

How do injured cells communicate with the surviving cell monolayer?

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SUMMARY

Mechanically scratching cell monolayers relieves contact inhibition and induces surviving cells near the wound edge to move and proliferate. The present work was designed to test whether surviving cells passively respond to newly available space, or whether cells are actively stimulated by signals from injured cells nearby. We monitored intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) while scratching confluent monolayers of bovine pulmonary endothelial cells and mouse mammary epithelial cells. Within seconds after wounding, a transient elevation of $[\text{Ca}^{2+}]_i$ was observed in surviving cells. In endothelial cells, the $[\text{Ca}^{2+}]_i$ elevation propagated into the monolayer for a distance of 10 to 12 cell rows at a speed of 20 to 28 $\mu\text{m}/\text{second}$. The amplitude of the wave of $[\text{Ca}^{2+}]_i$ was reduced as it propagated into the monolayer, but the velocity of the wave was nearly constant. Cells that experienced the $[\text{Ca}^{2+}]_i$ elevation had intact plasma membranes, and survived for over 24 hours post wounding. Removing extracellular Ca^{2+} decreased the amplitude by two-thirds and reduced the propagation rate by half, suggesting that Ca^{2+} influx contributed to the increased $[\text{Ca}^{2+}]_i$. To determine how $[\text{Ca}^{2+}]_i$ waves were stimulated, we blocked extracellular communication by fluid perfusion or intercellular communication by breaks in the monolayer.

In bovine pulmonary artery endothelial cultures, the $[\text{Ca}^{2+}]_i$ wave passed over breaks in the monolayer, and was prevented from traveling upstream in a perfusion chamber. Conditioned media from injured cells also elevated $[\text{Ca}^{2+}]_i$ in unwounded reporter cultures. In mouse mammary epithelial monolayers with established cell-cell contacts, the $[\text{Ca}^{2+}]_i$ wave passed over breaks in the monolayer, but was only partially prevented from traveling upstream during perfusion. These experiments showed that mechanical wounds lead to long distance, $[\text{Ca}^{2+}]_i$ -dependent communication between the injured cells and the surviving cell monolayer through at least two mechanisms: first, extracellular release of a chemical stimulus from wounded cells that diffused to neighboring cells (present in both monolayers); second, transmission of an intercellular signal through cell-cell junctions (present in the mammary epithelial monolayers). Thus, mechanical injury provided a direct, chemical stimulus to nearby cells which have not themselves been damaged.

Key words: Intracellular calcium, Wound healing, Cell motility, Cell communication

INTRODUCTION

Mechanically wounding confluent cell monolayers induces movement and proliferation of cells into the denuded area (Conrad et al., 1993; Coomber and Gotlieb, 1990; Gotlieb et al., 1981; Kupfer et al., 1982; Nagasaki et al., 1992; Todaro et al., 1965). How do surviving cells at the edges of wounds learn about nearby injury? One perspective suggests that it is simply release from contact inhibition that permits cells to move. Contact inhibition has been studied in single motile fibroblasts that touch and repel (Abercrombie, 1980; Abercrombie and Heaysman, 1953), but these signals are likely to be different from signals produced upon release of contact inhibition in confluent monolayers by mechanical rupture of cells. Signals that are specifically produced by mechanical injury might participate in paracrine cell communication during wound healing. Mechanical wounding might remove inhibitory signals mediated by cell surface proteins, such as contactinhibin

(Gradl et al., 1995; Heimark and Schwartz, 1985; Wieser et al., 1990). Alternatively, mechanical wounding might produce stimulatory signals. We investigated signaling among cells in the proximity of mechanically ruptured cells, as would happen after trauma of whole tissue. We chose to study changes of $[\text{Ca}^{2+}]_i$ following multi-cell mechanical wounding since rapid responses to gross mechanical injury have not been well studied. In addition, $[\text{Ca}^{2+}]_i$ is involved in the regulation of both cell motility (Brundage et al., 1991; Gilbert et al., 1994; Hahn et al., 1992; Herman, 1993; Marks and Maxfield, 1990; Mascardo and Eilon, 1988) and proliferation (Byron and Villereal, 1989; Huang et al., 1989; Wahl and Gruenstein, 1993) and might directly regulate healing after injury.

There have been several studies of the $[\text{Ca}^{2+}]_i$ response to single cell stimulation with microneedles (Sanderson et al., 1994, and shear stress (Schwarz et al., 1992; Shen et al., 1992) (see also Diamond et al., 1994, and references therein). Previous studies showed that gentle mechanical stretching,

without membrane rupture, led to propagated waves of $[Ca^{2+}]_i$ in monolayers of endothelial cells, and tracheal, mammary and lens epithelial cells (Boitano et al., 1992; Churchill et al., 1996; Enomoto et al., 1992b, 1994; Hansen et al., 1993). Signaling from gentle mechanical stretching might be somewhat different from the more robust stimulation after mechanical injury where cellular factors might be released after membrane rupture (Churchill et al., 1996; Enomoto et al., 1994; Hansen et al., 1993; Palmer et al., 1996). These studies suggested that $[Ca^{2+}]_i$ would be a likely mediator of mechanical stimuli.

To evaluate communication between the wound and surviving cells we assayed $[Ca^{2+}]_i$ as a potential stimulatory signal upon release from contact inhibition. We determined: (1) whether there was an immediate increase in $[Ca^{2+}]_i$ upon mechanical wounding; (2) whether the elevated $[Ca^{2+}]_i$ propagated from cell-to-cell and did it result from Ca^{2+} release from internal stores or Ca^{2+} entry from the extracellular space; and (3) whether the elevated $[Ca^{2+}]_i$ signal was caused by release of factors that diffused from the wound or whether the $[Ca^{2+}]_i$ signal was propagated intercellularly through cell-cell junctions. In our endothelial cultures, we found that the primary stimuli were extracellular, fluid borne factors derived from the wound. In our epithelial cultures, we found that both extracellular factors and cell junctions were responsible for $[Ca^{2+}]_i$ signal propagation. Diffusible wound factors contributed to the $[Ca^{2+}]_i$ response in cell types with either low or high levels of intercellular junctions.

MATERIALS AND METHODS

Cell culture and $[Ca^{2+}]_i$ measurements

Calf pulmonary artery endothelial cells (CPAE), line CCL 209 (ATCC, Rockville, MD), were grown to confluency (2-3 days) on glass coverslips in DMEM medium (University of California Cell Culture Facility and Gibco Life Sciences, Grand Island, NY) supplemented with 5 mg/ml penicillin, 5 mg/ml streptomycin, 10% fetal calf serum, and 1.6 μ M insulin (St Louis, MO), except as noted where insulin was omitted. Monolayers were then cultured for 2 days in DMEM with penicillin and streptomycin but without serum or insulin to reduce the effects of exogenous growth factors on the wound response. Mouse mammary cells, line 31EG4 (a gift from Karen Zettl and Gary Firestone, University of California, Berkeley) (Zettl et al., 1992) were passaged in DMEM/F-12 supplemented with 5% FBS, 5 mg/ml insulin, and 50 mg/ml gentamycin. For experiments, cells were passaged onto glass coverslips and cultured for 3 to 5 days in DMEM supplemented with 2% FBS, 5 mg/ml insulin 50 mg/ml gentamycin and 1 μ M dexamethasone to induce cell junction formation. These culture conditions resulted in differentiation, with columnar epithelial morphology, high transepithelial resistance (Sjaastad et al., 1993), associated tight junction formation (Zettl et al., 1992), and stratified Golgi in the apical portion of the cell (not shown). CPAE cultures showed no transendothelial resistance, but had focused Golgi and cobblestone morphology (not shown).

