Growth factors but not gap junctions play a role in injury-induced Ca^{2+} waves in epithelial cells

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SUMMARY

This paper characterizes the early responses of epithelial cells to injury. Ca^{2+} is an important early messenger that transiently increases in the cytoplasm of cells in response to external stimuli. Its elevation leads to the regulation of signaling pathways responsible for the downstream events important for wound repair, such as cell migration and proliferation. Live cell imaging in combination with confocal laser scanning microscopy of fluo-3 AM loaded cells was performed. We found that mechanical injury in a confluent region of cells creates an elevation in Ca^{2+} that is immediately initiated at the wound edge and travels as a wave to neighboring cells, with $[Ca^{2+}]_i$ returning to background levels within two minutes. Addition of epidermal growth factor (EGF), but not platelet-derived growth factor-BB, resulted in increased $[Ca^{2+}]_i$, and EGF specifically enhanced the amplitude and duration of the injury-induced Ca^{2+} wave. Propagation of the Ca^{2+} wave was dependent on intracellular Ca^{2+} stores, as was demonstrated using both thapsigargin and Ca^{2+} chelators (EGTA and BAPTA/AM). Injury-induced Ca^{2+} waves were not mediated via gap junctions, as the gap-junction inhibitors 1-heptanol and 18α-glycyrrhetinic acid did not alter wave propagation, nor did the cells recover in photobleaching experiments. Additional studies also demonstrated that the wave could propagate across an acellular region. The propagation of the injury-induced Ca^{2+} wave occurs via diffusion of an extracellular mediator, most probably via a nucleotide such as ATP or UTP, that is released upon cell damage.

Movies available on-line

Key words: Ca^{2+} signaling, Corneal epithelium, EGF, Wound repair

INTRODUCTION

Wound repair is an intricate process that involves changes in cellular adhesion, migration and proliferation, as well as alterations in matrix composition (Moulin, 1995; Mutsaers et al., 1997; Brown et al., 1999; Richardson et al., 1999). By studying these mechanisms, progress has been made in the treatment of both normally healing wounds and those hindered by coexisting pathological conditions. For example, growth factors have long been recognized as important components of wound repair (Bennett and Schultz, 1993; Declair, 1999; Wells, 1999; Imanishi et al., 2000), and their administration enhances healing (Robinson, 1993; Schultz et al., 1992; Pierce and Mustoe, 1995; Rudkin and Miller, 1996). In some of the earliest studies, Leibowitz et al., were able to show that topical application of epidermal growth factor (EGF) increases the tensile strength of full thickness corneal wounds (Leibowitz et al., 1990). More recently, topical application of platelet-derived growth factor-BB (PDGF-BB), which is available as a gel (becaplermin), has been shown to be beneficial for the treatment of poorly healing ulcers in diabetic patients (Embil et al., 2000; Ladin, 2000).

These therapeutic advancements stem from the observation that alterations in growth factor and growth factor receptor expression, availability and localization are normal components of the healing process. For example, our laboratory has shown that 30 minutes after injury an increase in the expression of transforming growth factor-β1 (TGF-β1) mRNA occurs in corneal fibroblasts along the wound edge, and this is associated with an increase in TGF-β receptor binding. Furthermore, these elevations are enhanced by the addition of exogenous TGF-β1 (Song et al., 2000). Matrix-associated growth factors such as TGF-β1 and fibroblast growth factor-2 (FGF-2) have also been transiently detected along the corneal wound edge in vivo (Trinkaus-Randall and Nugent, 1998). Finally, Zieske et al., established that activation of the EGF receptor is required for epithelial cell migration and proliferation, events important for wound repair (Zieske et al., 2000).

Like many other biological processes, wound repair can not be characterized by a simple sequence of events. Evidence from numerous studies suggests that injury initiates activation and interaction of a multitude of intracellular signaling pathways, leading to induction of specific downstream events. Ca^{2+} is an important early messenger whose transient cytosolic oscillations can be induced by an extensive variety of external stimuli, such as binding of peptide factors to receptors in the plasma membrane or mechanical stimulation. The two main storage sites for Ca^{2+} are the endoplasmic/sarcoplasmic reticulum and the extracellular space, where the $[Ca^{2+}]$ is maintained at levels 10,000 fold higher than in the cytosol. Ca^{2+} concentration must normally be kept low in the cytosol because

numerous Ca^{2+}-binding proteins reside there. Ca^{2+} causes a change in activity of these cytosolic proteins, which include protein kinases and phosphatases that participate in regulation of the signaling pathways that determine cellular response (van Haasteren et al., 1999; Clapham, 1995; Berridge, 1997).

Several laboratories have reported that mechanical stimulation results in an immediate transient elevation in $[Ca^{2+}]_i$ that spreads quickly as a wave to neighboring cells. Much of this work employed the type of mechanical stimulation that involves distortion of a single cell rather than 'gross' mechanical injury resulting in rupture and/or removal of a group of cells. Some of the cell types studied include articular chondrocytes (Grandolfo et al., 1998), glial cells (Charles et al., 1991), pancreatic islet cells (Cao et al., 1997; Bertuzzi et al., 1999), osteoblastic cells (Jorgensen et al., 1997), and a variety of epithelial cells, including those originating from lens (Churchill et al., 1996), airway (Hansen et al., 1993), liver (Frame and de Feijter, 1997) and mammary (Enomoto et al., 1994) tissue. Investigators have focused on the underlying mechanisms for the multicellular Ca^{2+} wave, and some have suggested that it propagates via gap junctions, which are aggregates of intercellular channels comprised of connexins (Goodenough et al., 1996). Unlike the growth factor and growth-factor receptor changes mentioned previously, this Ca^{2+} response is immediate and initiated within milliseconds of injury. The study of this early and rapid event was facilitated by the use of confocal microscopy or rapid digital imaging microscopy in combination with special dyes that fluoresce upon Ca^{2+} binding (Grynkiewicz et al., 1985). Investigation of these early events may help decipher activation and regulation of the later events and final outcome of wound repair.

