Local Modulation of Intracellular Calcium Levels Near a Single-Cell Wound in Human Endothelial Monolayers

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An endothelial cell monolayer with a single mechanically lysed cell was used as a model to examine the extent, kinetics, and nature of local calcium mobilization in the neighborhood of a wound. Individual endothelial cells from confluent monolayers were mechanically lysed with a minuten needle coupled to a micromanipulator while producing no observable mechanical trauma to the neighboring cells. Changes in calcium levels in individual cells surrounding the wound site were monitored by epifluorescence microphotometry with the calcium-sensitive fluorophore indo-1. Individual cells adjacent to the wound site showed a substantial increase in their intracellular calcium levels, almost as high as the calcium levels attained by ionophore controls. The magnitude of intracellular calcium mobilization in confluent monolayers decreased with distance from the wound site, and those cells located at a radius greater than seven cells from the wound site showed no change in their calcium levels. Thus, lysis of a single cell resulted in calcium mobilization in approximately 200 neighboring cells. The time necessary for intracellular calcium to reach maximum levels also increased with distance from the wound site. Calcium mobilization was partly intracellular and was inhibited by disrupting cell–cell coupling or by increasing gap junction resistance by heptanol. This mobilization was greatly attenuated in subconfluent endothelial monolayers, and it was not observed in fibroblasts or smooth muscle cells; furthermore, the effect was defective in monolayers intentionally contaminated with smooth muscle cells. This study examines the extent and possible mechanisms of local endothelial activation near a microscopic endothelial wound.

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The endothelial cell plays a critical role in the maintenance of hemostasis within an injured blood vessel. The intact vascular endothelial surface is normally nonthrombogenic and will not support platelet adhesion, but platelets will adhere and aggregate at a wound site in the endothelium. It is known that endothelial cells participate in the hemostatic process in many ways, including modulation of the coagulation cascade and recruitment of platelets to the endothelial surface. Injury to the endothelial lining from mechanical or chemical trauma results in numerous responses that alter platelet and endothelial function; wounds to the endothelium result in the expression or secretion of vasoactive factors such as tissue factor and prothrombinase complex assembly, prostaglandins, von Willebrand factor, and platelet-activating factor. The wounded endothelium also exhibits increased affinity for fibrinogen and releases growth factors, such as basic fibroblast growth factor, to promote reendothelialization of the denuded area. Long-term responses to endothelial injury involve cell division and migration into the denuded area. Many of these responses appear to be wholly or partly controlled by calcium as a secondary messenger.

Previous studies examining the role of calcium in endothelial activation have demonstrated that calcium is correlated with the global release or expression of various vasoactive factors from entire endothelial monolayers. Many different stimuli other than mechanical trauma can activate the monolayer, including histamine, ATP, bradykinin, endotoxins, and thrombin. The effect of these stimuli is a multicellular mobilization of intracellular calcium within the entire monolayer. The release or expression of hemostatic factors has been quantified by assaying the culture superfusate and by examining monolayer extracts. These studies have shown that...
calcium is a vital secondary messenger for the proper monolayer responses to inflicted injuries, as evidenced by the elimination of endothelial function on removal of calcium or on inhibition of calcium entry into the cytosol.

Although much is known about the important role of intracellular calcium in endothelial function, relatively little is known about the local cell-to-cell events that transpire on endothelial wounding. The endothelium is known to have a complex and intricate system of endothelial cell–cell communication, and junctional transfer of small molecules has been demonstrated in cells lining the edge of a wound inflicted by policeman scraping to cells several layers away. Calcium mobilization has been observed in endothelial cells surrounding (but not contacting) a micropipette-induced scrape wound, leading to the suggestion that the presence of an extracellular "wound signal" is responsible for propagation of this calcium flux to nearby cells. Others, however, have postulated that a calcium flux transmitted to normal rat kidney cells touching a microelectrode-impaled cell occurs via gap junctions, with voltage-gated calcium channels responsible for calcium entry. Calcium entry in freshly dissociated capillary endothelial cells can occur via voltage-gated calcium channels, but evidence exists for calcium entry via receptor-operated rather than voltage-gated pathways in cultured endothelial cells. Divalent cations (such as Ni2+) are effective calcium channel antagonists capable of blocking both voltage-gated and receptor-mediated calcium entry pathways. Endothelial cells, smooth muscle cells, and fibroblasts form gap junctions with themselves and have extensive cell–cell transfer of small molecular tracers. Calcium entry pathways in endothelial cells and smooth muscle cells also form functional gap junctions with each other and are capable of heterocell transfer, which may be essential for proper vascular patency. Long-chain n-alkanols, such as heptanol, are able to increase gap junction resistance and lower cell–cell coupling without disturbing calcium homeostasis.

