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Isolated rat hepatocytes can signal to other hepatocytes and bile duct cells by release of nucleotides

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ABSTRACT **Intercellular communication among certain cell types can occur via ATP secretion, which leads to stimulation of nucleotide receptors on target cells. In epithelial cells, however, intercellular communication is thought to occur instead via gap junctions. Here we examined whether one epithelial cell type, hepatocytes, can also communicate via nucleotide secretion. The effects on cytosolic Ca^{2+} ($[Ca^{2+}]_i$) of mechanical stimulation, including microinjection, were examined in isolated rat hepatocytes and in isolated bile duct units using confocal fluorescence video microscopy. Mechanical stimulation of a single hepatocyte evoked an increase in $[Ca^{2+}]_i$ in the stimulated cell plus an unexpected $[Ca^{2+}]_i$ rise in neighboring noncontacting hepatocytes. Perifusion with ATP before mechanical stimulation suppressed the $[Ca^{2+}]_i$ increase, but pretreatment with phenylephrine did not. The P_2 receptor antagonist suramin inhibited these intercellular $[Ca^{2+}]_i$ signals. The ATP/ADPase apyrase reversibly inhibited the $[Ca^{2+}]_i$ rise induced by mechanical stimulation, and did not block vasopressin-induced $[Ca^{2+}]_i$ signals. Mechanical stimulation of hepatocytes also induced a $[Ca^{2+}]_i$ increase in cocultured isolated bile duct units, and this $[Ca^{2+}]_i$ increase was inhibited by apyrase as well. Finally, this form of $[Ca^{2+}]_i$ signaling could be elicited in the presence of propidium iodide without nuclear labeling by that dye, indicating that this phenomenon does not depend on disruption of the stimulated cell. Thus, mechanical stimulation of isolated hepatocytes, including by microinjection, can evoke $[Ca^{2+}]_i$ signals in the stimulated cell as well as in neighboring noncontacting hepatocytes and bile duct epithelia. This signaling is mediated by release of ATP or other nucleotides into the extracellular space. This is an important technical consideration given the widespread use of microinjection techniques for examining mechanisms of signal transduction. Moreover, the evidence provided suggests a novel paracrine signaling pathway for epithelia, which previously were thought to communicate exclusively via gap junctions.**

Biological responses to extracellular ATP have been described in virtually every major tissue (1). In some of these cell systems, the source and potential physiologic role of extracellular ATP are unclear. ATP can be released from mouse fibroblasts (2) or rat basophilic leukemia cells (3) to mediate the intercellular spread of $[Ca^{2+}]_i$ signals in an autocrine or a paracrine fashion. In epithelia, though, intercellular communication is thought to be mediated by direct cell-to-cell spread of second messengers via gap junctions. In hepatocytes, for example, both $[Ca^{2+}]_i$ and inositol 1,4,5-trisphosphate can cross gap junctions (4). Furthermore, hormonal stimulation of isolated hepatocyte couplets leads to highly coordinated $[Ca^{2+}]_i$ signals, including synchronized $[Ca^{2+}]_i$ oscillations (5); this synchronized response depends upon the presence of gap junctions. $[Ca^{2+}]_i$ signaling patterns are further organized in the intact liver (6, 7), and this also is thought to be due in part to gap junctional communication. Both hepatocytes and bile duct cells express ATP receptors, and stimulation of these receptors leads to an increase in $[Ca^{2+}]_i$ (8–11). However, the source of ATP that would stimulate these cell types *in vivo* is not established. Here we examined whether hepatocytes can release ATP, and whether this locally released ATP could provide a mechanism for spread of $[Ca^{2+}]_i$ signals to other, noncontacting isolated rat hepatocytes and isolated bile duct units.

MATERIALS AND METHODS

Animals and Materials. Male Sprague–Dawley rats (180–250 g; Camm Research Institute, Wayne, NJ) were maintained on Purina Rodent Chow under a constant light cycle and used for all experiments. ATP, grade III apyrase (E.C. 3.6.1.5), [Arg^8]vasopressin, phenylephrine, and propidium iodide were from Sigma; fluo-3/AM was from Molecular Probes; suramin was from Biomol (Plymouth Meeting, PA); and indodicarbocyanine (Cy5) was from Biological Detection Systems (Pittsburgh). All other chemicals were of the highest quality commercially available.

Preparation of Isolated Hepatocytes. Isolated rat hepatocytes were prepared in the Hepatocyte Isolation Core of the Yale Liver Center as described (5, 12). Briefly, rat livers were perfused with Hanks' A medium and then with Hanks' B medium containing 0.05% collagenase (Boehringer Mannheim) and 0.8 unit trypsin inhibitor (Sigma)/U tryptic activity. Livers were then excised, minced, and passed through serial nylon mesh filters, and the resultant cells were washed. These cells were suspended at a concentration of 7.5×10^5 cells per ml in Leibovitz L-15 medium (GIBCO) containing 10% fetal calf serum, 50 units penicillin, and 50 mg streptomycin/ml, and plated onto glass coverslips. Cells were incubated at 37°C and used 2–6 h after plating. When prepared in this fashion, ≈40% of isolated hepatocytes were not in contact with other hepatocytes, while ≈60% were in aggregates of two or more. Cell viability by trypan blue exclusion was measured 2 h after plating and exceeded 90%.