This paper characterizes the cellular Ca^{2+} response to injury in corneal epithelial cells. The cornea is a protective structure and offers an ideal system for the study of wound repair, because it is a simplified avascular and transparent tissue. It is divided into three regions, which are, from anterior to posterior, a squamous columnar epithelium that is multilayered, a sparsely populated stroma with a highly organized matrix and an endothelial monolayer (Trinkaus-Randall, 2000). Injury-induced Ca^{2+} waves have not been studied in the corneal epithelium and, unlike most previous studies, we chose to injure confluent monolayers of cells in a 'gross' manner to more closely approximate an actual wound. We imaged cells at a rapid rate (one image every 789 milliseconds) to capture the responses of this immediate change. Although growth factors are important in wound repair, the effect of growth-factor addition on the injury-induced Ca^{2+} wave has not been evaluated to date.

This study describes the cellular Ca^{2+} response of epithelial cells to mechanical injury, with or without the addition of exogenous growth factors, identifies the source of Ca^{2+} for the response and proposes a mechanism by which the signal may spread to neighboring cells. Addition of EGF, but not PDGF-BB (at the same concentration), resulted in increased $[Ca^{2+}]_i$, and EGF specifically enhanced the amplitude and duration of the injury-induced Ca^{2+} wave. We found that propagation of the wave was dependent on intracellular Ca^{2+} stores and was not mediated via gap junctions. We show evidence that propagation of the injury-induced wave occurs via diffusion of an extracellular mediator, such as the nucleotide ATP or UTP, that is released upon cell damage.

MATERIALS AND METHODS

Cell culture

Studies were performed using both an epithelial cell line and primary epithelial cells isolated from rabbit corneas. The human corneal epithelial cell line (HCE-T) was established by Araki-Sasaki et al., using a recombinant SV40-adenovirus vector (Araki-Sasaki et al., 1995). It displays properties similar to normal corneal epithelial cells. Fresh rabbit eyes were obtained from Pel-Freeze Biologicals (Rogers, AR), and the corneas were removed and rinsed in DMEM containing 1000 U/ml penicillin, 1000 μg/ml streptomycin and 50 U/ml nystatin (Gibco BRL/Life Technologies). After incubating the corneas with 1.2 mg/ml Dispase II (Boehringer Mannheim Corp., Indianapolis, IN) for one hour at 37°C, epithelial sheets were teased off and seeded onto 25 mm round glass (No. 1) coverslips (Trinkaus-Randall et al., 1988). All epithelial cells were cultured in Keratinocyte-SFM (KSFM) ($[Ca^{2+}] < 0.1$ mM) supplemented with 30 μg/ml bovine pituitary extract (BPE), 0.1 ng/ml EGF, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL/Life Technologies). Primary cortical astrocytes isolated from the hippocampus were cultured according to Cornell-Bell et al. (Cornell-Bell et al., 1990a).

Antibodies and reagents

All mouse monoclonal and rabbit polyclonal antibodies against connexin types 32, 43 and 50 were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). The fluorescent Ca^{2+} indicator fluo-3 AM and pluronic acid were from Molecular Probes, Inc. (Eugene, OR), as was the 5-carboxyfluorescein diacetate (5-CFDA) AM used in the photobleaching experiments. Thapsigargin, BAPTA/AM and tyrphostin AG1478 were obtained from Calbiochem (La Jolla, CA). The gap-junction inhibitors 1-heptanol and 18α-glycyrrhetinic acid (18α-GA) were bought from Sigma Chemical Company (St. Louis, MO), as were the nucleotides adenosine triphosphate (ATP) and uridine triphosphate (UTP), $CdCl_2$ and the phospholipase C inhibitor, U73122. EGF was purchased from Gibco BRL/Life Technologies, whereas the other growth factors (PDGF-BB and FGF-2) were from R & D Systems (Minneapolis, MN).

Confocal microscopy

Epithelial cells grown to confluency on 25 mm round glass (No. 1) coverslips were quiesced for 18 to 24 hours before experimentation in Keratinocyte-SFM lacking BPE and EGF. For all experiments, cells were incubated in an HEPES-buffered saline solution containing 137 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 3 mM $CaCl_2 \bullet 2H_2O$, 25 mM glucose and 10 mM HEPES (Cornell-Bell et al., 1990b). Cells were incubated in 4 μM fluo-3 AM, a dye that fluoresces upon binding Ca^{2+}, supplemented with 0.02% pluronic acid in DMSO for 30 minutes at 37°C. After rinsing two times in HEPES-buffered saline, the live cells were placed in an open chamber (Molecular Probes, Inc., Eugene, OR) with 500 μl of HEPES solution and positioned on the stage of a Zeiss LSM 510 Axiovert confocal laser scanning microscope equipped with an Argon laser. For each experiment, cells were scanned for at least five to 10 seconds before the addition of growth factor and/or injury to establish a base line fluorescence reading. All perturbations were made while continuously scanning the cells every 789 milliseconds. A circular wound 200 to 400 μm in diameter was made and the response was recorded for a maximum of 200 seconds. To prevent the influx of extracellular Ca^{2+}, cells were incubated in either a Ca^{2+}-free HEPES-buffered saline solution (137 mM NaCl, 5 mM KCL, 4 mM $MgCl_2$, 1 mM EGTA, 25 mM glucose and 10 mM HEPES) for 30 minutes prior to use or were incubated in 10 μM $CdCl_2$ for 10 minutes prior to wounding. To prevent the utilization of intracellular Ca^{2+} stores, cells were incubated with 1 μM thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, for 30 minutes prior to imaging. To determine whether gap junctions were functioning, cells were incubated in either 2 mM 1-heptanol or 20 μM 18α-GA for 30 minutes before injury. In addition, because

photobleaching has been demonstrated to be one of the most reliable methods for determining the presence of functioning gap junctions (Lee et al., 1994), 5-CFDA AM-loaded cells (5 μM) were bleached using the Argon laser and then followed for refilling over 30 minutes.