For $[Ca^{2+}]_i$ measurements, cells were loaded at room temperature with 0.25-0.5 μ M of the acetoxymethyl ester of fura-2 and 0.1% of the dispersing agent, F-127 (Molecular Probes, Eugene, OR) for 45 minutes. Loading and imaging of fura-2 was performed in Hanks' balanced salt solution without bicarbonate or Phenol Red, but with 20 mM Hepes (HBSS). Chelated Ca^{2+} solutions were prepared by the addition of 5 mM EGTA to standard HBSS. $[Ca^{2+}]_i$ was measured by fluorescence ratio imaging of fura-2 loaded cells at 37°C. Briefly, the imaging system consisted of a low light Dage Silicon-Intensified

Target camera (Dage-MTI Inc., Wabash, MI), a Zeiss IM-35 inverted microscope (Carl Zeiss, Oberkochen, Germany) and a Gould FD5000 image processor (Gould Inc., Cleveland, OH). 350/385 nm ratio images were calibrated with in vitro Ca^{2+} -EGTA solutions as described (Sammak et al., 1992). Measurement of cell motility, cell lysis and death was undertaken on a Diaphot 300 with $\times 20$ and $\times 40$ CF fluor objectives, and was visualized with a cooled CCD camera (Photometrics, PXL KAF 1400, Tucson, AZ) controlled by IP Lab (Signal Analytics, Vienna, VA) run on an Apple Macintosh computer. We found that wound induced elevations were unusually high (3.8 μ M). The wound-induced fura-2 fluorescence ratio increase was close to, but distinguishable from the maximum ratio elicited by digitonin cell lysis (5-fold vs 8-fold). Therefore, wounding did not saturate fura-2. Since fura-2 most accurately reports $[Ca^{2+}]_i$ close to its K_d of 224 nM (Gryniewicz et al., 1985), the true wound-induced elevation might be underestimated. In addition, the in vitro calibrations we used produced higher maximum ratios than in vivo calibrations. This choice of calibration is more conservative and will underestimate measurements of high $[Ca^{2+}]_i$ and minimize differences at high $[Ca^{2+}]_i$ values (Sammak et al., 1992).

Wounding protocol

All monolayers were wounded in 37°C HBSS to avoid interactions of any wound-released factors with growth medium components. Coverslips (22 mm #1.5, Dow Corning, Midland, MI) were mounted with a silicone grease seal into 35 mm Petri dishes with an 18 mm hole in the bottom. Wounds were made in the monolayer by scratching by hand at a speed of about 2 mm/second with a fresh 18 G needle (tip diameter, 8 μ m), which removed a 4 to 6 cell-wide swath (about 100-200 μ m). Monolayers were observed on the inverted microscope during fluorescence ratio imaging with a Nikon CF fluor $\times 20$ objective, NA 0.75. The needle removed most cells from the wound, although some dead and injured cells remained behind at the edges. Dead cells were distinguished in phase contrast microscopy by their lucidity and were limited to the first row of cells at the wound edge. Injured and dying cells in the first row lost their ability to regulate Ca^{2+} and were ignored in measurements of $[Ca^{2+}]_i$.

Fluid perfusion, preparation of wound-conditioned medium and microinjection

Perfusion rates were set with a gravity fed inlet (pressure head, 2 feet) that supplied fluid flow along the bottom of the Petri dish at a constant rate. An aspirator outlet at the top of the culture dish prevented overflow. Flow rates, measured microscopically with a pulse of fura-2 in the perfusate, were 110 μ m/second (4 times as fast as the $[Ca^{2+}]_i$ wave). Perfusion conditions were identical for the endothelial and epithelial cell cultures. $[Ca^{2+}]_i$ was not affected by the gentle starting or stopping of flow, nor by constant perfusion. Linear wounds were made across the direction of fluid flow and the asymmetric wave propagation distance from the upstream and downstream side of the wound was measured. Wound-conditioned medium was produced from confluent monolayers on 22 mm coverslips that were starved for 5 days to remove exogenous growth factors. Sacrificial coverslips were washed, scraped with a rubber policeman into 100 μ l HBSS, and were immediately applied to reporter cultures. Crude conditioned media were applied without further processing to more closely reflect the conditions with in situ wounded monolayers. Reporter endothelial cells were serum starved for 2 days, loaded with fura-2, and monitored for $[Ca^{2+}]_i$ during application of lysate. Microinjection of rhodamine dextran was performed by pressure injection using a Narishige (Japan) micromanipulator and microinjector. Borosilicate glass micropipettes with filaments were pulled with a Sutter pipette puller. Injections were performed after wounding with a diamond scribe to produce a reference scratch, and a series of images were taken with the cooled CCD imaging system over the subsequent 24 hours.

RESULTS

Mechanically wounding the cell monolayer induces surviving cells to elevate $[\text{Ca}^{2+}]_i$

Gentle mechanical stimuli of endothelial cells has been shown to elicit a $[\text{Ca}^{2+}]_i$ response (Ando et al., 1994; Drumheller and Hubbell, 1991; Shen et al., 1992), but mechanical trauma has not been well studied. We wounded cultured monolayers of pulmonary artery endothelial cells and monitored $[\text{Ca}^{2+}]_i$. Wounds were 40 to 100 μm wide (2 to 5 cells) and 1 cm long. Most cells in the path of the needle were completely removed from the coverslip and were ruptured as determined by uptake of trypan blue (not shown). The needle did not leave ragged tears in the surviving monolayer, nor did it pull on the adherent monolayer sheet.

To determine whether traumatic mechanical stimulus elicited a $[\text{Ca}^{2+}]_i$ response, fura-2 loaded monolayers were wounded on the microscope during fluorescence ratio imaging. As shown in Fig. 1A-F, $[\text{Ca}^{2+}]_i$ elevated in the cells at the wound edge within seconds of wounding (1B). The peak elevation was 3.8 μM (Table 1). The elevation spread to neighboring cells (1C-D) before decreasing to a plateau and eventually returned to baseline. A few cells in the first row elevated $[\text{Ca}^{2+}]_i$ maximally until fading away (1F). The elevation was transient, and the location of the peak elevation in $[\text{Ca}^{2+}]_i$ moved away from the wound for a limited distance.

The peak $[\text{Ca}^{2+}]_i$ elevation was progressively lower at larger distances from the wound edge (Fig. 2A), up to 10 to 12 cell rows from the wound edge, beyond which there was no $[\text{Ca}^{2+}]_i$ signaling (Table 1). In all cases, the peak elevation moved from the linear wound edge as a discrete straight-lined front.

The propagation of the Ca^{2+} elevation was wave-like in that its spatial form remained constant but its amplitude decayed over time. The wavefront in Fig. 1 propagated at a rate of 28 $\mu\text{m}/\text{second}$ and the shape of the peak was maintained during propagation (Fig. 2A). Wavespeed decayed slightly but the decrease was not significant ($P < 0.05$) (Table 1). In the absence of extracellular Ca^{2+} , propagation still occurred, but the wave speed was dependent on the concentration of Ca^{2+} in the medium since addition of 5 mM EGTA reduced wave speed to one half (Table 1). EGTA also reduced peak $[\text{Ca}^{2+}]_i$ threefold, although the response was not eliminated (Figs 1G-J, 2B). Wavespeed was constant in EGTA (Table 1). Therefore, propagation of the response through the monolayer involved activation of Ca^{2+} influx as well as coordinated release of Ca^{2+} from intracellular stores (Berridge, 1993; Berridge and Dupont, 1994; Harootunian et al., 1991).