Preparation of Isolated Bile Duct Units. Bile duct units, which are polarized and physiologically intact segments of bile duct epithelia, also were prepared in the Hepatocyte Isolation Core of the Yale Liver Center as described (13). After livers were perfused with Hanks' B medium as described above, the portal tissue residue was mechanically separated from parenchymal tissue first by shaking and then by forcing the tissue through a syringe to dissociate the remaining hepatocytes. The tissue was then minced in solution C, which contained α-MEM supplemented with 0.066% collagenase, 0.033% Pronase, 0.006% DNase, 3% fetal calf serum, 0.1% bovine serum albumin, and penicillin/streptomycin at 100,000 units per 100 mg/liter. The minced tissue was then shaken at 37°C for 30 min, minced again, and sequentially filtered. Fragments remaining on the filters were digested for an additional 30 min

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Abbreviations: $[Ca^{2+}]_i$, cytosolic calcium; Cy5, indodicarbocyanine.

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in solution C, and then digested again for 30 min in solution D, in which 0.036% hyaluronidase was substituted for Pronase. Fragments were again filtered and those remaining on the 30 μm filter were collected in 4–10 ml of Leibovitz L-15 medium or α-MEM supplemented with 0.1 mM insulin, 3% fetal calf serum, 2 mM L-glutamine, 50 g/ml gentamicin, and 10^5 units per 100 mg/liter penicillin/streptomycin. Fragments were then plated on coverslips, coated with a thin layer of Matrigel (Collaborative Research), and incubated at 37°C in α-MEM at 95% O_2/5% CO_2. This isolation procedure results in aggregates of bile duct cells that are 30–100 μm in diameter and originate from small interlobular bile ducts (13). After 20–24 h, the medium was replaced with Leibovitz L-15 medium and hepatocytes isolated as described above were plated on the same coverslips for 2–4 h.

Microinjection Experiments. Micropipettes were used for mechanical stimulation and microinjection. The micropipettes were commercially obtained from Eppendorf and had an i.d. of less than 0.5 μm. An Eppendorf series 5171 micromanipulator was used for positioning and an Eppendorf series 5242 microinjector was used for pressure microinjections. For injections, micropipettes were loaded with 150 mM KCl, 1 mM Hepes, and 100 nM Cy5.

Confocal Microscopic Measurements of Cytosolic Calcium. Rat hepatocytes and bile duct units were isolated and plated onto glass coverslips as described above, and then loaded with the $[Ca^{2+}]_i$-sensitive fluorescent dye fluo3-AM (6 μM) for 20 min at 37°C in Leibovitz L-15 medium containing 10% fetal calf serum. The coverslips were transferred to a chamber on the stage of a Zeiss Axiovert microscope, and then the cells were perifused at 37°C with a 20 mM Hepes-buffered solution and observed using a Bio-Rad model MRC-600 confocal imaging system. A krypton-argon laser was used to excite the dye at 488 nm and emission signals above 515 nm were collected (5, 14). Cells were stimulated under one of the conditions described below and the resulting $[Ca^{2+}]_i$ signals were videorecorded with a Panasonic model TQ3031F optical memory disc recorder at a rate of 1 frame s^{-1}. Changes in $[Ca^{2+}]_i$ over time were monitored in 5 × 5 pixel (4.1 × 4.1 μm) square regions within individual hepatocytes. Among 23 consecutive responding hepatocytes, fluo-3 fluorescence increased by 66 ± 5% (mean ± SEM), whereas fluorescence increased by only 3 ± 2% in 27 consecutive nonresponding cells, so that increases in fluorescence by >20% were taken to indicate an increase in $[Ca^{2+}]_i$. The increases in fluorescence we observed in responding hepatocytes are similar to the change in $[Ca^{2+}]_i$ previously reported in isolated rat hepatocytes stimulated with 10 μM ATP, where $[Ca^{2+}]_i$ increased from ≈210 nM at baseline to ≈430 nM (15). In some experiments, $[Ca^{2+}]_i$ signals were instead detected using confocal line scanning microscopy (5, 16). For these studies, a collection rate (i.e., scan rate) of either 1.25 sec^{-1} or 2.5 sec^{-1} was used.

In selected experiments, the fluorescent nuclear stain propidium iodide (10 μM) was present in the perifusion medium to identify disrupted or dead cells (17). In these studies, cells were excited with both the 488 nm and 568 nm excitation lines of a krypton-argon laser. Confocal images were recorded at two separate emission wavelengths, the first one centered at 522 nm and the second one greater than 585 nm, to detect fluorescence from fluo-3 and propidium iodide, respectively. This approach permitted $[Ca^{2+}]_i$ signaling (i.e., fluo-3 fluorescence) and propidium iodide uptake to be monitored simultaneously.

In other selected experiments, propidium iodide (10 μM) was present in the medium while cells were microinjected with an intracellular-like buffer containing 100 nM Cy5 as a marker of successful injection. In these studies, hepatocytes were excited at 488, 568, and 647 nm. Confocal images were recorded at three separate wavelengths, the first one centered at 522 nm, the second one greater than 585 nm, and the third greater than 660 nm. This approach permitted $[Ca^{2+}]_i$ signaling, propidium iodide uptake, and successful microinjection of Cy5 to be monitored simultaneously.