Image analysis

To evaluate Ca^{2+} dynamics over time, changes in the average fluorescence of the entire field of cells being imaged was plotted over time. The LSM 510 Imaging Software was used to determine average fluorescence of a 512 μm × 512 μm field for each 789 millisecond time point. The data were transferred to Microsoft Excel to perform calculations and plot the graph. To calculate the percentage change in average fluorescence with respect to the first time point (F_0) reading, the following equation was applied to each 789 millisecond time point (F):

$$\text{Percent change in average fluorescence} = ((F - F_0)/F_0) \times 100 \,.$$

The results were then plotted. For experiments in which cells were wounded, the cleared region was not included when calculating average fluorescence of the field. When Ca^{2+} oscillations of individual cells were plotted over time, the specific region of interest, that is, the cell, was outlined and the equation shown above used for calculating percentage change in the average fluorescence of that cell.

Migration

Epithelial cells grown to confluency on 100 mm tissue culture dishes were quiesced for 18 hours before use. Migrations were performed using Costar Transwell inserts (6.5 mm diameter) containing tissue-culture-treated polycarbonate membranes (8 μm pore size). Binding buffer was used for both diluting growth factors and resuspending the cells and was prepared as follows: 0.05% gelatin and 25 mM HEPES in KSFM. 600 μl of binding buffer, with or without growth factor, was added to each of the bottom chambers. Pure binding buffer was used as a negative control for migration, whereas 10% FCS in binding buffer was used as a positive control. Trypsinized cells were pelleted and resuspended in a volume of binding buffer that resulted in a final concentration of 125,000 cells/100 μl, and 100 μl of the cell suspension was added to each of the top chambers. The migration was carried out at 37°C and, after eight hours, migrated cells were fixed with methanol for 10 minutes at room temperature. Nonmigrated cells were swabbed from the tops of the membranes, and after permeabilizing the migrated cells with 0.1% Triton X-100 in PBS for one minute they were stained with 5 μg/ml propidium iodide (Molecular Probes, Eugene, OR) for 10 minutes at room temperature. The polycarbonate membranes were removed and mounted onto glass slides with a drop of SlowFade Antifade (Molecular Probes, Eugene, OR). For each membrane, the total number of cells was counted in each of 10 random fields (one field covering an area of 0.37 mm^2) and an average and standard deviation calculated.

Western blot analysis

To determine whether connexins were present, cells were cultured to confluency in 100 mm tissue culture dishes and washed once with PBS. Lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, 1 mM EGTA, 0.5% N P-40, 2 mM PMSF, 2 mM Na_3VO_4) was added and the cells were removed using a cell scraper. The cell lysate was sheared using a syringe fitted with a 20 gauge needle and then centrifuged for five minutes. The supernatant was collected and protein content was determined using the BCA protein assay (Pierce, Rockford, IL) following the manufacturer's instructions. Electrophoresis was performed using 12% SDS-polyacrylamide mini-gels (1.5 mm thickness). 10 μg of protein was loaded per lane and compared to positive control cell lysates (for connexins 32 and 43) (provided by Zymed Laboratories, Inc.). Proteins were transferred to a PolyScreen PVDF membrane (NEN Life Science Products, Inc., Boston, MA) and nonspecific binding was blocked by incubating the membranes in 5% BSA in TBST overnight at 4°C. Membranes were

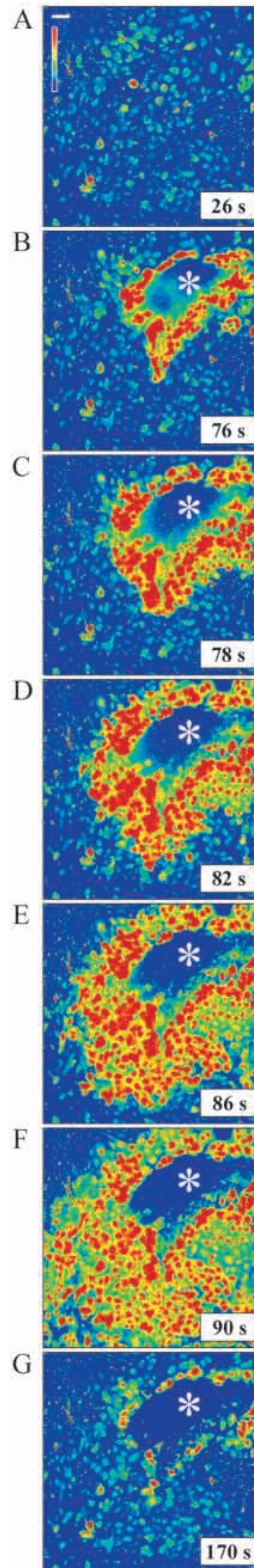

Fig. 1. Injury induces an intercellular Ca^{2+} wave. Cells were loaded with 4 μM fluo-3 AM for 30 minutes and washed two times. An initial baseline image is shown in (A). In response to a circular wound 250 μm in diameter made at 76 seconds (asterisk), cells immediately adjacent to the injury site displayed an increase in intracellular Ca^{2+} (B). The elevation in Ca^{2+} propagated as a radial pattern to neighboring cells (C-F). The response diminished over time and the intracellular Ca^{2+} levels of most cells decreased to original background levels within minutes (G). Intensity scale is shown in A, with red indicating highest Ca^{2+} levels and blue indicating lowest Ca^{2+} levels. The horizontal white bar in A represents 50 μm. Images are representative of 30 independent experiments. This series of images is taken from Movie 1 (http://jcs.biologists.org/supplemental).