Wound-induced $[\text{Ca}^{2+}]_i$ was higher than the maximal response to exogenous Ca^{2+} -mobilizing stimuli. Maximal concentrations of ATP (100 μM) elevated $[\text{Ca}^{2+}]_i$ from a baseline of between 50 to 100 nM to 1.2 μM (not shown, see also

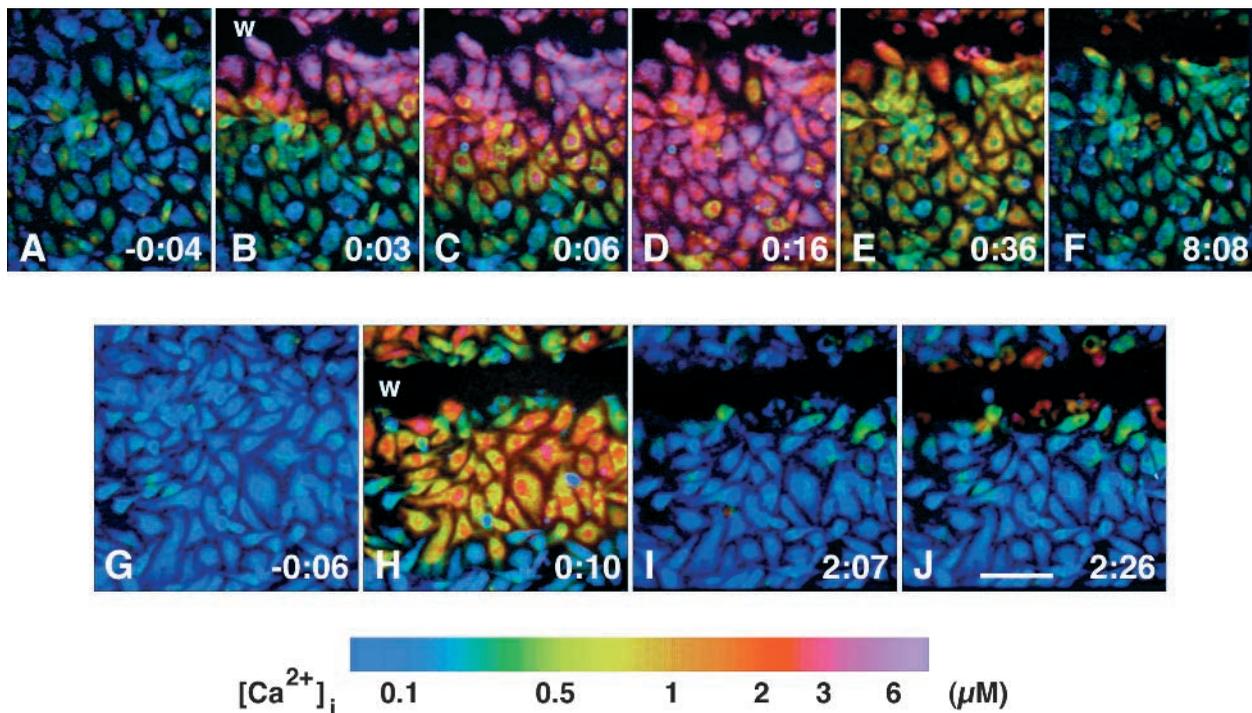


Fig. 1. $[\text{Ca}^{2+}]_i$ transiently increases in cells near the wound. (A-F) Basal $[\text{Ca}^{2+}]_i$ was initially 30-50 nM (pseudocolor display is calibrated as $[\text{Ca}^{2+}]_i$ in μM) and within seconds of wounding (W) $[\text{Ca}^{2+}]_i$ elevated to 4 μM in cells next to the wound. (C,D). The $[\text{Ca}^{2+}]_i$ elevation propagated to neighboring cells up to 10 cell rows away from the wound. (E) After the $[\text{Ca}^{2+}]_i$ peak, $[\text{Ca}^{2+}]_i$ decreased to a plateau of 0.5 to 1 μM within 1 minute and (F) returned to the pre-wounding baseline $[\text{Ca}^{2+}]_i$ within 8 minutes. Both the peak and the plateau $[\text{Ca}^{2+}]_i$ were progressively lower at greater distances from the wound. When cells were wounded in the presence of 5 mM extracellular EGTA, (G-J) the peak $[\text{Ca}^{2+}]_i$ elevation was attenuated (H) and $[\text{Ca}^{2+}]_i$ returned to baseline more rapidly (I). Many cells in the first row appeared to have damaged Ca^{2+} homeostatic mechanisms (H) since they failed to release Ca^{2+} stores after wounding in EGTA, and later (J), after replacement with HBSS containing Ca^{2+} , were unable to regulate basal $[\text{Ca}^{2+}]_i$ levels. Failed $[\text{Ca}^{2+}]_i$ homeostasis and sustained elevations are a signature of cell death. Therefore, subsequent $[\text{Ca}^{2+}]_i$ measurements were made away from the first row to avoid dying cells. Bar, 100 μm .

Table 1. Endothelial cell $[Ca^{2+}]_i$ wave characteristics

Condition	Peak $[Ca^{2+}]_i$ in row 2 (μM) mean \pm s.e.	Propagation rates ($\mu m/s$)		Range of $[Ca^{2+}]_i$ wave (# cell rows from wound)
		2-5 cell rows from wound	5-8 cell rows from wound	
Bath Ca^{2+} 26 wounds	3.8 ± 0.3	28 ± 5.2	20 ± 4.2	10 ± 0.5
Bath EGTA 16 wounds	1.3 ± 0.1	12 ± 1.7	11 ± 3.0	12 ± 2.0
Ca^{2+} and EGTA different at $P < 0.05$?	Yes	Yes	Yes	No

Three characteristics of the $[Ca^{2+}]_i$ wave were determined, the peak amplitude, the wave speed at two distances from the wound and the range of the wave. The peak $[Ca^{2+}]_i$ elevation was measured in 10 to 30 cells in the second row from the wound edge ($n=26$ wounds). The propagation rates were measured between the second and fifth row, and the fifth and eighth row from the edge ($n=13$). The range of the wave was determined by counting the number of cells (approximately 20 μm per cell) that elevated $[Ca^{2+}]_i$ above 500 μM . We found that chelating extracellular Ca^{2+} with EGTA significantly reduced the peak $[Ca^{2+}]_i$ amplitude by 2/3 ($n=16$), and the wave speed by half ($n=8$), but did not change the range of the $[Ca^{2+}]_i$ wave ($P < 0.05$, t -tests). In normal Ca^{2+} the propagation rates were reduced away from the wound (28 to 20 $\mu m/s$) but this decrease was not statistically significant ($P > 0.05$). In EGTA, the wave speed was constant throughout the field of view.

Weintraub et al., 1992). In comparison, fluid flow from pipettes, which triggers stretch-activated Ca^{2+} channels (Lansman et al., 1987; Resnick and Gimbrone, 1995), elevated $[Ca^{2+}]_i$ to $1.3 \pm 0.28 \mu M$ (3 experiments, $n=35$ cells). Therefore, the wound-induced $[Ca^{2+}]_i$ elevation of $3.8 \pm 0.3 \mu M$ was significantly different from other stimuli and was not quantitatively accounted for by our measurements of either fluid flow or by ATP stimulation of membrane receptors. $[Ca^{2+}]_i$ measurements of 1 μM and below are almost linearly proportional to fura-2 fluorescence ratios and are well discriminated, but $[Ca^{2+}]_i$ measurements near 3 μM vary nonlinearly with fluorescence ratios and tend to be depressed as the dye approaches

saturation (see Materials and Methods). Therefore, our measurements will tend to underestimate the difference between ATP or flow stimulation and wounding.