Experimental Design. Hepatocytes were perifused either with buffer alone or with buffer containing ATP (10 μM, 2 min), phenylephrine (10 μM, 2 min), apyrase (50 units/ml, 0.5 min), suramin (100 μM, 2 min), or propidium iodide (10 μM, 2 min), and then cells were mechanically stimulated by using a micropipette to depress the top of the cell membrane by ≈50% of the cell diameter for 2–5 sec. For cells examined in the presence of apyrase or suramin, controls were taken from the same coverslip before addition of the antagonist. Treatment with apyrase was followed either by a washout period of 10 min, and then repeated mechanical stimulation, or by addition of vasopressin (100 nM). The same protocol was applied in experiments in which the hepatocytes were cocultured with bile duct cells. In other experiments, hepatocytes were perifused with 10 μM phenylephrine followed by 10 μM ATP.

Statistics and Data Analysis. Values listed are mean ± SEM. Statistical comparisons were made using Student's *t* test, and P values < 0.05 were taken as significant.

RESULTS

Hepatocytes were mechanically stimulated with a micropipette, either by gradually deforming the cell membrane or by microinjection of an intracellular-like solution. Each stimulus induced an increase in $[Ca^{2+}]_i$, first in the stimulated cell and then in neighboring isolated hepatocytes that were not in contact with the stimulated cell. This pattern of response was seen in 103 experiments using hepatocytes isolated from 12 different livers (Fig. 1). A $[Ca^{2+}]_i$ increase was seen in nearly all cells within 100 μm of the stimulated hepatocyte, and then the fraction of cells that responded decreased with increased distance in a reproducible pattern. In nine consecutive experiments, for example, $[Ca^{2+}]_i$ signals were detected in 82 ± 7% of cells within 50 μm, 73 ± 9% of cells within 50–100 μm, 45 ± 7% of cells within 100–200 μm, and 13 ± 11% of cells within 200–300 μm from the stimulated hepatocyte. $[Ca^{2+}]_i$ increases were occasionally seen in hepatocytes greater than 300 μm away as well. The time delay between stimulation of one cell and response in another cell generally increased with increasing distance between the two cells, so that the $[Ca^{2+}]_i$ signal appeared to spread from cell to cell at a speed of 28.5 ± 3.6 μm/sec (measured in 64 cells from four consecutive experiments). A similar pattern of intercellular $[Ca^{2+}]_i$ signaling was seen when hepatocytes were stimulated in Ca^{2+}-free medium fortified with 1 mM EGTA (n = 11 experiments). These findings demonstrate that intercellular $[Ca^{2+}]_i$ signaling can occur in hepatocytes independent of gap junctional coupling.

To investigate whether ATP mediates this form of intercellular signaling, we examined the effect of desensitization of hepatocyte ATP receptors. Perifusion with 10 μm ATP induced a $[Ca^{2+}]_i$ increase in virtually all hepatocytes, and then prevented a subsequent, mechanically induced $[Ca^{2+}]_i$ increase in stimulated cells and their neighbors in the continued presence of ATP (n = 14 experiments). To control for depletion of intracellular Ca^{2+} stores, we examined the effects of pretreatment with the α_{1B}-adrenergic agonist phenylephrine (10 μM), since purinergic and α_{1B}-adrenergic receptors both increase $[Ca^{2+}]_i$ through mobilization of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores in hepatocytes (1, 18). Perifusion with phenylephrine did not prevent a $[Ca^{2+}]_i$ increase induced by subsequent stimulation with ATP (n = 5 experiments), and also failed to suppress mechanically induced $[Ca^{2+}]_i$ signaling in the continued presence of phenylephrine (n = 12 experiments). These findings suggest that mechanically induced $[Ca^{2+}]_i$ signals in hepatocytes are blocked by desensitization of ATP receptors, but not by types of receptor-mediated increases in $[Ca^{2+}]_i$ that do not affect ATP receptors.