Some basic and very simple questions still remain unanswered regarding local responses to endothelial injury. For example, it is unknown how large an area is activated by a single cell wound and if the mechanism of this activation is an effect of an extracellular signal or an intracellular messenger with direct cell–cell communication. The single-cell wounding model presented herein allows measurement of calcium mobilization in individual cells near a microscopic wound; the importance of established cell–cell coupling in calcium modulation is also investigated. Because calcium is known to regulate many aspects of endothelial function, we measured the local calcium mobilization in indo-1–loaded cells surrounding a single cell wound by use of epifluorescence microphotometry. We addressed the importance of calcium entry pathways in endothelial activation by using the antagonist Ni2+, and we analyzed the importance of established cell–cell coupling for endothelial calcium mobilization by increasing gap junction resistance by using heptanol, as well as by using subconfluent monolayers and monolayers disrupted by calcium chelation.

Methods

Cells and Cell Culture

Endothelial cells. Human venous endothelial cells were obtained from fresh umbilical cords by enzyme digestion with pronase (0.3 mg/ml, Calbiochem, La Jolla, Calif.) and were maintained at 37°C, 5% CO2 in medium 199 (M199) supplemented with 20% fetal bovine serum (GIBCO, Grand Island, N.Y.), 100 units/ml heparin (Sigma Chemical Co., St. Louis, Mo.), 50 μg/ml ascorbic acid (Sigma), and 100 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, Mass.). Cells from passages 1–4 were seeded onto gelatin-coated glass coverslips. After reaching confluency, the cells were grown for 1–2 days to ensure established cell–cell junctions, and the cells were maintained in medium without growth factors or heparin to reduce the possibility of altered endothelial function.

Studies involving subconfluent monolayers had lower initial seeding densities and were maintained in growth factor–free and heparin-free medium. The cells were seeded onto gelatin-coated glass coverslips and were allowed to grow until 20%, 50%, or 80% confluency had been reached (as determined by cell counts compared with fully confluent cell counts). Isolated cells and cells that had not yet attained a cobblestone appearance were chosen from 20% and 50% confluent monolayers for wounding, and only noncobblestone cells were chosen from 80% confluent monolayers (as isolated cells were not present). Cells that had attained a cobblestone morphology, for example, those cells at the center of a colony, were not examined.

Smooth muscle cells. Human venous smooth muscle cells were obtained by traumatizing a decapitated umbilical cord with hemostats, followed by collagenase digestion (type II, Sigma). The cells were maintained in M199 supplemented with 10% fetal bovine serum and 50 mg/ml ascorbic acid. All smooth muscle cells were from passages 1–2. Identification of contaminants smooth muscle cells in endothelial monolayers was performed by the use of rhodamine-labeled Ulex europaeus I lectin (Vector Labs, Burlingame, Calif.), which labels human endothelial cells but does not label nonendothelial cell types.

Fibroblasts. Human fibroblasts were obtained from collagenase digestion of minced neonatal foreskin tissue. Cells from passages 3–6 were maintained in Dulbecco's modified Eagle's medium supplemented after passages with 10% fetal bovine serum.

Wounding Protocol and Inhibitors

Lysis of single cells was performed by puncturing the cell with a minutien needle (Fine Science Tools, Belmont, Calif.) coupled to a three-dimensional mi-
cromanipulator (Narashige, Greenvale, N.Y.). The needle is a fine stainless steel pin with a 3-5-μm diameter tip, which permits lysis of a single cell without apparently traumatizing the surrounding cells; studies with rhodamine-labeled dextran (40S and 70S, Sigma) showed no transient permeabilization in the surrounding cells after lysis of this single cell. All wounding experiments were conducted at 37°C in M199 with 10% fetal bovine serum.

