Fat-storing cells as liver-specific pericytes: spatial dynamic of agonist-stimulated intracellular calcium transients.

Fat-storing Cells as Liver-specific Pericytes

Spatial Dynamics of Agonist-stimulated Intracellular Calcium Transients

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Abstract

Liver perisinusoidal fat-storing cells (FSC) show morphological and ultrastructural characteristics similar to pericytes regulating local blood flow in other organs. In the present study we have analyzed whether FSC respond to local vasoconstrictors such as thrombin, angiotensin-II, and endothelin-1 with an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) coupled with effective cell contraction. All agonists tested induced a rapid and dose-dependent increase in $[Ca^{2+}]_i$ followed by a sustained phase lasting several minutes in confluent monolayers of Fura-2–loaded human FSC. Pharmacological studies performed using different Ca^{2+} channel blockers indicated that, at least for thrombin and angiotensin-II, the sustained phase is due to the opening of voltage-sensitive membrane Ca^{2+} channels. To analyze the temporal and spatial dynamics of Ca^{2+} release in response to these agonists, we performed experiments on individual Fura-2–loaded human FSC using a dual wavelength, ratiometric video imaging system. The rise in $[Ca^{2+}]_i$ was exclusively localized to the cytoplasm, particularly in the branching processes. Increases in $[Ca^{2+}]_i$ more than fourfold were associated with a simultaneous and transient reduction of cell area indicating reversible cell contraction. Our results indicate that the Ca^{2+}-dependent contraction of human FSC in vitro may reflect a potential role in regulating sinusoidal blood flow in vivo. (*J. Clin. Invest.* 1992. 90:642–646.) Key words: fat-storing cells • intracellular free calcium • cell contraction • thrombin • angiotensin-II • endothelin-1

Introduction

Liver fat-storing cells (FSC[1]; also known as perisinusoidal stellate cells, lipocytes, Ito cells) have recently been shown to play important roles in retinol metabolism and hepatic fibrogenesis (for review see 1, 2). A possible role of FSC as liver-specific pericytes has also been hypothesized (1–3). Indeed, FSC show morphological and ultrastructural characteristics similar to pericytes regulating blood flow in other organs. These include: perisinusoidal and interhepatocellular branching processes containing massive 5-nm actin-like filaments and encircling neighboring sinusoids (4), a contact surface between stellate cells and nerve endings (5), and the expression of the α-smooth muscle actin gene (6).

The present study was undertaken to verify whether FSC, like other perivascular contractile cells, respond to local vasoconstrictors such as thrombin, endothelin-1, and angiotensin-II. For this purpose, we have analyzed the variations of intracellular free calcium concentration ($[Ca^{2+}]_i$) in monolayers and in individual agonist-stimulated FSC isolated from normal human liver. In addition, we have studied the reversible changes of cell area coupled with agonist-stimulated intracellular calcium transients by using a dynamic video imaging technique of cellular fluorescence.

Methods

Isolation, culture, and characterization of human FSC. Human FSC were isolated from wedge sections of normal human liver unsuitable for transplantation. After extensive washings in saline, liver tissue was finely minced using a razor blade, placed in a sterile flask containing 0.5% pronase (103 proteolytic U/mg; Calbiochem Corp., San Diego, CA), 0.05% type IV collagenase (Sigma Chemical Co., St. Louis, MO), and 10 μg/ml of DNAse (bovine pancreas; Calbiochem) in 100 ml of HBSS without calcium and magnesium, and agitated at 37°C for 30 min. The resulting cell suspension was filtered through a 105-μm nylon gauze. The undigested tissue retained into the gauze was further digested using 0.05% pronase, filtered, and pooled with the remainder of the cell suspension. The combined digest was washed four times at 450 *g* for 10 min in HBSS containing 10 μg/ml of DNAse and the final pellet was finally resuspended in 25 ml of the same solution. FSC were separated from other liver nonparenchymal cells by ultracentrifugation over gradients of stractan (Larex-LO; Larex International Co., Tacoma, WA) as described elsewhere (7, 8). FSC were recovered from the interface between the 1.053 stractan gradient and the medium. Cells recovered at this level (1.4×10^6 cells/g of tissue) were highly viable and ~ 90% pure.