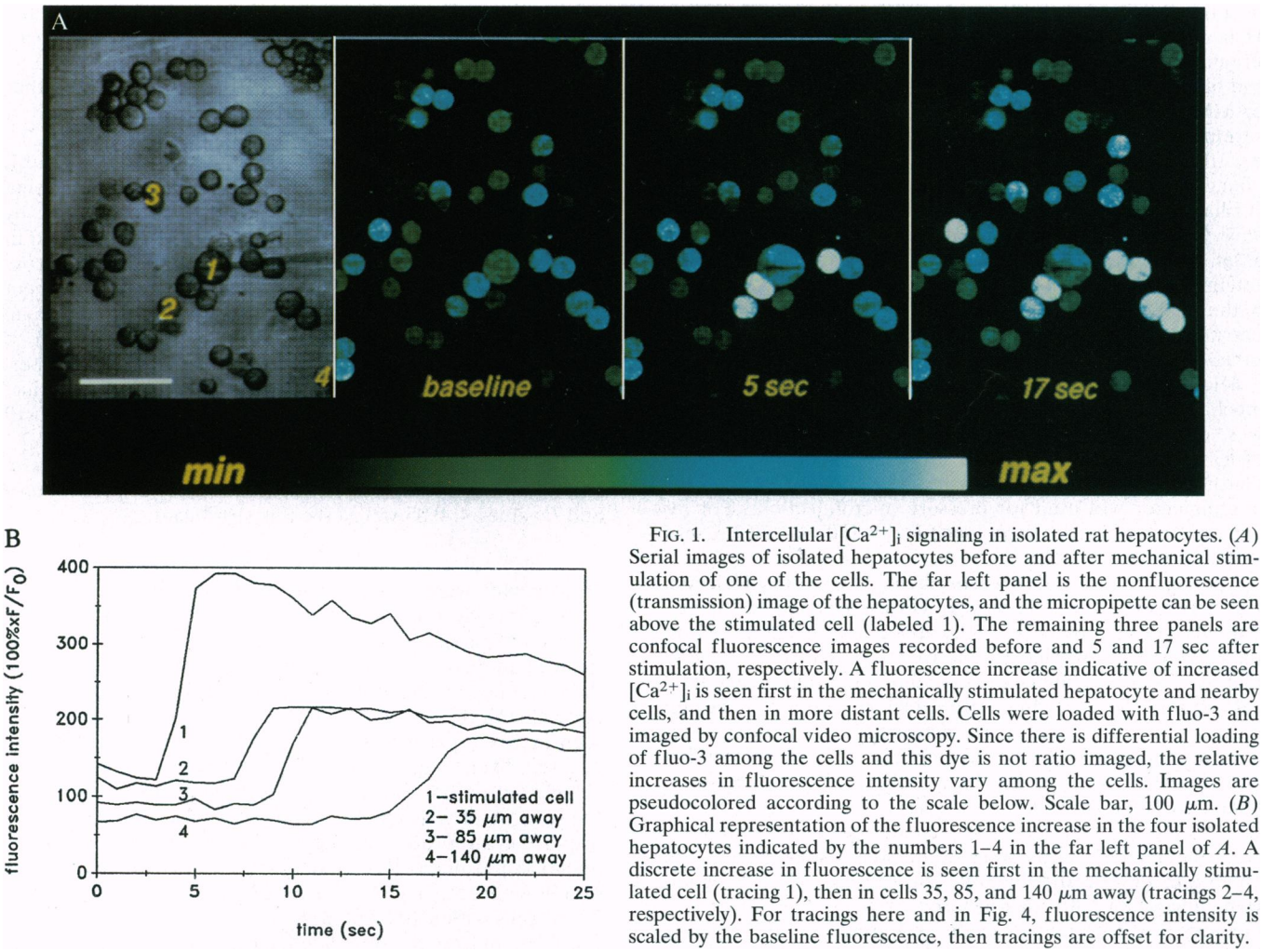

FIG. 1. Intercellular $[Ca^{2+}]_i$ signaling in isolated rat hepatocytes. (*A*) Serial images of isolated hepatocytes before and after mechanical stimulation of one of the cells. The far left panel is the nonfluorescence (transmission) image of the hepatocytes, and the micropipette can be seen above the stimulated cell (labeled 1). The remaining three panels are confocal fluorescence images recorded before and 5 and 17 sec after stimulation, respectively. A fluorescence increase indicative of increased $[Ca^{2+}]_i$ is seen first in the mechanically stimulated hepatocyte and nearby cells, and then in more distant cells. Cells were loaded with fluo-3 and imaged by confocal video microscopy. Since there is differential loading of fluo-3 among the cells and this dye is not ratio imaged, the relative increases in fluorescence intensity vary among the cells. Images are pseudocolored according to the scale below. Scale bar, 100 μm. (*B*) Graphical representation of the fluorescence increase in the four isolated hepatocytes indicated by the numbers 1–4 in the far left panel of *A*. A discrete increase in fluorescence is seen first in the mechanically stimulated cell (tracing 1), then in cells 35, 85, and 140 μm away (tracings 2–4, respectively). For tracings here and in Fig. 4, fluorescence intensity is scaled by the baseline fluorescence, then tracings are offset for clarity.

To further investigate whether ATP mediates this form of intercellular signaling, we examined the effects of the ATP/ADPase apyrase (19) and the competitive P_2 receptor antagonist suramin (1, 3) on the spread of the $[Ca^{2+}]_i$ signals. $[Ca^{2+}]_i$ increased in most cells within 50 μm of a mechanically stimulated hepatocyte, whether or not apyrase was present (Fig. 2*A*). However, apyrase reduced the fraction of responding cells 50–100 μm away by more than one-half, and virtually no cells responded that were over 100 μm away ($P < 0.001$; Fig. 2*A*). In experiments where apyrase was removed and cells were mechanically restimulated, the response rate among cells 50–100 μm away increased from 33 ± 8% to 60 ± 9% ($P = 0.02$), a value not significantly different from the pre-apyrase response rate ($P > 0.3$). The response rate among cells over 100 μm away increased from 1 ± 1% to 22 ± 7% ($P < 0.001$), which is marginally less than the pre-apyrase response rate ($P = 0.048$). In the presence of apyrase, there also was an increase in the time delay between stimulation of one cell and response in another (data not shown). These findings demonstrate that apyrase retards intercellular $[Ca^{2+}]_i$ signaling induced by mechanical stimulation in hepatocytes, which supports the hypothesis that this form of signaling is mediated by ATP release. In addition, apyrase did not prevent $[Ca^{2+}]_i$ signals induced by addition of vasopressin (100 nM) to the medium ($n = 3$ experiments), demonstrating the specificity of apyrase for nucleotide Ca^{2+} agonists. We also examined the effects of suramin on ATP- and mechanically induced $[Ca^{2+}]_i$ signals. The lowest concentration of ATP that increased $[Ca^{2+}]_i$ in isolated hepatocytes was 20 nM, to which 3 of 76 cells (4%) responded. In the presence of 100 μM suramin, though, the threshold dose of ATP was increased to 200 nM, to which 4 of 50 cells (8%) responded. In experiments where hepatocytes were mechanically stimulated, suramin reduced by more than one-half the fraction of responding cells within 50 μm of the stimulated hepatocyte, and virtually no cells responded that were over 50 μm away ($P < 0.0001$; Fig. 2*B*). These findings show that suramin retards intercellular $[Ca^{2+}]_i$ signaling induced by mechanical stimulation in hepatocytes, which further suggests that this form of signaling is mediated by ATP release.