Is the $[Ca^{2+}]_i$ elevation indicative of cell death or transient membrane tearing?

It was possible that the wound-induced $[Ca^{2+}]_i$ elevation was an epiphenomenon of cell death. In this case, the $[Ca^{2+}]_i$ elevation would have been irrelevant for altering cell behavior after injury. It was also possible that the elevated $[Ca^{2+}]_i$ resulted from survivable, transient openings of cell membranes due to mechanical stimulation (McNeil et al., 1985). We therefore performed experiments to determine whether cells that exhibited elevated $[Ca^{2+}]_i$ remained viable subsequent to $[Ca^{2+}]_i$ elevation.

We tested whether cells that exhibited increased $[Ca^{2+}]_i$ took up large dyes that are normally impermeant through cell membranes. Monolayers were wounded in the presence of 0.2% trypan blue, 2 $\mu g/ml$ propidium iodide (a DNA binding dye that stains nuclei of lysed cells), or 100 $\mu g/ml$ rhodamine dextran (which is trapped in the cytoplasm; see Clarke and McNeil, 1992), washed and then observed for dye retention.

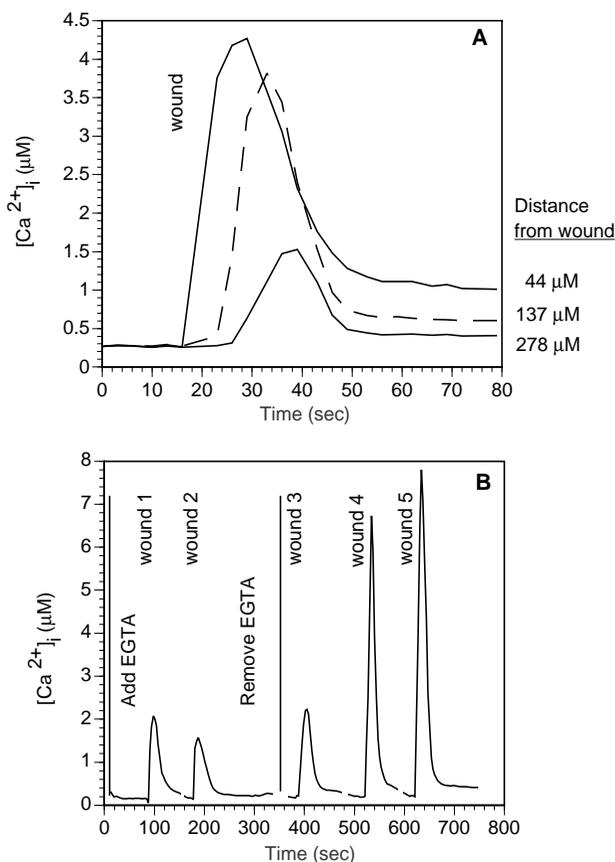


Fig. 2. The $[Ca^{2+}]_i$ elevation is transient and propagates a limited distance. (A) The time course for $[Ca^{2+}]_i$ elevation at 44, 137, and 278 μm from the wound edge shows that at greater distances from the wound, both the peak and the plateau $[Ca^{2+}]_i$ were reduced, and that the peak $[Ca^{2+}]_i$ elevation appeared at progressively longer times. $[Ca^{2+}]_i$ was measured in 30-35 cells equidistant from the linear wound edge. For each data point, the $[Ca^{2+}]_i$ was averaged in 40 by 360 μm^2 rectangles parallel to the wound, encompassing 30-35 cells. The waveform was maintained throughout propagation even though the amplitude was damped and approached unstimulated values past 300 μm (about 10 cell diameters) from the wound edge. The increase and the return to basal $[Ca^{2+}]_i$ occurred first in cells nearest to the wound, consistent with propagation of $[Ca^{2+}]_i$ elevation as a damped wave. (B) Chelation of extracellular Ca^{2+} reduced the wound-induced $[Ca^{2+}]_i$ elevation by 3-fold. A series of independent wounds were made in a monolayer during addition and washout of EGTA (5 mM in HBSS). $[Ca^{2+}]_i$ in the second row of cells increased only to 1 to 2 μM demonstrating that both intra- and extracellular sources of Ca^{2+} were mobilized. A few minute after EGTA was washed out, wounding caused peak $[Ca^{2+}]_i$ to increase transiently to $>7 \mu M$.

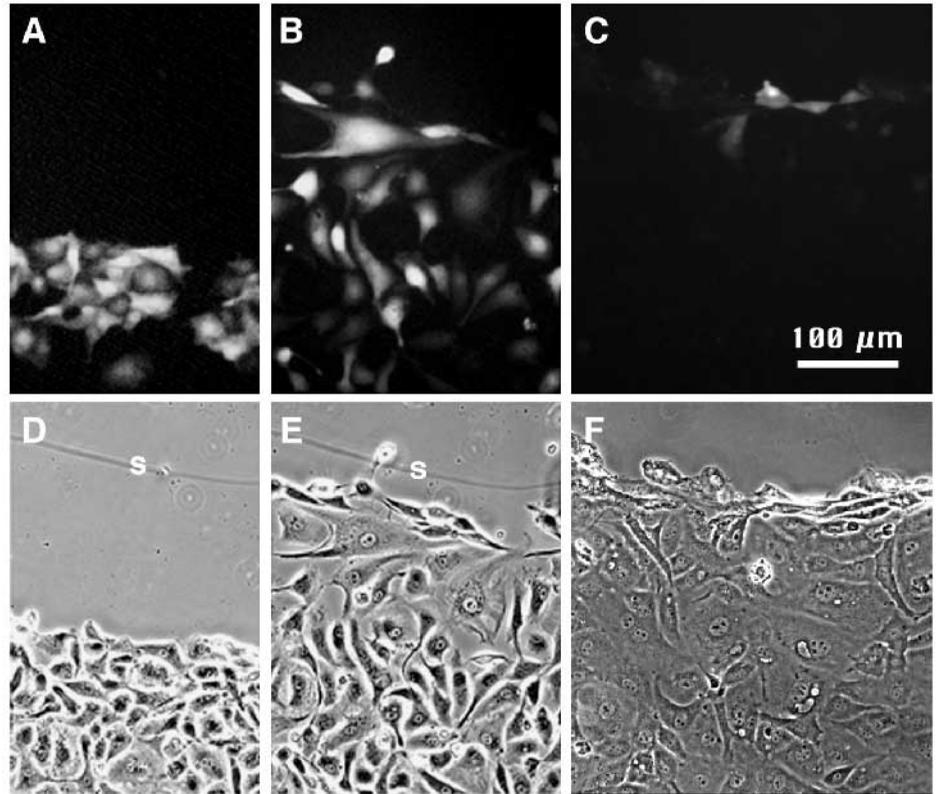


Fig. 3. The $[\text{Ca}^{2+}]_i$ wave did not cause subsequent cell rupture nor did wound-induced cell rupture cause the elevation of $[\text{Ca}^{2+}]_i$. (A) Cells were pressure microinjected with rhodamine-dextran immediately after wounding. Wounding was performed with a diamond scribe which left a reference mark (S) observable in phase contrast (D,E). (B) After 24 hours, the same field was relocated, and more than 90% of microinjected cells were observed to have survived and to have moved into the wound. (C,D) Cells were wounded in HBSS containing rhodamine dextran. Immediately after washout, 28% of cells in the first row retained dye, but no cell past the first row took up dye, suggesting that the $[\text{Ca}^{2+}]_i$ wave was not the result of rupture of cell membranes.