Cells were cultured in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY) supplemented with 0.6 U/ml insulin, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM

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1. *Abbreviations used in this paper:* AII, angiotensin-II; $[Ca^{2+}]_e$, extracellular calcium; $[Ca^{2+}]_i$, intracellular free calcium concentration; ET-1, endothelin-1; FSC, fat-storing cells; KHH, Krebs-Henseleit-Hepes; SFIF, serum-free insulin-free; THR, thrombin.

sodium pyruvate, antibiotic antifungal solution, and 20% fetal bovine serum. Experiments described in this study were performed on cells between first and second passage using two individual cell lines. Human FSC in primary culture were identified by immunostaining for intermediate filaments, surface antigens, and by transmission electron microscopy. Cells plated onto sterile tissue culture chambers (Lab-tek Div., Miles Laboratories Inc., Naperville, IL) were washed twice with PBS, dried overnight at room temperature, and fixed in acetone at 4°C for 5 min. Monoclonal antibodies specific for vimentin (V9; Dakopatts, Glostrup, Denmark), α-smooth muscle actin (1A4; BioGenex, San Ramon, CA) human desmin (D-33; Dakopatts), pan-cytokeratin (Lu5; Boehringer Mannheim GmbH, Mannheim, Germany), Ki-M1P, directed against the monocyte/macrophage antigen CD68 (9), and polyclonal antibodies against factor VIII-related antigen (Dakopatts), and porcine desmin (Sigma Chemical Co.) were applied onto cells and detected with the alkaline anti-phosphatase anti-alkaline phosphatase (APAAP) method (10). Cells demonstrated intense staining for vimentin, and slightly for α-smooth muscle actin and porcine desmin. The negative staining for CD-68, factor VIII-related antigens, and cytokeratin, demonstrated the absence of contaminating mono/macrophagic, endothelial, and epithelial cells, respectively. Transmission electron microscopy studies, performed as described elsewhere (11), revealed the presence of numerous large lipid droplets in the cytoplasm associated with abundant bundles of myofilaments.

Fluorimetric analysis of intracellular free calcium in human FSC monolayers. Human FSC were grown to confluence on 14 × 14 mm plastic (Aclar, Pottsville, PA) cover slips in complete culture medium, and then incubated in serum-free insulin-free (SFIF) medium for 24 h. Loading of the cells with the fluorescent Ca^{2+} indicator Fura-2-AM (Calbiochem) was achieved by incubating the cells for 45 min at 37°C with 1 μM Fura-2-AM. The loading medium was then replaced with 1 ml of fresh SFIF medium and the cells were incubated for additional 20 min at 37°C, followed by rinsing with ice-cold Krebs-Henseleit-Hepes (KHH) containing (in mM): Na^+ 140.7, K^+ 5.3, Cl^- 132.4, Ca^{2+} 1.0, Mg^{2+} 0.81, glucose 5.5, Hepes 20.3, with 0.1% fatty acid–free bovine serum albumin, pH 7.4. The cover slips were placed diagonally in a square quartz cuvette so that the excitation and emission paths were at a 45° angle to the cover slip. The cuvette, containing 2 ml of KHH buffer, was maintained at 37°C. Thrombin (THR; from human plasma, Boehringer Mannheim) endothelin-1 (ET-1; Novabiochem AG, Läufelfingen, Switzerland) or angiotensin-II (AII; Novabiochem) were directly added to the cuvette, and fluorescence was continuously recorded, under constant stirring, by a Johnson Foundation Biomedical Instrumentation Group fluorometer (Philadelphia, PA) using a single-wavelength excitation (340 nm)/emission (500 nm). In some experiments, cell monolayers were pretreated with 3 mM EGTA (Sigma) or with two Ca^{2+} channel blockers, namely bepridil and nifedipine (both purchased from Calbiochem). Calibration was performed for each cover slip as indicated elsewhere (12). Cell autofluorescence was evaluated in parallel cover slips both by measuring fluorescence of unloaded cells and by quenching Fura-2 fluorescence with 1 mM $MnCl_2$ after the addition of ionomycin. In both cases cell autofluorescence was negligible and, in addition, it was not modified by addition of the agonists, as measured in monolayers not loaded with Fura-2.