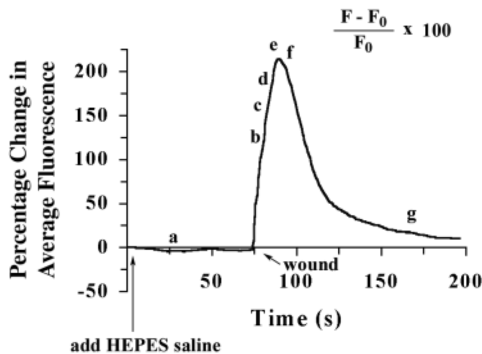

Fig. 2. Representative plot of the change in fluorescence that occurs after injury. The average fluorescence of a 512 μm × 512 μm field of cells was recorded every 789 milliseconds. Background fluorescence, F_0, was subtracted and the percentage change in fluorescence with respect to F_0 was calculated for each time point and plotted as described in Materials and Methods. The increase in fluorescence is immediate and peaks within 15 seconds of injury and then diminishes more slowly over time to original background levels within 100 seconds. The graph is labeled with letters that refer back to the images shown in Fig. 1.

probed with a primary antibody of the appropriate concentration in TBST containing 1% BSA for one hour at room temperature with gentle agitation. After washing the membranes three times (10 minutes each) in 1% BSA in TBST, they were incubated with horseradish-peroxidase-conjugated secondary antibody in TBST containing 1% BSA (Amersham Pharmacia Biotech, Piscataway, NJ). After washing the membrane with TBST, the chemiluminescence enzymatic reaction was carried out according to the manufacturer's instructions (NEN Life Science Products, Inc., Boston, MA): the blots were exposed to film and the film was developed.

RESULTS

Mechanical injury induces a Ca^{2+} wave that rapidly propagates to neighboring cells

To evaluate the changes in intracellular Ca^{2+} levels that occur immediately after mechanical injury, cells grown to confluency on glass coverslips were incubated in fluo-3 AM for 30 minutes and rinsed two times. A series of initial background images were obtained for each experiment (Fig. 1A), and then a circular wound of 250 μm in diameter was made within the field being scanned. The response was monitored. Cells directly adjacent to the injury site displayed an immediate elevation in $[Ca^{2+}]_i$ (Fig. 1B). This Ca^{2+} response traveled as a wave, moving away from the injury site in a radial pattern at a rate of 13 μm/second (this is an average of six individual experiments) (Fig. 1C-E). A decrease in intensity and wave propagation rate was observed with increasing distance from the injury site, and $[Ca^{2+}]_i$ returned to normal background levels within one to two minutes of injury (Fig. 1F,G). Furthermore, the injury-induced Ca^{2+} elevations are general to epithelial cells, as the intercellular waves propagated in parallel experiments using primary corneal epithelial cells and another human corneal epithelial cell line (gift from May Griffith, University of Ottawa Eye Institute). The overall changes in the average fluorescence of the field of cells being imaged was plotted over time (Fig. 2).

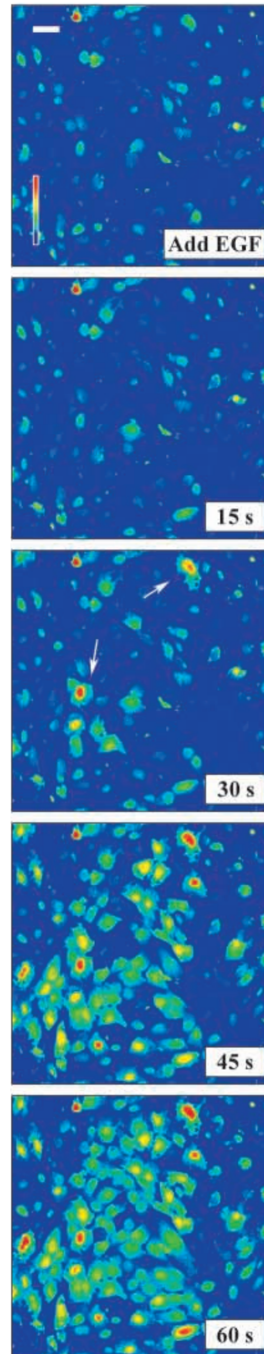

Fig. 3. EGF initiates a delayed Ca^{2+} response in non-wounded cultures. Cells were loaded with fluo-3 AM, washed and background images collected. 25 ng/ml of EGF was added, and the response was monitored for one minute. A response was not detected until 30 seconds after EGF addition (arrows). The horizontal white bar in the top figure represents 50 μm. The intensity scale is also shown, with, red indicating the highest Ca^{2+} levels, and blue indicating the lowest Ca^{2+} levels. Images are representative of 10 independent experiments.

The injury is represented by the dip in the graph and is followed by an immediate increase in $[Ca^{2+}]_i$ that diminishes slowly over time back to the original prewound levels (Fig. 2).