Unperturbed cells in control monolayers rarely took up these dyes. In wounded monolayers, cells that were further than one row away from the wound edge did not retain trypan blue, propidium iodide (not shown) or rhodamine dextran (Fig. 3), while about 25% of cells in the first row were stained; When rhodamine dextran was present during wounding, 11 ± 4 (mean \pm s.d.) cells per field in the first row were stained at 15 minutes after wounding and no cells retained dye in the second through tenth row ($n=4$ wounds; 40 cells per row, both sides; 400 cells per field). Of the total number of cells that experienced the $[\text{Ca}^{2+}]_i$ elevation, less than 4% took up dye, and these cells were most likely injured by direct mechanical breakage, not the elevation of $[\text{Ca}^{2+}]_i$.

Ruptured cells in row one were prevented from elevating $[\text{Ca}^{2+}]_i$ by addition of 5 mM EGTA unlike cells in rows 2-12 $[\text{Ca}^{2+}]_i$ (Fig. 1H). It is possible that cells in the first row were blocked from elevating $[\text{Ca}^{2+}]_i$ because membrane tears permitted entry of EGTA. When normal extracellular Ca^{2+} was restored, ruptured cells experienced an uncontrolled elevation of $[\text{Ca}^{2+}]_i$ (Fig. 1J). This too might be due to unregulated entry of Ca^{2+} through membrane tears. Therefore, this subpopulation of cells could not regulate $[\text{Ca}^{2+}]_i$ as other cells could. Fluorescence intensity was eventually lost from these first row cells, presumably due to efflux of fura-2 through torn membranes (Poenie et al., 1987; Steinhardt et al., 1994). All other cells returned to basal $[\text{Ca}^{2+}]_i$ levels after wounding and maintained low $[\text{Ca}^{2+}]_i$ for hours afterwards.

It was also possible that cells in rows 2-12 that elevated $[\text{Ca}^{2+}]_i$ might have been fatally injured or the $[\text{Ca}^{2+}]_i$ elevation itself could have caused delayed cell death (Dubinsky, 1993; Randall and Thayer, 1992). To determine the long term fate of cells that experienced the wound-induced $[\text{Ca}^{2+}]_i$ elevation,

cells in the first several rows after wounding were microinjected with rhodamine dextran and were followed for 24 hours. 90% of dextran-injected cells remained adherent and motile after 24 hours (Fig. 3B,E). By this time cells at the edge had entered S phase as determined by bromodeoxyuridine stain (unpublished work), indicating that cells were also proliferative after wounding. Therefore, the $[\text{Ca}^{2+}]_i$ wave did not kill cells.

These studies showed that the wound-induced elevation of $[\text{Ca}^{2+}]_i$ resulted from several different mechanisms. Cells adjacent to the wound were either mechanically killed (about 25% of cells in the first row lost Ca^{2+} homeostatic capability, took up trypan blue and propidium iodide, and eventually floated away) or exhibited transient membrane ruptures (1% of row one cells took up rhodamine-dextran but had intact membranes at one hour post wounding, not shown). In contrast, row 2-12 cells exhibited large, transient changes of $[\text{Ca}^{2+}]_i$ that were apparently not due to cell death or transient membrane tearing. The following set of experiments investigate the generation of the $[\text{Ca}^{2+}]_i$ wave in rows 2-12 adjacent to the wound.

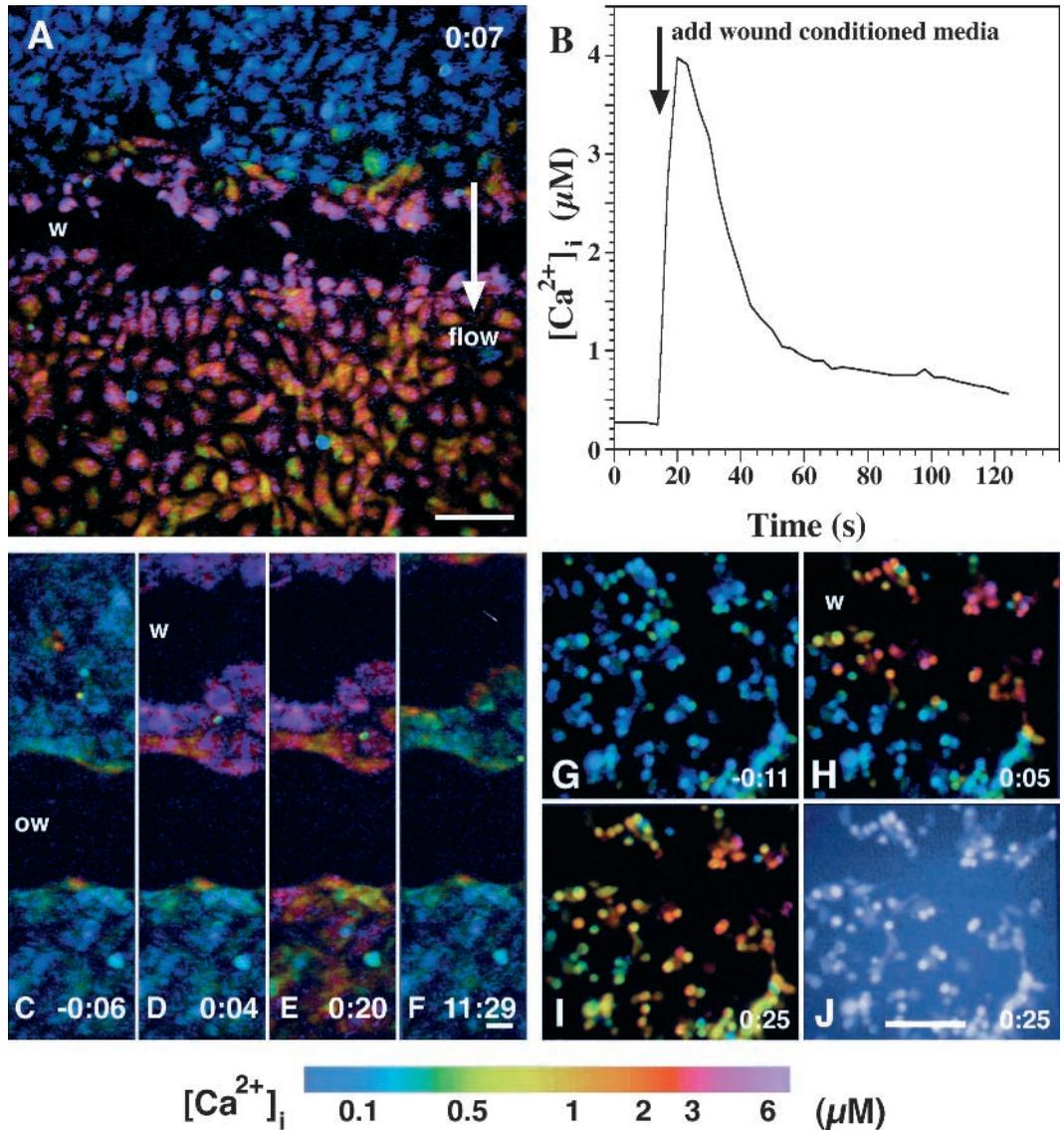
Soluble wound-derived factors mobilize $[\text{Ca}^{2+}]_i$