Digital video imaging of intracellular free calcium in individual FSC. For these experiments human FSC were seeded at low density and grown in complete culture medium on round glass cover slips (25 mm diameter, 0.2 mm thick) for 72 h, and then incubated for 24 h in SFIF medium. Cells were loaded with 10 μM Fura-2-AM in Hepes-$NaHCO_3$ buffer containing 140 mM NaCl, 3 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, with 0.1% fatty acid–free bovine serum albumine, pH 7.4, at 37°C for 45 min (13). After loading, the cover slips were washed and stored at room temperature until used (always < 45 min after loading). The cover slips were then placed in a perfusion chamber mounted on the stage of a Diaphot-TMD epifluorescence inverted microscope (Nikon Co., Tokyo, Japan) equipped with a xenon lamp. Fura-2–loaded cells were visualized with a Nikon CF ×100 oil immersion or ×40 objectives (Nikon Co.). A filter cassette with a dichroic mirror (DM 400) and a barrier filter (BA 510) were used. Excitation wavelengths were alternated between 340 and 380 nm with an automated filter changer. Neutral density filters were used to diminish Fura-2 photobleaching. Video images were obtained with an extended ISIS-M camera (Photonic Science, Roberts Bridge, East Sussex, UK) and the resultant analogic video signal from the camera was digitalized with an 8-bit analogue-to-digital converter. Images were collected every 5 s for a standard time of 5–6 min. Agonists were added directly to the perfusion chamber immediately after recording the $[Ca^{2+}]_i$ basal value. $[Ca^{2+}]_i$ was calculated using "Tardis" software (Joyce Loebl, Newcastle, UK) after the creation of ratio images (340:380) by dividing original images from which the background (cell removed) had been subtracted on a pixel-to-pixel basis. The calibration was performed according to Cheung et al. (14), calculating β, R_{min}, and R_{max} for each preparation and using a K_d for Fura-2 of 224 according to Grynkiewics et al. (15). To measure cell area, spatial calibration was performed by measuring division on a graticule under the same optical conditions as the rest of the experiments.

Results and Discussion

Fig. 1 illustrates the changes in $[Ca^{2+}]_i$ induced by exposure of human FSC monolayers to THR, AII, and ET-1 in a series of representative experiments. For all agonists tested, the onset of responses was virtually immediate and peak increments of $[Ca^{2+}]_i$, over a resting level of 90–100 nM, were transiently reached within 15–20 s, declining rapidly to a sustained phase which was maintained for several minutes. When otherwise identical experiments were performed in virtual absence of extracellular calcium ($[Ca^{2+}]_e$) the peak height of the calcium transient induced by all agonists was reduced on average by 5–10% and the sustained phase was almost completely abrogated. These observations indicate that the peak effect induced by these vasoconstrictors is mainly due to intracellular release of Ca^{2+} from cytosolic stores, whereas the sustained phase depends on stimulated influx. In the presence of 1.0 mM $[Ca^{2+}]_e$ the sustained phase induced by exposure to THR and AII was abolished by pretreating cell monolayers with two different membrane Ca^{2+} channel blockers, namely bepridil (a phenethylamine type of Ca^{2+} channel blocker) and nifedipine (a 1,4-dihydropyridine type of Ca^{2+} channel blocker), suggesting that the stimulated entry of extracellular calcium responsible for the sustained phase is likely due to the opening of voltage-dependent transmembrane Ca^{2+} channels. Indeed, the two Ca^{2+} channel blockers used, belonging to series with different molecular structure, have been shown to specifically block the same putative voltage-sensitive Ca^{2+} channels (16). Conversely, pretreatment with nifedipine or bepridil did not affect the sustained phase induced by exposure to ET-1 in the presence of 1.0 mM $[Ca^{2+}]_i$ failing to support a role for voltage-dependent Ca^{2+} channels or, at least, for those phenethylamine- and 1.4-dihydropyridine-sensitive.

In Fura-2–loaded human FSC monolayers the $[Ca^{2+}]_i$ increase induced by THR, AII, and ET-1 was dose-dependent as shown in Fig. 2.

The results of this first set of experiments indicated that human FSC respond to local vasoconstrictor agonists with a rapid increase in $[Ca^{2+}]_i$ as other perivascular contractile cells. In addition, the morphology and the dynamics of $[Ca^{2+}]_i$, and the pharmacological effects of voltage-dependent Ca^{2+} blockers for the three agonists used, are similar to those previously shown in smooth muscle cells (17), and in other organ-specific pericytes such as glomerular mesangial cells (18–21).