In selected experiments, isolated bile duct units were cocultured with isolated hepatocytes. Mechanical stimulation of individual hepatocytes resulted in increased $[Ca^{2+}]_i$ in 15 of 23 (64%) nearby bile duct units, which is similar to the response rate of 60% that is observed among bile duct cells in bile duct units stimulated with exogenous ATP (11). In the presence of apyrase, in contrast, mechanical stimulation of nearby hepatocytes resulted in increased $[Ca^{2+}]_i$ in only 2 of 19 (11%) nearby bile duct units. A range of $[Ca^{2+}]_i$ signaling patterns was seen in the bile duct cells, including sustained and transient $[Ca^{2+}]_i$ increases and $[Ca^{2+}]_i$ oscillations (Fig. 3). This range of signaling patterns is similar to that elicited by direct stimulation of bile duct cells with ATP (11). Together, these findings suggest that the release of nucleotides by hepatocytes can stimulate nearby bile duct cells.

To determine whether nucleotide release was due to loss of cell integrity, we examined fluo-3-loaded hepatocytes in the presence of propidium iodide (17). Intercellular spread of $[Ca^{2+}]_i$ signals was detected without nuclear labeling by propidium iodide in 15 separate experiments (Fig. 4*A*). In con-

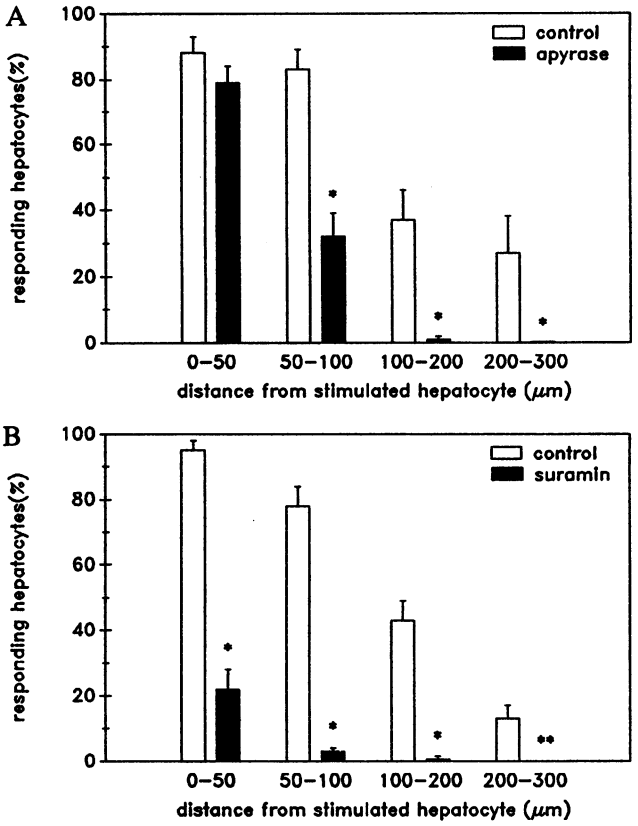

FIG. 2. Effects of (*A*) apyrase (50 units/ml) and (*B*) suramin (100 μM) on the spread of $[Ca^{2+}]_i$ signals in isolated rat hepatocytes. Under control conditions, most cells respond that are within 100 μm of the stimulated cell, and some cells respond that are up to 300 μm away. In the presence of apyrase, $[Ca^{2+}]_i$ signals are seen in significantly fewer cells between 50–100 μm away, and in virtually no cells over 100 μm away (*, $P < 0.001$). In the presence of suramin, $[Ca^{2+}]_i$ signals are seen in significantly fewer cells within 50 μm of the stimulated hepatocyte, and in virtually no cells over 50 μm away (*, $P < 0.0001$; **, $P < 0.005$). Values are mean ± SEM ($n = 13$ control and 27 apyrase experiments from four coverslips, and $n = 35$ control and 44 suramin experiments from five coverslips).

trast, intentional disruption of an hepatocyte ($n = 3$) caused easily detectable nuclear labeling within 10 sec, which was associated with variable $[Ca^{2+}]_i$ signaling in nearby cells (Fig. 4*B*). In a related series of experiments, individual fluo-3-loaded hepatocytes were microinjected in the presence of propidium iodide with an intracellular-like solution containing Cy5. In four separate experiments, individual hepatocytes were successfully microinjected (as demonstrated by intracellular appearance of Cy5 fluorescence), and the injection was associated with intercellular spread of $[Ca^{2+}]_i$ signals without propidium iodide uptake into the injected cell (Fig. 4*C*). These results suggest that mechanical stimulation of an isolated hepatocyte induces intercellular $[Ca^{2+}]_i$ signaling in nearby, noncontacting hepatocytes without compromising the integrity of the stimulated cell. These results also demonstrate that microinjection itself can serve as a sufficient stimulus to induce this form of $[Ca^{2+}]_i$ signaling.