Cells were incubated in 20 mM NiCl2 (Aldrich Chemical Co., Milwaukee, Wis.) for 30 minutes to inhibit receptor-operated calcium entry channels. Cell–cell coupling was disrupted by adding 3.5 mM 1-heptanol (Aldrich) for 15 minutes (short-term) or 25–30 minutes (long-term) before wounding. Alternatively, cell–cell coupling was disrupted by incubation in 0.9 mM ethylene glycol-bis(β-aminoethyl ether)-N,N′,N″,N″′-tetraacetic acid (EGTA) (Aldrich) for 20–30 minutes (long-term) before wounding to disrupt cell–cell adhesion. To chelate extracellular calcium without disrupting cell–cell coupling, the cells were incubated in 0.9 mM EGTA for 5–10 minutes (short-term) before wounding. The pH of the medium supplemented with NiCl2, heptanol, or EGTA was adjusted to 7.4 before incubation. Cells incubated in 3.5 mM heptanol and 20 mM NiCl2 for 30 minutes showed unaltered viability compared with controls, as determined by Trypan blue exclusion.

Coupling of endothelial cells or the lack thereof was demonstrated by scrape wounding monolayers at various stages of confluency in the presence of 0.2% Lucifer yellow CH (Aldrich) by using the technique of Larson and Haudenschild.16 Cells were incubated for 5 minutes after wounding, washed with medium, selected as detailed above, and visualized with epifluorescence microscopy.

Epifluorescence Microphotometry

Cells were loaded with 5 μM indo-1 AM (Molecular Probes, Eugene, Ore.) for 15 minutes. The cells were then washed with medium and were incubated for 45 minutes or longer to ensure ester hydrolysis to form indo-1. Cells loaded with indo-1 showed a diffusely stained cytoplasm, with no visible sequestering of the fluorophore. The emission intensities from the calcium fluorophore were monitored by the use of two photomultiplier tubes (EMI Thorn, Fairfield, N.J.), each equipped with a bandpass filter (410/5 and 478/5 nm, Ealing Optics, South Natick, Mass.). The photomultiplier tubes were arranged in a T format onto the camera port of one of two microscopes (Diaphot, Nikon, Garden City, N.Y., and IM35, Zeiss, Dallas, Tex.); the microscope was equipped with an epifluorescence filter set and dichroic mirror specific for use with indo-1 (Nikon, Zeiss). The fluorescence ratio at 410 and 480 nm (I410/I480) was obtained by dividing the output voltages from the photomultiplier tubes by use of a voltage signal division chip (No. AD532JD, Analog Devices, Richardson, Tex.). This arrangement, which is similar to the one described elsewhere,36 allowed continuous on-line microphotometric monitoring of fluorescence ratios and intracellular calcium concentrations averaged over one field of view.

Only the overall calcium concentration within a single cell was monitored; this apparatus did not give spatial resolution of calcium mobilization within an individual cell but rather the overall fluorescence intensities averaged from a single cell when the field of view was appropriately limited. Release of calcium from intracellular stores or propagation of a calcium wave front across a cell, for example, was not detectable by this system, but rather changes in a cell’s overall calcium mobilization were detected.

Segregation of calcium mobilization on a cell-to-cell basis was made possible by closing the field diaphragm on the microscope until only a single cell was illuminated. The overall calcium concentration within this single cell, which was monitored as a target cell, was lysed at a specified distance from the monitored cell. The space between the target cell and the monitored cell was specified by positioning the micromanipulator a predetermined distance away from the monitored cell before lysing. The objective diaphragm was also closed enough to eliminate ultraviolet light–induced cellular activation. Target cells were lysed, and the fluorescence ratio of the illuminated cell was recorded with a strip chart recorder connected to the division chip signal output. The exact time of cell lysis was noted by spiking the chart recorder. A new uninjured area of the monolayer was selected, and the position of the manipulator was also shifted slightly to give a different distance between the new monitored cell and the new target cell. By repeatedly selecting new uninjured cells and changing the micromanipulator’s position, data were gathered for individual cells at various distances from a single-cell wound. Because only single cells were illuminated, neighboring cells were not fluorescing, and thus mobilization on a single-cell basis was obtained.