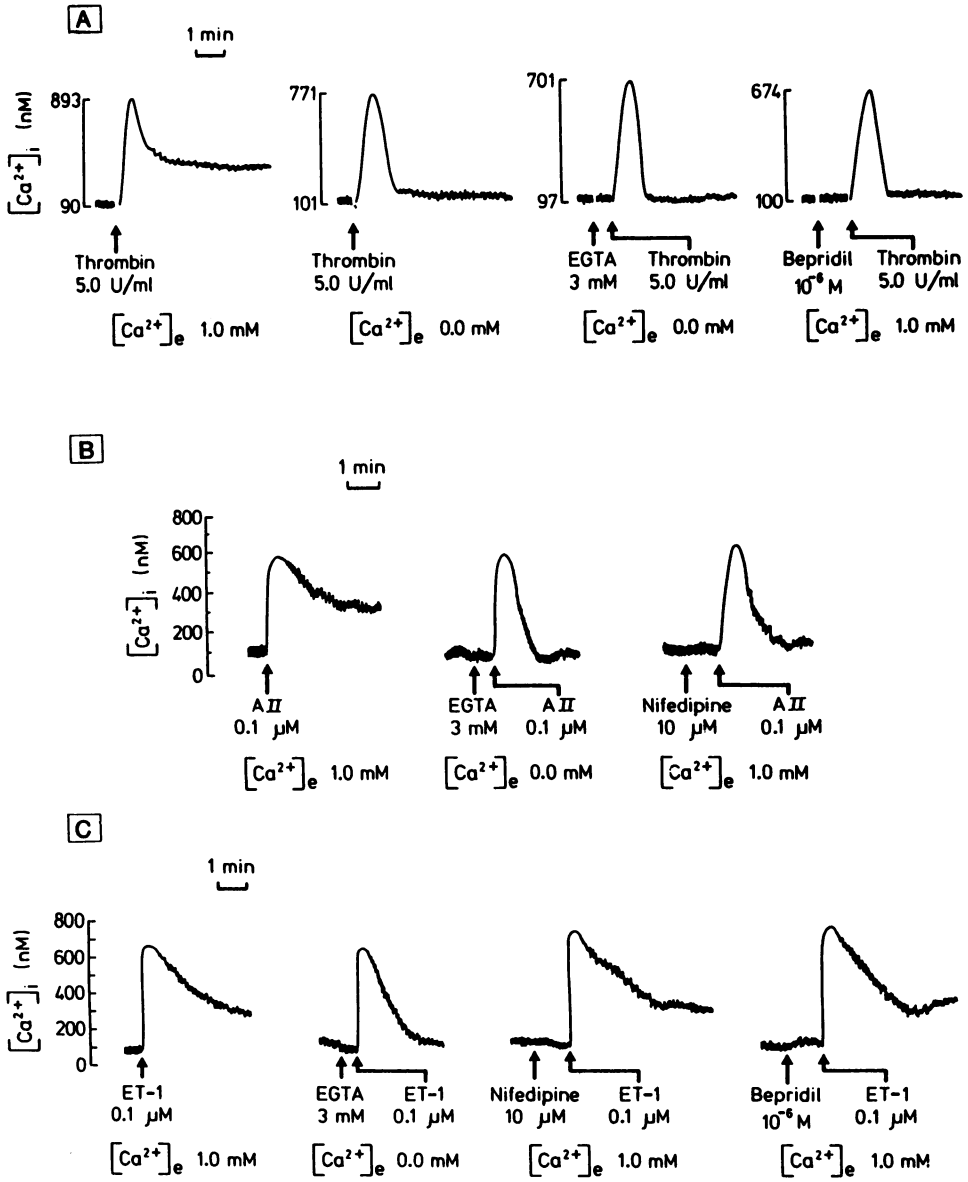

Figure 1. Changes in cytosolic free calcium concentration ($[Ca^{2+}]_i$) induced by exposure of Fura-2–loaded human FSC monolayers to different vasoconstrictor agonists. Human FSC monolayers were loaded with Fura-2-AM as described in Methods. (*A*) Addition of thrombin at a final concentration of 5.0 NIH units/ml induced a rapid $[Ca^{2+}]_i$ peak increment followed by a sustained phase lasting several minutes. This plateau was virtually abolished by repeating the experiments in the absence of extracellular calcium ($[Ca^{2+}]_e$) with or without pretreatment with 3.0 mM EGTA. Similarly, the sustained phase was abolished by pretreating the cells with 10^{-6} M bepridil, a phenethylamine type of calcium channel blocker, in the presence of 1.0 nM $[Ca^{2+}]_e$. Similar results were obtained pretreating the cells with 10 μM nifedipine, a dihydropyridine-sensitive Ca^{2+} channel blocker (not shown). (*B*) Similar $[Ca^{2+}]_i$ increases were induced by the addition of angiotensin II at a final concentration of 0.1 μM. Analogously, the sustained phase was abolished either by performing the experiment in the virtual absence of $[Ca^{2+}]_e$ or by pretreating the cells with 10 μM nifedipine. (*C*) Addition of 0.1 μM endothelin-1 induced analogous $[Ca^{2+}]_i$ increases with abrogation of the sustained phase in the absence of $[Ca^{2+}]_e$. However, both nifedipine and bepridil were ineffective in abolishing the sustained phase.

Since an elevation of $[Ca^{2+}]_i$ in smooth muscle cells results in activation of contractile proteins (22) and FSC have been hypothesized to play a role in the local regulation of sinusoidal hemodynamics, we have also analyzed cell contractility in response to the same agonists able to increase $[Ca^{2+}]_i$. For this purpose we employed a dual wavelength, ratiometric video imaging system that allows to study variations of $[Ca^{2+}]_i$ along with changes of cell area in individual Fura-2–loaded cells. Similarly