EGF enhances the injury-induced Ca^{2+} wave

A number of growth factors, including EGF, bind to and activate tyrosine-kinase-linked receptors and facilitate wound repair. The Ca^{2+} response to EGF is well documented and functions through receptor activation of the phospholipase C/IP_3 signaling pathway. Addition of EGF in an HEPES solution to fluo-3 AM-loaded HCE-Ts results in $[Ca^{2+}]_i$ changes as the EGF diffuses randomly across the field. Unlike the injury response, the response to EGF is not detected until 20 to 40 seconds after its addition (Fig. 3). EGF causes a dose-dependent increase in average fluorescence over time, whereas HEPES-buffered saline alone does not elicit a response (Fig. 4A). As EGF concentration was increased from 12.5 to 50 ng/ml, there was an increase in the number of cells responding, with an accompanying decrease in the delay time and an increase in the intensity of the response (Fig. 4A). The EGF-induced response was inhibited by incubating cells for two hours in 1 μM tyrphostin AG1478, an inhibitor of EGF receptor tyrosine kinase activity (Fig. 4A). The response is EGF specific, as other growth factors added at similar concentrations, such as PDGF-BB and FGF-2, failed to elicit a significant Ca^{2+} response (Fig. 4A). This was observed despite the fact that the PDGF-BB receptor, along with several of the EGF receptor types, was detected by western blot analysis (data not shown). (The presence of FGF receptors was not evaluated.) Another example of the

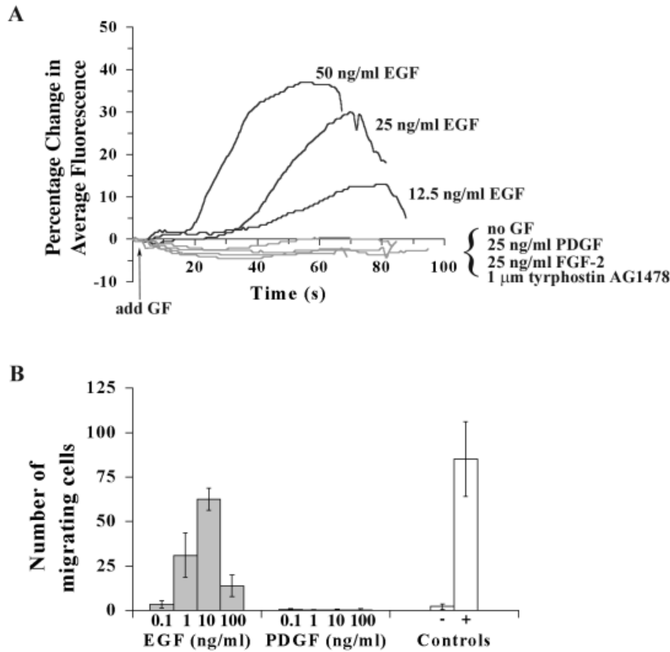

Fig. 4. EGF elicits a specific Ca^{2+} response. (A) A series of concentrations of EGF were evaluated, with images acquired every 789 milliseconds and data plotted as a percentage change in average fluorescence. The darker lines represent the response to EGF. The higher the concentration of EGF, the higher the number of cells recruited and the faster the response time. Cells incubated in 1 μM tyrphostin AG1478 for two hours before EGF addition did not respond. HEPES-buffered saline, lacking any growth factor (negative control) or containing 25 ng/ml PDGF or FGF-2, did not induce a detectable response. Images are representative of three independent experiments. (B) HCE-Ts migrated to EGF, with a maximal response seen at a concentration of 10 ng/ml EGF. In contrast, the same cells did not migrate to PDGF within the concentration range of 0.1 to 100 ng/ml PDGF. One representative experiment is shown in which the number of cells migrating per 10× field (average of 10 fields) was calculated (+/–, s.d.). Cells did not migrate in the absence of growth factor (negative control, (–)), but migrated extensively in the presence of 10% FBS (positive control, (+)).

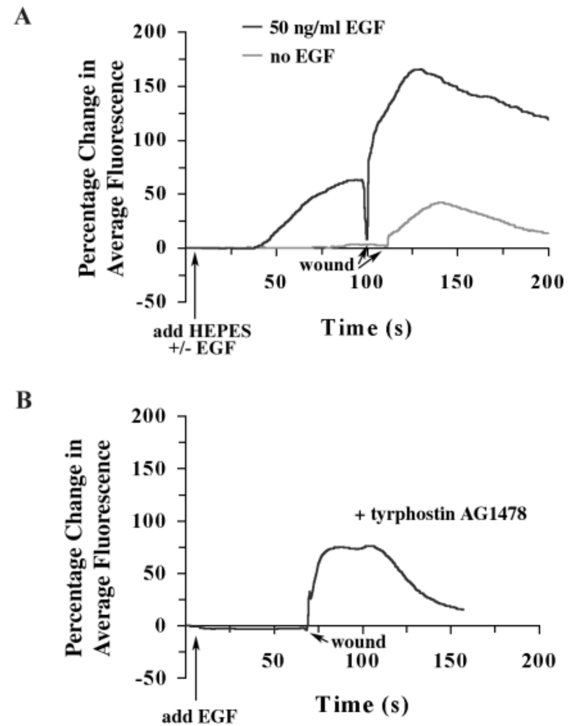

Fig. 5. EGF augments the injury-induced Ca^{2+} wave. Percentage change in average fluorescence is plotted for cells pre-treated in the presence or absence of 50 ng/ml EGF or tyrphostin AG1478. (A) Cells pretreated with EGF 90 seconds before injury produce a wave that is enhanced in duration, magnitude and peak fluorescence. (B) Cells pretreated with 1 μM tyrphostin AG1478 did not respond to EGF but did display an injury-induced wave. The graph is representative of three individual experiments.

specificity is the migration of HCE-Ts to EGF in a dose-dependent manner; HCE-Ts failed to migrate in the presence of PDGF (Fig. 4B).

To determine the effect of EGF on the injury-induced Ca^{2+} wave, fluo-3-AM-loaded cells were wounded during the EGF-induced Ca^{2+} response. Cells were wounded 60 to 100 seconds after exposure to 50 ng/ml EGF. Under these conditions, the intensity of the Ca^{2+} response to injury was enhanced four-fold. Not only was there an increase in overall amplitude and a prolonged duration of the injury response, but the percentage change from the time of the wound to the peak response was enhanced 2.5-fold (Fig. 5A). To determine whether activation of the EGF receptor was required for injury-induced propagation, cells were incubated for two hours in 1 μM tyrphostin AG1478 prior to the addition of EGF and injury (Fig. 5B). EGF was added to the cells and 60 seconds later the cells were injured. The initial response to EGF was inhibited, but a wave did propagate to surrounding cells following injury, indicating that two independent mechanisms of Ca^{2+} regulation are potentially present.