DISCUSSION

Here we showed that mechanical stimulation of individual hepatocytes causes an increase in $[Ca^{2+}]_i$ in the stimulated cell as well as in neighboring, noncontacting hepatocytes and bile duct cells. Since most of the hepatocytes were not physically connected, the intercellular $[Ca^{2+}]_i$ signaling reported here is likely due to a diffusible extracellular mediator released upon mechanical stimulation. Since desensitization of ATP receptors blocks and apyrase inhibits this signaling, the extracellular mediator may be ATP. However, apyrase hydrolyzes ADP and UTP as well (19). Furthermore, hepatocyte ATP receptors are sensitive to both ADP (20) and UTP (15), and bile duct cell ATP receptors are sensitive to UTP (10, 11). Therefore, an alternative possibility is that ADP or UTP is the intercellular mediator of the mechanically induced $[Ca^{2+}]_i$ waves we observed here. Further identification of this nucleotide mediator may require subtype-specific receptor antagonists that are not yet available. The current work demonstrates, though, that mechanical stimulation of individual hepatocytes causes release of nucleotides, which are able to induce $[Ca^{2+}]_i$ signals in nearby hepatocytes and bile duct cells.

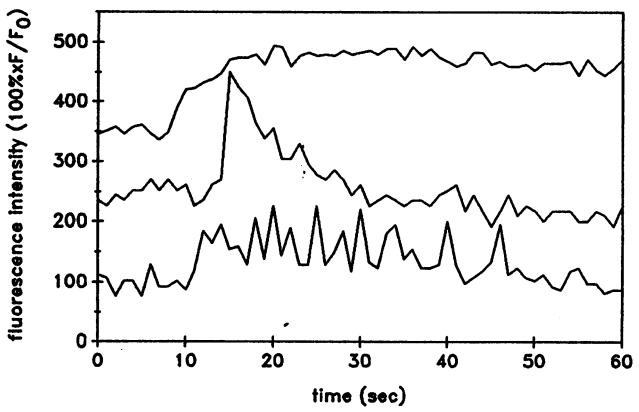

FIG. 3. Range of $[Ca^{2+}]_i$ signaling patterns detected in bile duct cells cocultured with mechanically stimulated hepatocytes. Patterns include a sustained increase in $[Ca^{2+}]_i$ (top tracing), a transient $[Ca^{2+}]_i$ increase (middle tracing), and repetitive $[Ca^{2+}]_i$ increases (i.e., $[Ca^{2+}]_i$ oscillations; bottom tracing). Tracings are from individual bile duct cells within bile duct units isolated 24 hr earlier and were obtained by confocal fluorescence video microscopy as in Fig. 1. Tracings are offset for clarity. Results are representative of patterns observed in cells from nine separate bile duct units.

Intercellular $[Ca^{2+}]_i$ waves induced by endocrine hormones such as vasopressin propagate among adjacent hepatoyctes via gap junctions (4, 5). The speed at which $[Ca^{2+}]_i$ signals spread among noncontacting hepatocytes in this study averaged under 30 μm/sec. This is comparable to the speed of intercellular $[Ca^{2+}]_i$ waves induced by picomolar-range concentrations of vasopressin in the isolated perfused rat liver (6, 21), but considerably slower than the wave speed of greater than 100 μm/sec that can be seen in isolated hepatocyte couplets (5) and intact liver (6) stimulated with nanomolar-range concentrations of vasopressin. $[Ca^{2+}]_i$ signals mediated by nucleotide release may spread at very different speeds *in vivo*, though. For example, if hepatocytes secrete the ATP found in bile (22), then bile flow would enhance the spread of this nucleotide downstream but would retard it upstream. In addition, hepatocytes express apical ecto-ATPases (23), which could inhibit the spread of ATP throughout the intrahepatic biliary tree.

What is the physiological correlate of mechanical stimulation? Osmotic challenges may provide a common form of mechanical stress to hepatocytes *in vivo* since the composition of portal blood can vary. Such osmotic challenges can change cell volume, which in turn can induce $[Ca^{2+}]_i$ signals in hepatocytes (24). Stimulation of integrin receptors via cell-matrix interactions provides another type of mechanical stimulus that occurs *in vivo*. For example, mechanical loading of integrin subunits increases $[Ca^{2+}]_i$ in a mouse hepatoma cell line (25). It remains to be established whether mechanical stimulation increases $[Ca^{2+}]_i$ in the stimulated hepatocyte directly as a result of that stimulus or indirectly as a result of ATP-induced autocrine stimulation. ATP release via vesicular exocytosis is well-established (1) but other mechanisms of