We considered two potential pathways for propagation of the wave of $[\text{Ca}^{2+}]_i$ from the site of injury. The propagation of the $[\text{Ca}^{2+}]_i$ wave might have been via the extracellular medium (e.g. by agonists released from the wound site, see Enomoto et al., 1992a; Osipchuk and Cahalan, 1992). Alternatively, the route might have been via cell-dependent pathways, either via the cytoplasm (e.g. by diffusion of Ca^{2+} or inositol 1,4,5-trisphosphate through gap junctions (Boitano et al., 1992; Charles et al., 1992; Cornell-Bell et al., 1990; Drumheller and Hubbell, 1991; Nedergaard, 1994; Sanderson et al., 1990; Xia

Fig. 4. In pulmonary artery endothelial cells, extracellular factors are necessary and sufficient for $[Ca^{2+}]_i$ propagation, and cellular routes do not contribute to the mechanism of propagation. (A) Fluid flow alters wave propagation. To block an extracellular propagation pathway without disturbing cellular routes, fluid was perfused from top to bottom at four times the rate of unperturbed wave propagation. The average upstream propagation was 1.9 ± 0.3 rows (including damaged cells in the first row) and downstream was greater than 21 rows (limited by the field of view), more than double the range without perfusion. The $[Ca^{2+}]_i$ wave did not propagate upstream at all in 6 of 15 wounds.

(B) Conditioned medium from injured cells elevated $[Ca^{2+}]_i$ in unwounded reporter cells. Reporter cells were loaded with fura-2 and monitored for $[Ca^{2+}]_i$ during application of medium conditioned by injured cells. Addition of HBSS did not produce an elevation of $[Ca^{2+}]_i$, while application of conditioned medium produced an elevation of $[Ca^{2+}]_i$. The elevation had the same magnitude and duration as in monolayers that were directly wounded (cf Fig. 2A).

(C-F) An old wound (OW) provided a break in the monolayer to block cellular routes of $[Ca^{2+}]_i$ wave propagation without disturbing extracellular, fluid based routes. No cytoplasmic bridges were revealed by fluorescence or by phase contrast microscopy. A new wound (W) was made and the $[Ca^{2+}]_i$ elevation propagated across the break in the monolayer showing that cell contacts were not required for $[Ca^{2+}]_i$ wave propagation and that extracellular routes are sufficient. (G-J) Monolayers treated for 40 minutes in EGTA broke cell-cell contacts. Cells were returned to normal HBSS for more than 20 minutes and then wounded. The elevation propagated from cell cluster to cell cluster, supporting fluid borne routes rather than cellular pathways for elevating $[Ca^{2+}]_i$. Bars: 100 μ m (A); 25 μ m (F); 100 μ m (J).



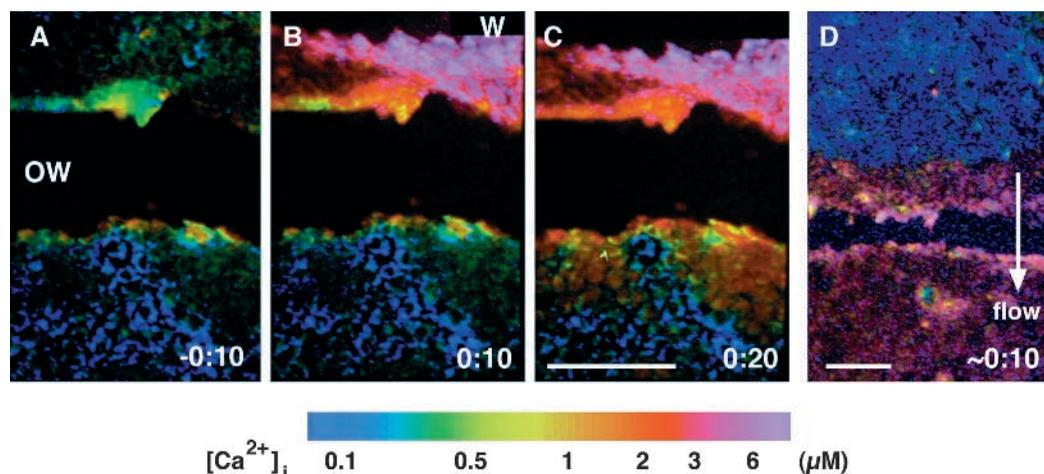
and Ferrier, 1992), or via electrical or mechanical stimulation of the membrane (e.g. stretch-activated channels; see Enomoto et al., 1992b; Shen et al., 1992). We performed four complementary experiments to examine the necessity and sufficiency of each propagation pathway.

We first tested whether soluble factors might be released from the wound site into the medium. To block extracellular fluid-mediated pathways (without interfering with cellular pathways), cultures were exposed to constant fluid flow while being wounded across the direction of flow (Fig. 4A). $[Ca^{2+}]_i$ elevated downstream more than twice as far as usual, as would be expected if a diffusible factor were involved in $[Ca^{2+}]_i$ wave propagation. The propagation front was a straight edge, parallel to the wound edge. In addition, there was no upstream propagation beyond the first row of cells in most wounds (average

range in 15 wounds: 1.9 ± 0.3 rows upstream compared to more than 25 rows downstream, limited by the field of view). If cell-cell junctions or direct mechanical pulling by the needle contributed to propagation along with extracellular pathways, fluid perfusion would not have reduced the upstream wave propagation to zero. This experiment suggested that soluble factors were necessary for the elevation of $[Ca^{2+}]_i$ in wounded endothelial monolayers.

Second, to test whether wound-derived factors were sufficient for elevating $[Ca^{2+}]_i$, we collected HBSS that was conditioned by wounding monolayers with a scraper and applied this conditioned medium to unwounded reporter cultures loaded with fura-2. Addition of wound-conditioned medium produced an elevation that was comparable in magnitude and dynamics to the $[Ca^{2+}]_i$ elevation produced by wounding (peak $[Ca^{2+}]_i$

Fig. 5. In mammary epithelial cells, both cell junctions and extracellular factors contribute to the propagation of $[\text{Ca}^{2+}]_i$. (A-D) A new wound (W) was made next to an old wound (OW) as in Fig. 4. The elevation of $[\text{Ca}^{2+}]_i$ propagated across the break in the monolayer, but the cells responded heterogeneously, in clusters. (D) Perfusion, under conditions identical to Fig. 4A, showed that the elevation of $[\text{Ca}^{2+}]_i$ propagated further downstream when cultures were wounded during perfusion. However, a substantial upstream propagation remained, suggesting that both extracellular and intercellular routes were also important for propagation in this cell culture. Bars, 100 μm .



$=3.2 \pm 0.4 \mu\text{M}$, $n=4$; cf. Figs 2A and 4B). Pipetting HBSS from unwounded cultures in a similar manner did not elevate $[\text{Ca}^{2+}]_i$, nor did contact with debris. Although flow-induced shear stress is sufficient to elevate $[\text{Ca}^{2+}]_i$ (above), these experiments show that simple mechanical shearing by the needle or by the addition of fluid is not necessary for initiating the $[\text{Ca}^{2+}]_i$ wave. We conclude that endogenous factors released from wounded monolayers are sufficient to elevate $[\text{Ca}^{2+}]_i$ without the mechanical disturbance of a needle scraping the coverslip or direct cell-cell contact with the injured cells.