to what was observed in the experiments performed using cell monolayers, exposure of individual FSC to THR, AII, and ET-1 resulted in a rapid and transient increase of $[Ca^{2+}]_i$ over a resting level of 140–150 nM. When more than one cell per field was observed, it was evident that the number of cells responding and the extent of response were variable, ranging from 0 (nonresponders) to 10-fold increase in $[Ca^{2+}]_i$. In addition, agonist-induced Ca^{2+} transients did not begin simultaneously in different cells but varied up to 60 s, indicating different activation times. Video-imaging analysis revealed that the increase of $[Ca^{2+}]_i$ begins in discrete areas located at the cell periphery, particularly in the branching processes, with subsequent spreading to the cell cytoplasm, often with a typical "calcium wave" pattern. Among the three agonists tested, THR was the most effective in terms of the number of responding cells and extent of $[Ca^{2+}]_i$ increase. Indeed, a clear response was observed in 20 out of the 24 cell cells analyzed, with an average delta increase of ~ 600 nM. Fig. 3 shows four selected time-sequence frames from a representative experiment performed using 0.3 NIH units of THR. By analyzing the frame-to-frame variations of cell area versus the changes of $[Ca^{2+}]_i$ (Fig. 4) it was evident that the calcium increase induced by THR was coupled with a simultaneous and transient reduction of cell area, indicating reversible cell contraction. Similarly, reversible changes of cell area coupled with $[Ca^{2+}]_i$ increases (more than fourfold) were observed when FSC were stimulated with both ET-1 and AII, although the number of responding cells and the extent of response were lower than those induced by THR.

In summary, the results of this study demonstrate that in human FSC vasoconstrictor agonists induce intracellular calcium transients coupled with cell contraction analogously to what was observed in other better characterized pericytes. Al-

