_i$ was elevated further in the injected cell but was unchanged in the adjacent cell. Presumably the cells were no longer coupled. (*D* and *E*) $[Ca^{2+}]_i$ recovered almost completely in the uninjected cell and to a small extent in the injected cell. (*F*) $[Ca^{2+}]_i$ levels in three cells in contact after 4-hr culture in Ca^{2+}- and PO_4^{-3}-free Krebs' saline. $[Ca^{2+}]_i$ is highest at the cellular periphery except at the contact region. (*G*) Injection of $CaCl_2$ increased $[Ca^{2+}]_i$ diffusely in the injected cell and also increased $[Ca^{2+}]_i$ in the adjacent cell, in which the increase was most marked in the periphery. (*H–J*) Twenty-five seconds later $[Ca^{2+}]_i$ continued to rise.

response curve. We also found that injected $CaCl_2$, which could approach a concentration of 1 μM, increased $[Ca^{2+}]_i$ of adjacent cells. In Ca^{2+}-containing medium the time course of the rise of $[Ca^{2+}]_i$ in adjacent cells appeared consistent with simple diffusion. The increase in $[Ca^{2+}]_i$ in adjacent cells was completely prevented by octanol, which blocks gap junctions in many cell types, including hepatocytes (ref. 55 and unpublished work). Rises in $[Ca^{2+}]_i$ in uninjected cells occurred even when the extracellular Ca^{2+} concentration was buffered to levels below 50 nM. However, in low-Ca^{2+} medium the changes in $[Ca^{2+}]_i$ distribution no longer appeared consistent with simple diffusion. In these experiments it is possible that in addition to Ca^{2+} diffusion, injected Ca^{2+} caused release of InsP_3, which diffused to coupled cells and, as with direct InsP_3 injection, caused localized increase in $[Ca^{2+}]_i$.

Sufficiently high $[Ca^{2+}]_i$ causes junctional conductance to decrease in a variety of tissues (27–30). The levels of $[Ca^{2+}]_i$ reportedly required to reduce junctional conductance, 5–500 μM, are well above the highest that are induced hormonally in hepatocytes (43). Thus, Ca^{2+} may affect coupling in liver only in pathological conditions, such as when the surface membrane is ruptured allowing $[Ca^{2+}]_i$ to rise towards extracellular levels.

In liver, electrical coupling occurs over a distance of at least 500 μm (56), which is comparable to the length of the hemiacinus (≈20 cells in series, each with a diameter of 20–25 μM). Thus the distance InsP_3 and Ca^{2+} can diffuse when their concentrations are raised locally by hormone action is likely to be limited primarily by intracellular buffering and might extend along a significant portion of the hemiacinus.

In hepatocytes, hormonal activation of the signal-transduction system that leads to degradation of phosphatidylinositol 4,5-bisphosphate causes release of diacylglycerol and InsP_3 (36–38). Diacylglycerol activates protein kinase C, which phosphorylates the main gap junctional protein at the same site phosphorylated by cAMP-dependent protein kinase (57, 58), presumably leading to similar increases in junctional conductance. InsP_3 induces mobilization of intracellular Ca^{2+} stores into the cytoplasm (36, 39, 40), and elevation of $[Ca^{2+}]_i$ causes translocation of protein kinase C to the plasma membrane (59). Thus agonist-induced activation of protein kinase C and Ca^{2+} mobilization act synergistically (59). Activation of protein kinase C and mobilization of intracellular Ca^{2+} are both required for a full hormonal response in hepatocytes (60). One final effect of activation of this transduction system is glycogenolysis (42).

Because gap junctions between hepatocytes are permeable to InsP_3 and Ca^{2+}, the gap junctions would permit an averaging of the concentration of these second messengers among coupled cells. At the least this property would tend to equalize hormonal response in spite of differences of receptor density or metabolic competence. Interestingly, although glycogen stores are equal throughout the hemiacinus of well-fed animals (61), the hemiacinus is a heterogeneous cellular system, where zone 1 (surrounding the terminal portal vein) is more active in glycogenolysis and zone 3 (surrounding hepatic venules) is more active in glycolysis (62), and each zone presumably expresses more receptors for the hormones that activate their respective metabolic processes. At low levels of hormonal input the zones could act independently, but at high levels of stimulation either zone could be recruited by intercellular diffusion of second messengers to the process normally predominating in the other zone.

Finally, intercellular Ca^{2+} diffusion in liver may play a role in propagation of contraction of the bile canaliculi. Increase in $[Ca^{2+}]_i$ causes active contractions of the canaliculi (63), which facilitate bile flow (64). Gap junctions, by equalizing the distribution of Ca^{2+} and $InsP_3$, could lead to more synchronous and effective pumping of bile.

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