Third, to test whether intercellular routes were required for the wound-induced $[\text{Ca}^{2+}]_i$ wave, we broke cell-cell-contacts (without interfering with extracellular pathways) by double-wounding. Forty minutes after $[\text{Ca}^{2+}]_i$ had returned to steady state around an old wound, a new wound was made a few cell diameters away (Fig. 4D). The new $[\text{Ca}^{2+}]_i$ elevation jumped across the old wound (Fig. 4E), showing that cell-cell contacts were not necessary for the propagation of the $[\text{Ca}^{2+}]_i$ wave. Lack of bridging strands across the old wound was confirmed by fluorescence (Fig. 4C-F) and phase-contrast video microscopy (not shown).

Fourth, we provided a barrier to cell-cell communication by detaching cells from each other with a 40 minute treatment in HBSS containing 5 mM EGTA. Communication among these cells could not have depended on cell-cell contact since clusters of cells were not connected to each other by cytoplasmic strands as detected by fura-2 fluorescence (Fig. 4J), or by phase contrast microscopy. After cells rounded up, EGTA was removed, and cells were incubated for 10-30 minutes in normal HBSS before wounding to promote refilling of intracellular Ca^{2+} stores. Basal $[\text{Ca}^{2+}]_i$ levels were normal in these cells (Fig. 4G), and upon scratching with a needle, an elevation of $[\text{Ca}^{2+}]_i$ propagated from the wound (Fig. 4H-I). Cell-cell contact was not necessary for propagation of the $[\text{Ca}^{2+}]_i$ elevation in our pulmonary artery endothelial cell cultures.

Propagation of $[\text{Ca}^{2+}]_i$ waves in mouse mammary epithelial cells depends on both cellular and extracellular pathways

Experiments in pulmonary artery endothelial cells in the previous sections showed that diffusion of soluble, wound-

derived factors in the extracellular space were almost exclusively responsible for the $[\text{Ca}^{2+}]_i$ elevations. In contrast, in several cell types where gap junctions have been established, intercellular rather than extracellular pathways mediate $[\text{Ca}^{2+}]_i$ waves (Boitano et al., 1992; Charles et al., 1992; Cornell-Bell et al., 1990; Drumheller and Hubbell, 1991; Nedergaard, 1994; Sanderson et al., 1990; Xia and Ferrier, 1992). We therefore evaluated whether the extracellular communication pathway was present in the mouse mammary epithelial cells, line 31EG4. These cells have well developed tight junctions (Zettl et al., 1992) and also exhibit evidence of gap junctional communication among cells at the wound edge since cells that are mechanically injured in solutions containing propidium iodide can pass the dye to adjacent uninjured cells in the monolayer between 10 and 60 minutes post-wounding (G. Giorgi and T. Machen, unpublished observations). In pulmonary endothelial cell cultures propidium iodide does not pass from cells at the wound edge to adjacent cells (not shown). When monolayers of 31EG4 cells were wounded, a $[\text{Ca}^{2+}]_i$ wave propagated with an irregular wavefront, unlike the straight propagation front seen in endothelial cell cultures. The propagation was rapid among cells in clusters but there were pauses and variable $[\text{Ca}^{2+}]_i$ amplitudes among cells in different clusters (Fig. 5B,C).

We performed two experiments under conditions identical to the endothelial cell experiments above to measure the contribution of fluid- and cell-mediated $[\text{Ca}^{2+}]_i$ propagation. First, an old wound provided a break in the contiguous monolayer, and a new, parallel wound made a few cell diameters away produced a $[\text{Ca}^{2+}]_i$ elevation that jumped over the break (Fig. 5A-C). Therefore, soluble factors contributed to the propagation of $[\text{Ca}^{2+}]_i$ in this cell type as well. Second, we wounded mammary cell monolayers in the presence of fluid flow under conditions identical to the endothelial perfusion experiments (Fig. 4A). Under these conditions, the elevation of $[\text{Ca}^{2+}]_i$ propagated farther downstream, suggesting that soluble factors were sufficient for producing a $[\text{Ca}^{2+}]_i$ elevation in mammary epithelium. In contrast to endothelial cells, though, there was some upstream propagation that was suggestive of cell-cell mediated communication as well (Fig. 5B,C). Therefore, mechanical trauma stimulated mammary epithelium via at least

two routes: the production and extracellular diffusion of soluble factors (as observed in endothelial cells) and by intercellular communication dependent on cell contact.

DISCUSSION

In this study we investigated whether cells near wounds receive signals from mechanically injured cells. We serum starved confluent cell monolayers to remove exogenous stimulators and scratched a linear path through the monolayer to relieve contact inhibition and open space on the coverslip for cells to move. Our aim was to determine whether there were wound-derived factors that diffused to adjacent cells and by what means do $[Ca^{2+}]_i$ -dependent signals emanate from the wound site. Previous work has shown that mechanical signals can be propagated from cell-to-cell via gap junctions (Sanderson et al., 1994) and extracellular factors (Enomoto et al., 1992a). Mechanically stimulated $[Ca^{2+}]_i$ waves have been reported in other tissues including osteoblasts (Xia and Ferrier, 1992), mammary epithelial cells (Enomoto et al., 1992b), aortic endothelial cells (Demer et al., 1993), venous endothelial cells (Drumheller and Hubbell, 1991), neuron co-cultures with glial cells (Charles, 1994) or astrocytes (Parpura et al., 1994), and neuroepithelioma cells (Palmer et al., 1996). Here, we report $[Ca^{2+}]_i$ waves stimulated by mechanical injury in pulmonary endothelial cells and mammary epithelial cells and show that the mechanism of propagation depends on the release of wound-derived factors.

Immediately following mechanical scratching of the monolayer, $[Ca^{2+}]_i$ elevated in surviving cells 2-12 rows distant from the edges of wounds. The $[Ca^{2+}]_i$ elevation was transient, propagated a limited distance through the monolayer, and produced a graded response, falling off to baseline within 12 cell rows from the wound. The source of Ca^{2+} was from both extracellular and intracellular sources, since addition of bath EGTA reduced, but did not eliminate, the elevation. The speed of the $[Ca^{2+}]_i$ wave was relatively constant over time, but was reduced 2-fold in the absence of extracellular Ca^{2+} .

The propagated $[Ca^{2+}]_i$ elevation is *prima facie* evidence for a cellular response to mechanical injury. However, the $[Ca^{2+}]_i$ elevation could be considered an epiphenomenon if the cells that experienced the elevation did not survive. Cell survival was demonstrated by injecting cells with rhodamine dextran and demonstrating viability at 24 hours (Fig. 3). Immediately after wounding, all but a few of the remaining undamaged cells remained intact as determined by the lack of uptake of the impermeant dyes rhodamine-dextran and propidium iodide, and the retention of the cytoplasmic dye, fura-2. In cells that took up dye, the $[Ca^{2+}]_i$ elevation was dependent on Ca^{2+} influx (presumably through tears) while intact cells did not require Ca^{2+} influx (Fig. 1). Additionally, the presence of extracellular Ca^{2+} was not sufficient to elevate $[Ca^{2+}]_i$ during wounding when wound factors were removed by perfusion (Fig. 4A). Therefore, the elevation of $[Ca^{2+}]_i$ was not the result of passive leakage through membrane tears and the $[Ca^{2+}]_i$ elevation did not kill cells.