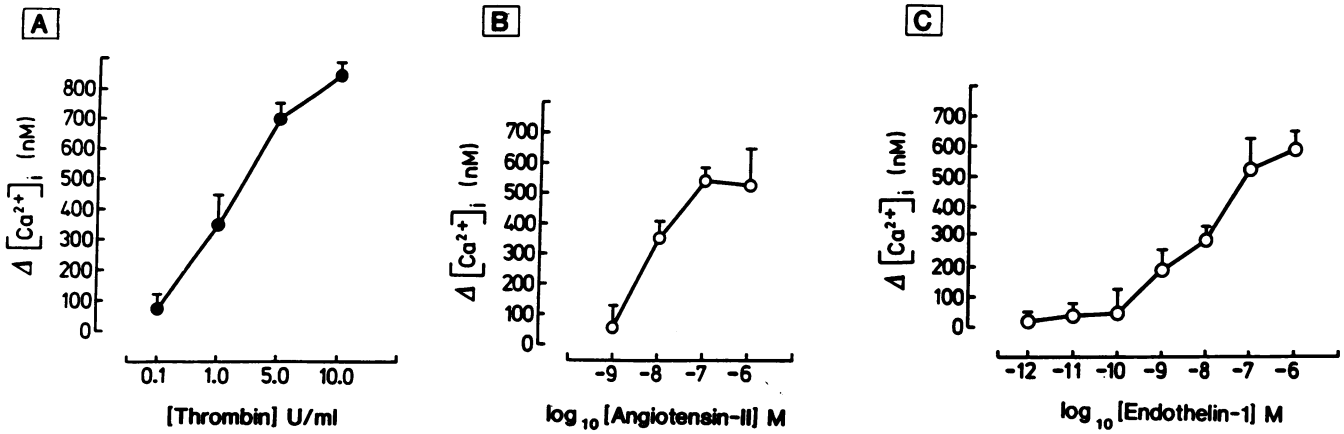

Figure 2. Dose-response curves for the effect of different vasoconstrictor agonists on $[Ca^{2+}]_i$ peak increase in monolayers of Fura-2–loaded human FSC. Human FSC monolayers were loaded with Fura-2 as described in Methods. (*A*) Thrombin. (*B*) Angiotensin II. (*C*) Endothelin-1. Data are means±SD for three individual determinations.

though the in vivo biologic relevance of these observations remains speculative, the results of this in vitro study strongly support the hypothesis that FSC, beyond their morphological appearance, may function as perisinusoidal contractile cells. The presence of a functionally active contractile apparatus encircling the sinusoids suggests that these vascular structures might constitute a major regulatory site of intrahepatic blood flow. The responses induced in FSC by endothelium-derived