Injury-induced Ca^{2+} wave propagation depends on intracellular Ca^{2+} stores

The two major Ca^{2+} storage sites are the extracellular space and the intracellular compartments, most important of which is the endoplasmic reticulum (ER). Experiments were performed to determine the source of Ca^{2+} for the intercellular Ca^{2+} wave in our injury model. Fig. 6A is a schematic diagram of our injury model that defines the cells we describe as being immediately adjacent to the injury site. When the imaging experiments were conducted in the absence of extracellular Ca^{2+}, mechanical injury continued to produce a Ca^{2+} wave. However, cells immediately adjacent to the injury site did not participate in the response (Fig. 6B). We hypothesize that the response in the adjacent cells is mediated by a different mechanism than in the cells that are further away. In support of this, experiments conducted in the presence of either BAPTA/AM, an intracellular Ca^{2+} chelator, or thapsigargin, an inhibitor of the Ca^{2+}-ATPase pump, resulted in a different response pattern from that observed in the absence of extracellular Ca^{2+} (Fig. 6C). In the presence of thapsigargin, propagation of the injury-induced Ca^{2+} wave was completely inhibited, and the only population of cells that responded were those located along the immediate wound edge. These results indicate that cells exposed to direct mechanical injury, that is, cells along the wound edge, depend on a different source of Ca^{2+} from those that are participating in the Ca^{2+} wave at a

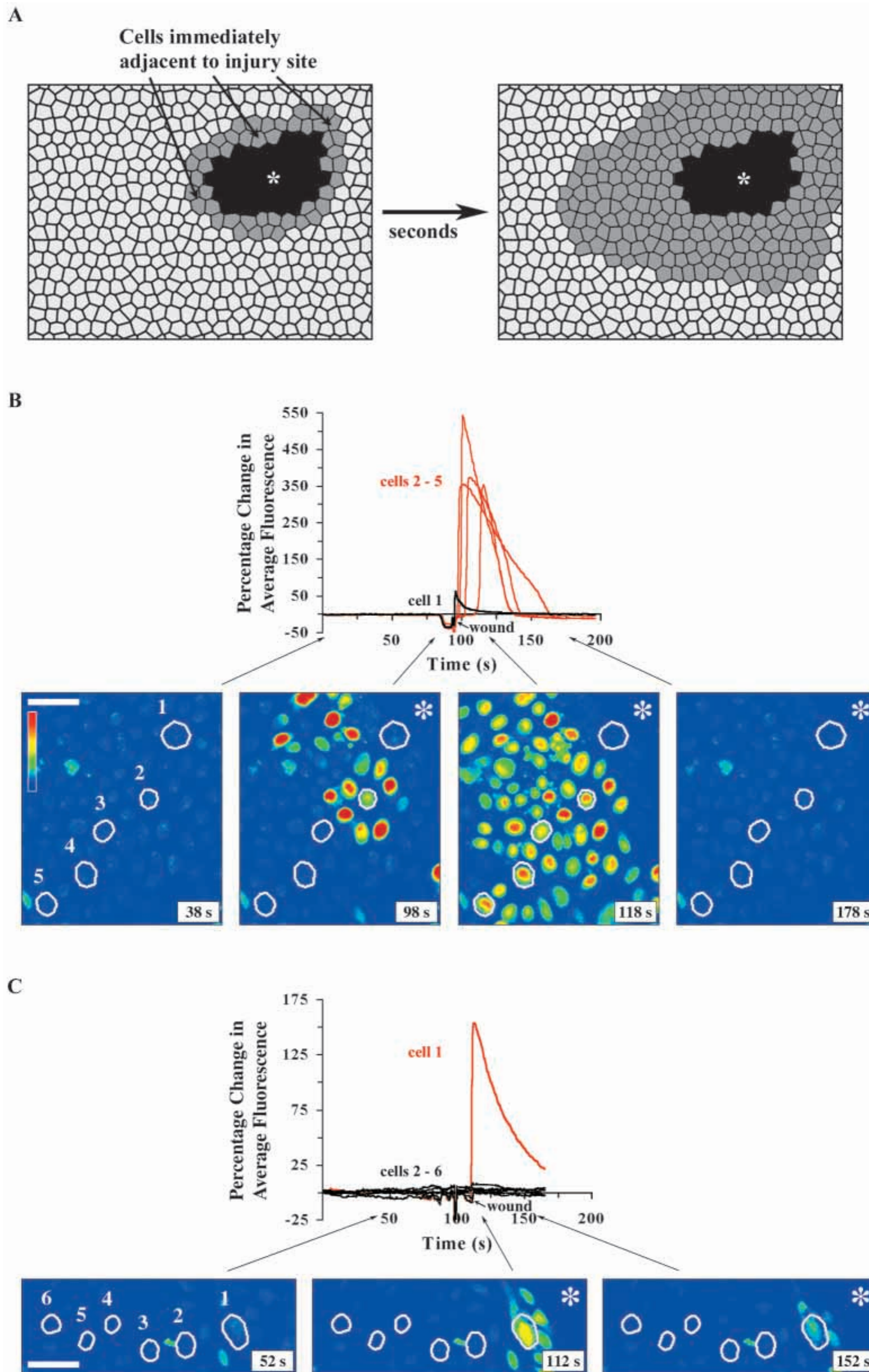

Fig. 6. Characterization of the source of Ca^{2+} during injury. (A) A schematic diagram of the wound model, illustrating that the cells located along the immediate wound edge respond first (left image), followed seconds later by propagation of a Ca^{2+} wave to neighboring cells (right image). For B and C, the average fluorescence of individual cells at various distances from the injury site (asterisk) was recorded and plotted as a percentage change in average fluorescence. (B) Absence of extracellular Ca^{2+} does not inhibit propagation of the wave but prevents an injury response, depicted as an increase in intracellular Ca^{2+}, for cells immediately adjacent to the injury site. (C) Cells treated with 1 μM thapsigargin do not exhibit propagation of a Ca^{2+} wave upon injury (asterisk). Only cells immediately adjacent to the injury site show release of Ca^{2+}. The images in B and C are taken from Movie 2 and Movie 3, respectively (http://jcs.biologists.org/supplemental). The horizontal white bar represents 50 μm. The intensity scale is shown, with red indicating the highest Ca^{2+} levels and blue indicating the lowest Ca^{2+} levels. Images are representative of five individual experiments.