Calcium calibration of the indo-1 fluorescence ratios was performed semiquantitatively. The fluorescence ratios were normalized such that a 0% change in the ratio corresponded to the unchanged basal level of calcium within the cell, and a 100% change was the change in fluorescence ratio given by incubation with 1.5 μM 4-bromo-A23187 (Sigma). The 0% point was obtained for a nonstimulated cell before wounding its neighbor; the 100% point was obtained by averaging the fluorescence ratios of intact monolayers after perfusion with A23187 at the conclusion of each set of experiments. Approximately 15–20 cells were averaged to obtain the 100% point. Although cell-to-cell variation in indo-1 loading may exist, this effect was minimized by monitoring the 100% intensity ratio: the very high Ca2+ levels saturate the fluorophore, and very little variation in the 100% ratio was observed. The difference between the 100% and 0% ratios was large, so that individual variances in cell loading of indo-1 did not influence the calibration.
Local Intracellular Calcium Modulation

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Figure 1. Line plot of calcium mobilization kinetics of endothelial cells in complete medium. 0% Ca\(^{2+}\) mobilization implies no change in calcium levels from basal conditions; 100% Ca\(^{2+}\) mobilization is the fluorescence ratio given by incubation with A23187. •, Cells adjacent to the injury; ○, two cells away; □, three cells away; △, four cells away; ◼, seven cells away; ●, ≥10 cells away. Standard deviations are given in Table 1.

Results

Figure 1 shows results for calcium mobilization of individual endothelial cells from confluent monolayers after lysis of a single cell. The cells developed sustained calcium levels that decreased in magnitude with increasing distance from the target cell. Not only did the magnitudes of the calcium mobilization decrease with increasing distance, but also the onset of mobilization and the time to reach the maximum also increased. Cells adjacent to the wound showed a large, almost immediate mobilization of calcium that began 1.1 seconds after wounding and reached a maximum of 90% at 2.6 seconds after wounding. The next layer of cells (two cells away) did not begin calcium mobilization until 1.6 seconds after wounding, and they did not reach a maximum of 61% until 8.8 seconds. Table 1 lists the times of onset and maxima obtained corresponding to Figure 1.

Experiments were performed to determine if this calcium mobilization originated from intracellular stores or from calcium fluxes across plasma membranes. Wounding experiments were repeated in medium supplemented with 0.9 mM EGTA to chelate all free extracellular Ca\(^{2+}\). Figure 2 shows the mobilization kinetics for individual cells in a brief exposure (<10 minutes) and a long-term exposure (>20 minutes) to calcium-free medium. The calcium mobilization after brief calcium chelation is transient, unlike the mobilization depicted in Figure 1, and is much smaller in magnitude. The transient mobilization is detected only to a radius of four cells, and the onset of mobilization and time to reach maximum also increase relative to whole medium. Adjacent cells began mobilizing calcium 1.6 seconds after wounding and reached a maximum of 47% at 5.0 seconds. Cells two diameters from the lysed cell started mobilizing calcium 2.4 seconds after wounding and reached a maximum of 17% at 6.0 seconds after wounding (Table 2). Long-term exposure to calcium-free medium resulted in cell shape changes

Table 1. Calcium Mobilization in Confluent Endothelial Monolayers

<table>
<thead>
<tr>
<th>No. of cells from wound</th>
<th>Time of onset (sec)</th>
<th>Time of maximum (sec)</th>
<th>Maximum mobilization (%)</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.1±0.3</td>
<td>2.6±1.9</td>
<td>90±7</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>1.6±0.6</td>
<td>8.8±1.8</td>
<td>61±18</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>2.4±0.8</td>
<td>11±0</td>
<td>49±9</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>3.0±1.6</td>
<td>12±2</td>
<td>15±3</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>12±3</td>
<td>26±4</td>
<td>14±2</td>
<td>5</td>
</tr>
<tr>
<td>≥10</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

Values are mean ±SD.
*Calcium mobilization not detected.