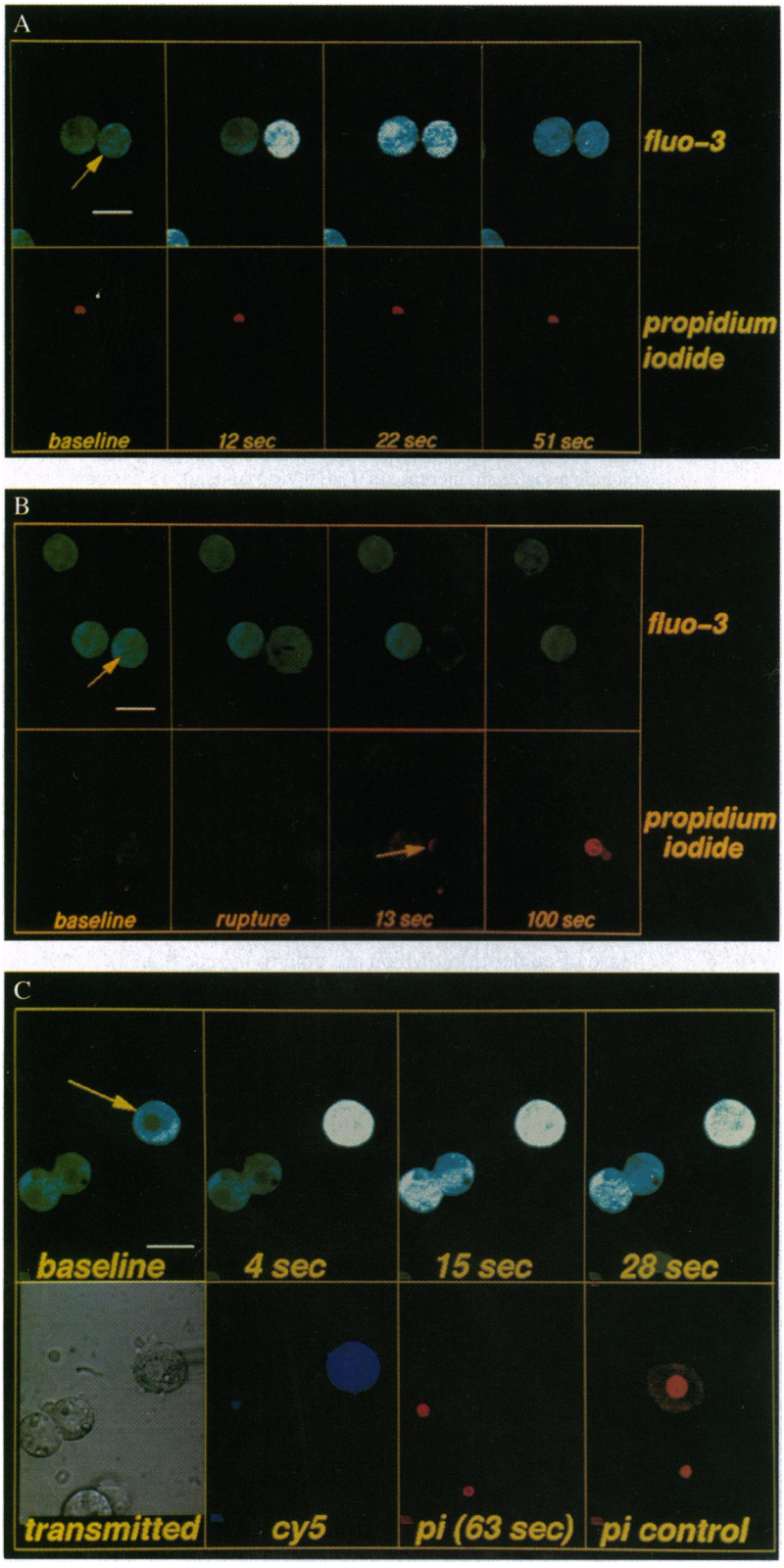

FIG. 4. Effects of mechanical stimulation on $[Ca^{2+}]_i$ signaling and cell viability in isolated rat hepatocytes. Cells were loaded with the $[Ca^{2+}]_i$-sensitive dye fluo-3 and the nuclear marker propidium iodide was present in the medium, and then the cells were observed during mechanical stimulation using double-label confocal fluorescence video microscopy. (*A*) Serial images obtained before and 12, 22, and 51 sec after mechanical stimulation of the cell indicated by the arrow. Transient increases in $[Ca^{2+}]_i$ are evident in the stimulated cell and its neighboring hepatocytes (*Top*), but stimulation does not lead to labeling by propidium iodide (*Bottom*). Nonspecific labeling of extracellular debris by propidium iodide is seen, though. Results are representative of those observed in $n = 15$ separate experiments. (*B*) Serial images obtained before, during, and 13 and 100 sec after mechanical rupture of an isolated hepatocyte. No increase in $[Ca^{2+}]_i$ is detected in the ruptured cell (arrow), and $[Ca^{2+}]_i$ signaling in its neighbors is weak and transient (*Top*). However, nuclear labeling of the ruptured cell by propidium iodide (*Bottom*) is seen within 13 sec (arrow) and is intense by 100 sec. (*C*) Microinjection induces intra- and intercellular $[Ca^{2+}]_i$ signaling in isolated rat hepatocytes, demonstrated by triple-label confocal fluorescence video microscopy. (*Top*) Fluo-3 fluorescence before and 4, 15, and 28 sec after microinjection of the cell indicated by the arrow. An increase in $[Ca^{2+}]_i$ is detected in the injected cell within 4 sec, and in nearby cells at the subsequent time points. Fluo-3-labeled images (*Top*) are pseudocolored using the scale shown in Fig. 1. *Bottom, Left* to *Right*: (*i*) the nonfluorescence (transmission) image of the cells, in which the microinjection pipette can be seen near the injected cell; (*ii*) the Cy5 image, demonstrating the cell that was successfully injected with an intracellular-like solution labeled with Cy5; (*iii*) the propidium iodide (*pi*) image taken 63 sec after microinjection, demonstrating that extracellular propidium iodide does not label the injected cell; and (*iv*) the propidium iodide image of a ruptured cell in a different field, which illustrates that propidium iodide fluorescence can be detected in damaged cells under these experimental conditions. Results are representative of those observed in $n = 4$ separate experiments. (Bars = 25 mm.)