There were several routes by which surviving endothelial cells might respond to nearby injury with a wave of $[Ca^{2+}]_i$. (1) One possibility was that cell membranes were ruptured. As discussed above, membrane rupture was not detected in cells

that were not in immediate contact with the wound site (Fig. 3). (2) Another possibility was that stretch-activated Ca^{2+} channels were stimulated by direct mechanical stress. This seemed unlikely since the upstream $[Ca^{2+}]_i$ wave was blocked by perfusion while mechanical stimulation should not be altered by perfusion (Fig. 4A). (3) Gap junction mediated diffusion of intracellular messengers such as inositol 1,4,5-trisphosphate can produce an intercellular wave of elevated $[Ca^{2+}]_i$ in response to non-penetrating mechanical stimulus (Charles et al., 1992; Sanderson et al., 1994). However, the $[Ca^{2+}]_i$ wave passed between cells separated by breaks in the monolayers (Fig. 4C,D and Fig. 5C,D), while a continuous endothelial cell monolayer was insufficient for supporting $[Ca^{2+}]_i$ wave propagation in the presence of fluid flow (Fig. 4A). It should be noted, though, that perfusion of mammary epithelial cultures did not totally eliminate cell-cell $[Ca^{2+}]_i$ waves, suggesting both extracellular and intracellular communication, consistent with a partial role for gap junctions in these cells. (4) Electrical stimulation of membranes has also been postulated (Enomoto et al., 1992b), whereby membrane depolarization is passed from cell-to-cell. However, mechanisms that posit cell-cell contact are not supported by the perfusion experiments (Fig. 4A) and are not necessary to explain the barrier experiments (Fig. 4C,D).

Diffusion of extracellular factors released from the wound site is the simplest explanation for the mechanism of propagation of the $[Ca^{2+}]_i$ wave that is consistent with our data. Soluble factors released from injured cells would be expected to diffuse over breaks in the monolayer (endothelial and epithelial cells; Figs 4C,D, 5B), would be carried farther downstream if cultures were perfused during wounding (endothelial and epithelial cells; Figs 4A, 5A), and would be able to quantitatively reproduce the $[Ca^{2+}]_i$ elevation if unwounded reporter cultures were treated with medium conditioned by mechanically injured cells (endothelial cells; cf Figs 2 and 4B).

Soluble, wound-derived factors might be acting via cell surface receptors. There is a large class of hormones that act locally in response to inflammation and injury that have been called autacoids (Garrison and Rall, 1990). A number of factors that are manufactured in endothelial cells are known to mobilize $[Ca^{2+}]_i$ (Himmel et al., 1993). These include ATP, substance P acetylcholine and endothelin, which are known to be released from endothelial cells in response to flow induced shear stress (Bodin et al., 1991; Milner et al., 1990, 1992). Platelet derived growth factor (PDGF) is released from endothelial cell cytoplasm after cell injury (McNeil and Ito, 1990; McNeil et al., 1989). Substance P, acetylcholine and serotonin are released during hypoxic damage to endothelial cells (Burnstock et al., 1988; Milner et al., 1989). Among the potential $[Ca^{2+}]_i$ agonists, ATP is interesting since it has been shown to produce $[Ca^{2+}]_i$ waves in mast cells (Osipchuk and Cahalan, 1992), mammary epithelial cells (Enomoto et al., 1994), and neuroepithelioma cells (Palmer et al., 1996) and has been found locally at concentrations sufficient for receptor activation.

If the elevation of $[Ca^{2+}]_i$ was due to diffusion of factors released from the wound site, it might seem that propagation of the $[Ca^{2+}]_i$ response should have been diffusive and not wave like. However, propagation depended on details of $[Ca^{2+}]_i$ mobilization as well as wound factor diffusion since the wave speed was reduced by 2 when extracellular Ca^{2+} was buffered

by EGTA (Table 1). Reduced Ca²⁺ influx would seem unlikely to alter the diffusion rate of soluble factors, but could have altered the cell stimulus by reducing secretion or binding of wound factors released from cells. Alternately, reducing Ca²⁺ influx could have altered the cellular response. For example, reduced influx could reduce the speed of the [Ca²⁺]_i wave by retarding elevation of priming levels of [Ca²⁺]_i necessary to trigger the release of intracellular stores, or by limiting the filling of intracellular stores. These effects would mean that longer times would be required for Ca²⁺ stores to reach a threshold for release into the cytoplasm to propagate the wave, and slow the wave down (Berridge, 1993; Berridge and Dupont, 1994). [Ca²⁺]_i waves in other systems have been shown to depend on extracellular Ca²⁺ (Girard and Clapham, 1993), although the requirement for extracellular Ca²⁺ is not universal (Rooney et al., 1990). The propagation likely does not depend on bulk diffusion of Ca²⁺ through the cell but instead involves successive activation of Ca²⁺ stores by Ca²⁺ and inositol 1,4,5-trisphosphate (Dupont and Goldbeter, 1994; Jafri and Keizer, 1994). Therefore, the most likely interpretation of how our [Ca²⁺]_i wave propagates is that the primary, extracellular signal moves away from the wound by diffusion, but that the cellular Ca²⁺ response is dependent on more complicated, regenerative mechanisms in the cell.

As noted above, the [Ca²⁺]_i propagation in endothelial cells seemed to be solely due to extracellular factors while the [Ca²⁺]_i response of mammary epithelial cultures was likely due to extracellular factors and gap junction communication: the [Ca²⁺]_i wavefront in endothelial cells moved out parallel to the wound edge in a straight line, while in epithelial cells, the response of cells was heterogeneous (Fig. 5C). The patchy [Ca²⁺]_i response corresponded to patches of cell with distinct cell packing and morphology in phase contrast images (not shown). In response to wounding, cells in a patch elevated [Ca²⁺]_i more quickly than surrounding areas. Although baseline [Ca²⁺]_i is slightly elevated in some patches (Fig. 5A), the change in [Ca²⁺]_i upon wounding is significantly greater in these patches compared to neighboring areas (Fig. 5C). The clustering of responsive cells suggested that there might be coordination among cells that are in contact, possibly via cell junctions.

In mammary epithelial cultures, fluid flow did not completely block upstream [Ca²⁺]_i wave propagation providing further evidence that additional, fluid-independent mechanisms of propagation likely exist in these cells. It is possible that tight junctions might restrict fluid flow to the top of the monolayer while wound factors might diffuse on the underside of the monolayer. However, diffusion on the underside would be limited by cell-substrate attachment. Attachment was similar in endothelial and epithelial cells, judging by monolayer deformation due to mechanical forces, and yet upstream [Ca²⁺]_i waves were different between the cell types. Thus, even though multiple mechanisms may coexist, extracellular communication from mechanical wounds are important in two cell types, each with different degrees of cell-cell contact.

The focus of the current work is on the stimulus-response coupling of cells to mechanical injury. How do cells near wounds receive information about nearby injury? Our principal observation is that mechanical injury of a cell monolayer can be converted to chemical information at a distance via diffusion of wound-factors through the extracellular medium. Elevation of [Ca²⁺]_i is one cellular response to these wound factors.

Therefore, [Ca²⁺]_i helps mediate the flow of information from the wound site into a limited number of cells surrounding the wound.

It remains to be determined whether or not the [Ca²⁺]_i elevation is biologically significant.

One possible function for the one-time release of factors from injured cells might be to contribute to the spatial control of movement and proliferation after tissue injury. The spatial limitations, due to dilution or degradation of the extracellular signal, might provide an important mechanism for producing a localized response of tissue to wounding; distant cells remain quiescent, while cells in the proximity of wounds move and grow. Mechanical injury provides one of the few known switches for the induction of motility and growth in quiescent cells. Characterizing this switch will lead to a better understanding of the regulatory control of cell movement and proliferation during wound healing. Elucidating this pathway may provide clues to the induction of angiogenesis where blood vessel growth is desirable (to improve wound healing), and to the inhibition of angiogenesis where growth is undesirable (in the neovascularization of solid tumors).

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