Table 2. Calcium Mobilization in Short- and Long-term Incubation in Calcium-Free Medium

<table>
<thead>
<tr>
<th>No. of cells from wound</th>
<th>Short-term time of onset (sec)</th>
<th>Short-term time of maximum (sec)</th>
<th>Maximum mobilization (%)</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6±0.7</td>
<td>5.0±4.2</td>
<td>47±15, 1.7±2.1*</td>
<td>8, 3*</td>
</tr>
<tr>
<td>2</td>
<td>2.4±1.2</td>
<td>6.0±2.4</td>
<td>17±7, 2.0±1.0*</td>
<td>12, 3*</td>
</tr>
<tr>
<td>3</td>
<td>4.3±3.1</td>
<td>7.0±1.6</td>
<td>3.4±4.7,†</td>
<td>3, 3*</td>
</tr>
<tr>
<td>4</td>
<td>5.0±0</td>
<td>15±1</td>
<td>2.0±3.0,†</td>
<td>3, 1*</td>
</tr>
<tr>
<td>≥5</td>
<td></td>
<td></td>
<td></td>
<td>3, 3*</td>
</tr>
</tbody>
</table>

Values are mean ±SD.
*Values for short-term, long-term.
†Calcium mobilization not detected.
and visible loss of cell–cell contact; at this stage, no calcium mobilization was detected in the surrounding cells, even in those cells adjacent to the lysed cell (Figure 2). Replacing the medium with complete medium at this point did not restore the calcium mobilization that was observed in Figure 1.

The role of receptor-mediated calcium entry pathways was probed by the addition of Ni²⁺, which resulted in diminished Ca²⁺ mobilization. Cells incubated in medium supplemented with 20 mM NiCl₂ exhibited transient calcium mobilization, onset of mobilization, and time to reach maximum, as shown in Figure 3. These results are very similar to those in short-term incubation in calcium-free medium (Figure 2).

The role of monolayer confluency and cell–cell coupling was also examined. Wounds were inflicted in complete medium, and Figure 4 shows the maximum calcium mobilization as a function of mono-

layer confluency and cell distance from the lysed cell. Nearly confluent monolayers (80%) had attenuated calcium mobilization compared with that of 100% confluent monolayers, and very subconfluent monolayers (50% and 20%) showed no detectable calcium mobilization. Studies with Lucifer yellow demonstrated transfer of the dye to cells four to six layers back from the wound edge in confluent scrape-loaded monolayers. Transfer of the dye in subconfluent monolayers was absent in 20% and 50% confluent monolayers, and dye transfer in 80% confluent monolayers was observed from zero to two layers back from the wound edge, demonstrating a reduction or lack of cell–cell coupling in these subconfluent monolayers.

The effect of heptanol on calcium entry is shown in Figure 5. Cells briefly incubated in medium supplemented with 3.5 mM heptanol (15 minutes) showed less Lucifer yellow transfer from the wound edge (two to three cell layers), and cells surrounding a single-cell injury showed attenuated calcium mobilization, with delayed onset and time of maximum relative to those in a heptanol-free medium. However, the mobilization was sustained compared with that of untreated cells (Figure 1). Longer incubations with 3.5 mM heptanol (25–30 minutes) inhibited dye transfer from the scrape edge (no cell layers) and also effectively inhibited calcium mobilization in neighboring cells, as shown in Figure 5.

Other cell types were not able to propagate this calcium mobilization, as detailed in Table 3. Monolayers of smooth muscle cells and fibroblasts, when wounded by single-cell lysis as in the endothelial wounding experiments, did not mobilize calcium even in cells adjacent to the injury (0% for all times and distances from the wound). Also, confluent monolayers of endothelial cells intentionally contaminated with smooth muscle cells did not propagate the cal-
Calcium mobilization beyond the contaminating cell type. Calcium mobilization in endothelial cells was unchanged up to the contaminating smooth muscle cell, whereas those endothelial cells beyond the smooth muscle cell showed no or attenuated mobilization.