more distal location. The graphs plotted in (Fig. 6B,C) illustrate the effects of these different experimental conditions on the responsiveness of individual cells located at various distances from the injury site.

Gap junctions are not involved in propagation of the injury-induced Ca^{2+} wave

Gap junctions are used by many cell types to coordinate cellular activity, therefore we evaluated whether the injury-induced Ca^{2+} wave relied on these intercellular channels. Connexins 32, 43 and 50 were detected in cell lysates from non-wounded cultures (Fig. 7). However, preincubation with either 20 μM 18α-GA or 2 mM 1-heptanol, both of which are capable of disrupting functional gap junctions, did not inhibit propagation of the Ca^{2+} wave following mechanical injury (Fig. 8A). In fact, immunocytochemistry results confirmed that

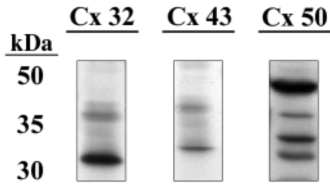

Fig. 7. Identification of connexins. 10 μg of protein was loaded into each lane of a 12% SDS-PAGE. Following electrophoresis, proteins were transferred to a PVDF membrane, and mouse monoclonal antibodies were used to probe for connexins 50, 43, or 32, all of which were detected. Positive lysates were run for connexins 43 and 32 to confirm identification. Known markers are indicated by their molecular mass (kDa) along the left edge. Results are representative of four independent experiments.

there was no significant clustering of gap junctional complexes along the plasma membrane (data not shown). A parallel set of experiments was performed using rat cortical astrocytes to test the efficacy of our system. These cells are known to propagate spiraling Ca^{2+} waves after the addition of the receptor agonist kainate (Cornell-Bell and Finkbeiner, 1991). Gap junctions are required for these spiral waves, and these waves were inhibited in the presence of 20 μM 18α-GA (data not shown). To further investigate these results, cells loaded with 5-CFDA were photobleached and followed over time. The bleached HCE-Ts did not refill over a 30 minute period, whereas the bleached rat cortical astrocyte rapidly refilled (83%), with a time for half-maximal refilling (τ) of about 65 seconds, as would be expected for cells connected by functioning gap junctions (Fig. 8B).

Additional experiments were conducted to verify that propagation did not occur via gap junctions. In one set of experiments (Fig. 8C), an initial linear wound was made in a confluent monolayer to create an acellular region 50 μm wide (see the two red lines delineating wound acellular region; Fig. 8C). The surrounding cells responded to the injury and were then allowed to recover for 30 minutes. After recovery, an injury was made (indicated by a red oval; Fig. 8C) near the linear acellular region, and the response was imaged using the protocol described in Fig. 1. This experiment enabled us to ask whether the wave could propagate across an acellular region. Briefly, after the 30 minute recovery period, background images were obtained with the linear wound in the field (Fig. 8C; 0 seconds) and then a circular wound was made nearby (Fig. 8C; 5 seconds). The injury-induced Ca^{2+} wave propagated in a normal radial pattern away from the injury site (Fig. 8C, 10 seconds and 20 seconds) and moved across the linear acellular space. In another series of experiments, subconfluent cells were injured in the same way as previously described (Fig. 1) and the wave crossed an acellular region 800 μm in length (data not shown). All of these results together demonstrate that gap junctions are not being used for wave propagation in corneal epithelial cells.