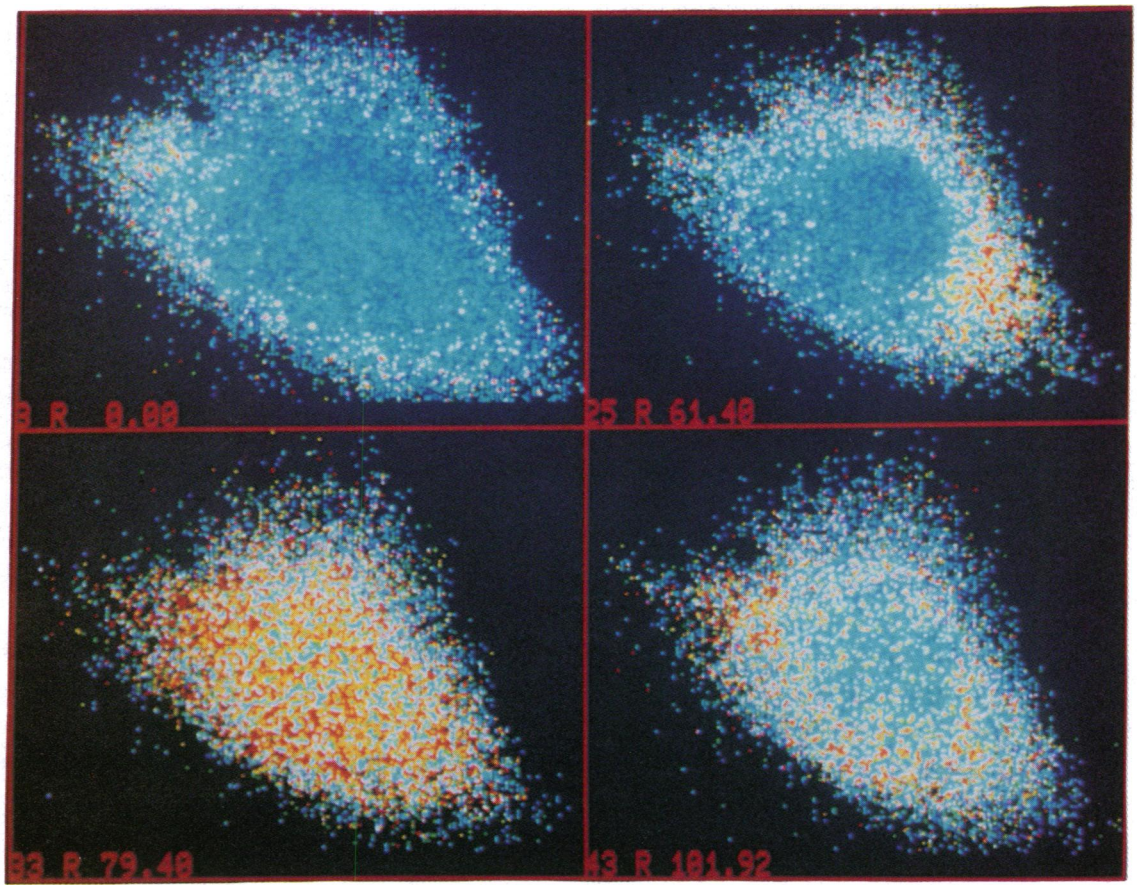

Figure 3. Time-sequence changes in intracellular calcium concentration in a single Fura-2–loaded human FSC responding to 0.3 NIH units/ml of thrombin (representative experiment). Fura-2 loading and experimental procedures are described in Methods. Frame number and time(s) after the addition of the agonist are shown in the left lower corner of each frame. Frame 3 (0.0 s) shows resting levels of $[Ca^{+2}]_i$. Frame 25 (61.4 s) shows that increase in $[Ca^{+2}]_i$ (indicated by the shift from green-blue to orange-red) starts at the cell periphery and rapidly progresses through the cytoplasm (frame 33, 79.4 s) as a "calcium wave." Frame 43 (101.9 s) shows the progressive return to basal values. Note the reduction of cell area associated with the increase in $[Ca^{2+}]_i$.

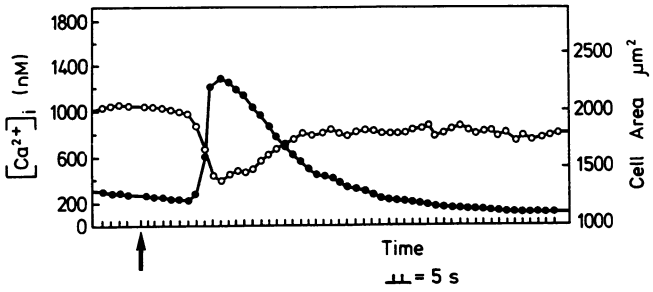

Figure 4. Increase in intracellular calcium concentration $[Ca^{2+}]_i$ is coupled with reversible cell contraction in an individual Fura-2–loaded human FSC. Data from the same representative experiment shown in Fig. 3. 0.3 NIH units/ml thrombin were added at the time point indicated by the black arrow. Ratio frames were collected every 5 s. *Closed circles,* $[Ca^{2+}]_i$ nM; *open circles,* cell area μm^2.

mediators, such as ET-1 and AII, raise the possibility of a local regulation of sinusoidal resistance operated by FSC-sinusoidal endothelium interactions.

It should be noted, however, that early passaged human FSC, as those employed in the present study, are characterized by an "activated" phenotype resembling "transitional" or "myofibroblast-like" cells rather than quiescent FSC retaining the original "storing" phenotype. This phenotypical transition, normally observed in cultures on plastic or glass (23) and in vivo during active fibrogenesis (24), is characterized by a progressively more intense staining for α-smooth muscle actin (25). Myofibroblast-like cells with prominent α-smooth muscle actin filaments have been described in fibrous septa, around sinusoids, and terminal hepatic venules of cirrhotic livers (26). In this clinical condition they are believed to be responsible for the contraction of maturing scar tissue and to contribute, by maintaining a contractile state, to the increased resistance to portal flow (27). Although the relevance of our findings to the situation in normal liver remains to be established, they are likely to be more representative of FSC contractile status in fibrotic liver. The potent effect of THR described in this study provides an example of the effect of vasoconstricting agents possibly involved in this process. In conclusion, our observations open new perspectives in the interpretation of mechanisms regulating intrahepatic blood flow and may contribute to the development of pharmacological strategies able to affect intrasinusoidal blood pressure.

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