**Discussion**

The present cell wounding model allows lysis of a single endothelial cell without apparent trauma to the surrounding cells, as no transient permeabilization to labeled dextran was observed. We opted to calibrate the indo-1 fluorescence ratio semiquantitatively by normalizing the change in the ratio as a percentage of basal and maximal cellular calcium concentrations rather than to calibrate the fluorescence quantitatively, as significant heterogeneity in cell loading and ester hydrolysis could result in inaccuracies. A 0% change in ratio corresponded to unchanged basal concentrations within the cell, and a 100% change was that ratio given by stimulation of 100% change was that ratio given by stimulation of 100% change was that ratio given by stimulation of 100%

Figure 1 shows extensive sustained calcium mobilization in cells in complete medium at various distances from target cells. Because these surrounding cells were not mechanically traumatized by the single-cell wounds, this calcium mobilization must be an effect of the single-cell injury itself. This activation extended to a radius of about seven cells from the lysed cell. As such, the functional wound (defined by cellular calcium mobilization) was much larger than the mechanical wound: a single-cell wound resulted in activation of endothelial cells to a diameter of 14 cells, or an area of approximately 200 cells as calculated with the formula

\[
\frac{1}{4} \pi d^2 \sqrt[4]{\pi d^2} = 196
\]

where \(d\) is the cell diameter and \(D = 14d\) is the diameter of the affected area.

The source for the increase in intracellular calcium appears to be derived partly from intracellular stores and partly from extracellular influx. In the absence of extracellular calcium or by inhibiting calcium entry pathways with an inorganic calcium channel blocker such as nickel, any observed calcium mobilization must be a result of \(\text{Ca}^{2+}\) release from intracellular stores. This mobilization was transient and much lower in magnitude, as shown in Figures 2 and 3. Thus, both intracellular and extracellular pools of calcium must be involved in the observed overall mobilization (Figure 1) by shuttling of \(\text{Ca}^{2+}\) across the cell membrane. However, we are unable to say what percentage of the calcium comes from intracellular stores because our calibration is semiquantitative.

The signaling mechanism for this calcium propagation between cells may be extracellular or intracellular. An extracellular mechanism might involve release of cytosolic agonists into the fluid phase; the observed calcium mobilization could then be a result of agonist diffusion to the surrounding cell with subsequent activation. Hence, an extracellular mechanism should not require cell–cell contact or coupling. An intracellular mechanism, however, should require cell–cell coupling for calcium mobilization propagation via diffusion of a messenger through cell gap junctions. Identification of whether extracellular or intracellular mediators are responsible for this mobilization was performed by using subconfluent monolayers, confluent monolayers treated with heptanol to increase gap junction resistance, and confluent monolayers incubated for long times in calcium-free medium to disrupt cell–cell coupling via disruption of cell–cell adhesion.

Apparently, an extracellular mechanism is not operating in this model to trigger calcium mobilization, as subconfluent monolayers did not exhibit calcium mobilization, even though the physical distance for diffusing agonists was unchanged. Calcium mobilization was not observed in 20% or 50% confluent monolayers; only after the monolayer reached 80% confluency and began to establish cell–cell coupling was a much reduced calcium mobilization detected. Evidence for the lack of cell–cell coupling in the subconfluent monolayers was obtained by scrape wounding in the presence of Lucifer yellow, which showed no dye transfer for the 20% and 50% confluent monolayers and minimal dye transfer for the 80% confluent monolayers. In these subconfluent monolayers, normal calcium mobilization was not observed (Figure 4). Moreover, when cell–cell contact in confluent monolayers was disrupted by long-term \(\text{Ca}^{2+}\) chelation (Figure 2, dashed lines), normal \(\text{Ca}^{2+}\) mobilization was not observed, even when extracellular calcium was restored to 1.8 mM. These studies with subconfluent monolayers and the cell–cell contact disruption suggest that diffusion of cellular agonists from the lysed cell does not induce mobilization and that established cell–cell contact is necessary for this phenomenon. However, Larson and Haudenschild have observed calcium mobilization in cells adjacent to but not contacting injured
single cells in subconfluent monolayers. Using confluent monolayers, they observed calcium mobilization in adjacent cells 1–2 seconds after wounding, consistent with our observations. Their injury model used a glass micropipette to scrape linear wounds into endothelial layers, so it may be that a multicellular lysis produces higher levels of diffusible wound signals than the single-cell lysis model presented here. Both mechanisms may indeed be present in large multicellular wounds.