ATP release from cells are controversial. Hypoxia induces ATP release from intact perfused hearts (26), isolated cardiac myocytes (27), and erythrocytes (27) without cell lysis or irreversible damage of the plasma membrane. In other work, it has been shown that two members of the ATP-binding cassette transporter superfamily can function as ATP channels: the multidrug resistance gene product *mdr*1 (28) and the cystic fibrosis transmembrane regulator (CFTR) (29, 30). The mechanism by which mechanical stimulation leads to nucleotide release from hepatocytes will require further investigation.

Cell lysis represents a distinct route by which ATP could be released into the extracellular space. ATP thus released could be an important signal that initiates compensatory metabolic changes in neighboring cells. To determine whether mechanical stimulation is a model for such signaling, propidium iodide uptake was used as a monitor for cell membrane damage (17).

The molecular weights of ATP and propidium iodide are similar (507 and 668, respectively), so that mechanical effects on the cell membrane that would permit ATP to leak from the cell should also permit entry of propidium iodide. However, mechanical stimulation of hepatocytes stimulated nucleotide release without propidium iodide labeling, which suggests that nucleotide release was not associated with loss of membrane integrity. We also found that microinjection of an intracellular-like solution provided sufficient stimulus to initiate nucleotide release without loss of cell integrity. This release of nucleotides can initiate $[Ca^{2+}]_i$ signaling pathways not only in nearby cells, but presumably in the mechanically stimulated cell as well. This is an important technical consideration, given the widespread use of microinjection techniques to study the effects of second messengers and receptor antagonists on signal transduction pathways in epithelia (31–35). Our findings thus suggest that the results of such studies must be interpreted with caution.

The current work provides a mechanism for autocrine and paracrine regulation of $[Ca^{2+}]_i$ signals by ATP or other nucleotides in liver. This type of regulation by ATP has been described previously in several nonepithelial cell types. In endothelial cells, for example, changes in blood flow can induce rapid release of ATP along with a transient rise in $[Ca^{2+}]_i$ (36). Similarly, in mouse fibroblasts ATP can mediate shear stress-induced increases in $[Ca^{2+}]_i$ (2). In rat basophilic leukemia cells, ATP can be released either by mechanical stimulation or by antigen activation, and this release subsequently mediates the intercellular spread of $[Ca^{2+}]_i$ signals (3). In epithelia, though, the form of $[Ca^{2+}]_i$ signaling characterized most extensively is that induced by endocrine hormones (16). Intercellular spread of these $[Ca^{2+}]_i$ signals is mediated by gap junctional communication, as has been shown both in hepatocytes (4, 5) and in pancreatic acinar cells (37). Thus, the mechanism described here provides a separate pathway for intercellular communication among epithelia. This pathway could merely represent an evolutionarily conserved mechanism for cell-to-cell signaling, since ATP receptors are present on elasmobranch hepatocytes, even though these cells lack receptors for vasotocin, angiotensin, or catecholamines (38). Alternatively, ATP release by hepatocytes may represent a way for these cells to modulate the function of other nearby hepatocytes and bile duct cells. Why would autocrine and paracrine signaling be important in liver? Hepatocytes and bile duct cells each express ATP receptors, and in both cell types the receptors link to mobilization of intracellular Ca^{2+} stores. Thus, stimulation of these receptors should regulate the same metabolic functions that are regulated by other $[Ca^{2+}]_i$-mobilizing hormones. In hepatocytes, these functions include glucose production (39), bile secretion (40), tight junction permeability (41), and canalicular contraction (41, 42). In particular, it has been shown that ATP stimulates canalicular contraction in isolated rat hepatocyte couplets (15), and that there is ATP in bile (22). Therefore, secretion of ATP into bile could stimulate canalicular contractions *in vivo*, which would serve to direct peristalsis as ATP flows downstream. Although there is no evidence that ATP induces similar contractions in bile duct epithelia, increased $[Ca^{2+}]_i$ activates Cl^- efflux in this cell type (10). Thus, another paracrine effect of ATP secreted from hepatocytes could be to stimulate secretion of fluid and electrolytes from bile duct cells into bile. Hepatocytes express *mdr*1 (43), so it might further be speculated that secretion of ATP could occur along with secretion of toxins and metabolites via this protein. This ATP could then serve to stimulate canalicular peristalsis in hepatocytes downstream, and then enhance fluid secretion by bile ducts, which together would promote clearance of the toxins or metabolites from the liver. Further studies will be needed to determine the molecular mechanism of nucleotide release from hepatocytes and to demonstrate that apical release of nucleotides promotes bile flow *in vivo*.

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