Further evidence for the requirement of cell–cell coupling is given by incubation of endothelial monolayers with heptanol. Exposure of endothelial monolayers to heptanol decreased Lucifer yellow transfer from a scrape wound (thereby demonstrating junctional restriction) and also affected calcium mobilization in response to a single-cell wound in a time-dependent manner. Brief incubation with 3.5 mM heptanol decreased the magnitude and onset of calcium mobilization (Figure 5), but the mobilization was sustained, similar to that of untreated calcium mobilization (Figure 1); longer exposure to heptanol almost completely blocked propagation of calcium mobilization to surrounding cells (Figure 5, dashed lines). Meda et al31 have demonstrated heptanol's ability to lower cell–cell coupling and thereby affect cell function. They showed that short-term incubation (15 minutes) with 3.5 mM heptanol dye uncoupled rat insulin–producing cells and attenuated their normal response to glucose without affecting cell calcium or secretory function. Our heptanol studies, in conjunction with our studies of subconfluent monolayers and of monolayers with disrupted cell–cell contacts by long-term calcium chelation (but with 1.8 mM calcium added back before single-cell lysis), demonstrate a requirement of established cell–cell contact with fully functioning gap junctions for propagation of calcium mobilization.

It was questioned if other cell types would exhibit calcium mobilization similar to that in endothelial cells. Experiments with monolayers of vascular smooth muscle cells and foreskin fibroblasts showed that these cell types did not mobilize calcium in this single-cell wounding model, even in cells adjacent to the wounded target cell (Table 3). Furthermore, endothelial monolayers cocultured with smooth muscle cells did not propagate this mobilization to different cell types: a smooth muscle cell did not mobilize calcium on lysis of an adjacent endothelial cell (and vice versa), even though calcium mobilization among the endothelial cells themselves was unaltered. These observations are perplexing, as cultured homogeneous monolayers of endothelial cells, smooth muscle cells, and fibroblasts form gap junctions with themselves and are extensively dye coupled23–26; endothelial and smooth muscle cells express the same connexin messenger RNAs37 and produce functional gap junctions between each other in coculture with heterocell transfer.27,28 However, others38 have demonstrated that these two cell types, when hyperpolarized with bradykinin or electrical pulse stimulation, are not dye or electrically coupled. Nevertheless, in this single-cell lysis model, calcium mobilization does not appear to occur in smooth muscle cells neighboring an endothelial wound or in monolayers of smooth muscle cells or fibroblasts.

The present study examined the role of calcium in cell–cell communication in microscopically wounded endothelial monolayers. Monolayers with a single mechanically lysed cell experienced a rise in intracellular calcium to a diameter of about 14 cells, or an area of approximately 200 neighboring cells. The signaling mechanism for the observed propagation of calcium mobilization in this model appeared not to be via diffusion of cell-released agonists but rather through a cell–cell junction–mediated mechanism, as mobilization was inhibited when cell–cell contact was disrupted, when gap junctional resistance was increased, and when junctional transfer of Lucifer yellow was inhibited. The response to single-cell lysis was dependent on receptor-operated calcium entry pathways. This response was highly specific to the endothelial cell because no mobilization was observed in smooth muscle cells adjacent to wounded endothelial cells and vice versa.

The conclusion that mediation of calcium entry occurs by intracellular signals rather than extracellular wound signals is at apparent odds with the conclusions of Larson and Haudenschild,17 who used multicellular wounds produced by a linear scrape induced with a micropipette. It is suggested that in the present single-cell wound, insufficient concentrations of extracellular wound signals are generated to result in calcium mobilization, but in multicellular wounds sufficient levels are achieved. It is further suggested that these endothelial-specific, junctionally dependent, and receptor-operated calcium entry pathway–dependent routes may operate in conjunction with endothelial stimulation by diffusible mediators in multicellular wounds to maintain hemostasis on a local cell-to-cell basis. In any case, the presence of an intracellular signal is indicated by the results of this study.

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