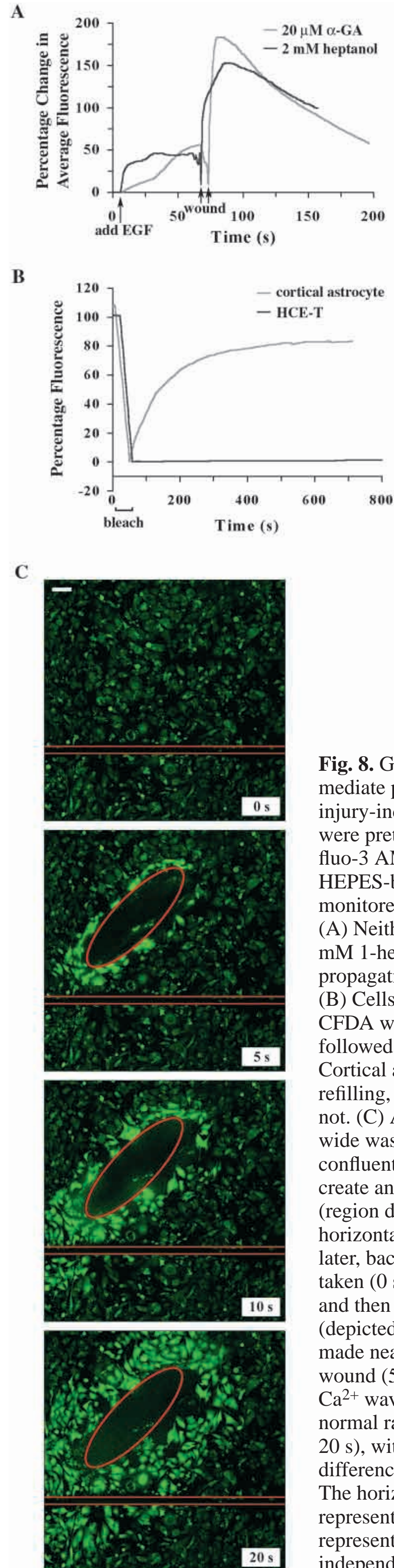

Fig. 8. Gap junctions do not mediate propagation of the injury-induced Ca^{2+} wave. Cells were pretreated and loaded with fluo-3 AM, washed with HEPES-buffered saline and monitored using CLSM. (A) Neither 20 μM α-GA nor 2 mM 1-heptanol prevented propagation of the Ca^{2+} wave. (B) Cells incubated with 5-CFDA were photobleached and followed for 30 minutes. Cortical astrocytes displayed refilling, whereas HCE-Ts did not. (C) A linear wound 50 μm wide was made within a confluent region of cells to create an acellular region (region delineated by two horizontal red lines). One hour later, background images were taken (0 s) at 10× magnification, and then a circular wound (depicted by a red oval) was made near the original linear wound (5 s). The injury-induced Ca^{2+} wave propagated in a normal radial pattern (10 s and 20 s), with no apparent difference in intensity or rate. The horizontal white bar represents 100 μm. Results are representative of three independent experiments.

Evidence for release of an extracellular soluble mediator upon mechanical injury

As gap junctions did not participate in the injury-induced Ca^{2+} wave, we tested the hypothesis that an extracellular soluble mediator was released upon injury and diffused away from the injury site. In one set of experiments, conditioned media was

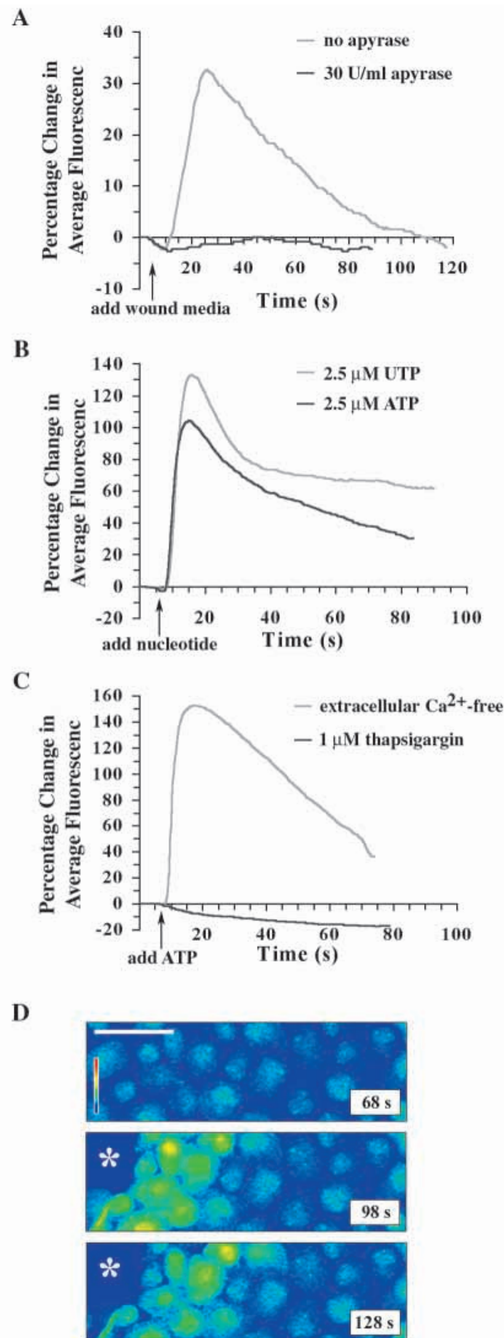

Fig. 9. Conditioned wound media induces a Ca^{2+} response that is inhibited with apyrase. (A) Conditioned media from injured corneal epithelial cells was added to fluo-3-AM-loaded epithelial cells and imaged using confocal microscopy. The response was inhibited when the wound media was treated with 30 U/ml apyrase for two minutes. (B) Cells respond to addition of 2.5 μM ATP or UTP with an immediate increase in $[Ca^{2+}]_i$. Percentage change in average fluorescence was calculated and plotted. (C) No elevation in intracellular Ca^{2+} was detected when ATP was added to non-wounded cells pretreated with thapsigargin. (D) Pretreatment of cells with 50 μM of the phospholipase-C inhibitor, U73122, prevented propagation of a Ca^{2+} wave following injury. Cells immediately adjacent to the injury site (asterisk) display an elevation in intracellular Ca^{2+}, and the injury response is similar to that exhibited by cells pretreated with 1 μM thapsigargin (see Fig. 6C). The horizontal white bar represents 50 μm. The intensity scale is shown, with red indicating highest Ca^{2+} levels and blue indicating lowest Ca^{2+} levels. Images are representative of three individual experiments.

collected from a group of HCE-Ts within seconds of the injury. When this media was added to a separate group of fluo-3-AM-loaded HCE-Ts, a significant increase in fluorescence (35%) was observed for all of the cells in the field (Fig. 9A). Because nucleotides such as ATP function as extracellular messengers, a parallel experiment was conducted where the conditioned wound media was incubated in the presence of 30 U/ml apyrase before addition to the cells. The addition of apyrase to the media inhibited any detectable elevations in intracellular Ca^{2+} (Fig. 9A). In addition, media collected immediately from injured cells possessed a 3.5 fold increase in the amount of extracellular ATP over control uninjured cells as determined using a luciferase assay.

To further validate this hypothesis, we showed that the epithelial cells respond to either ATP or UTP (Fig. 9B). The nucleotide was applied locally to a field of confluent cells, and as the nucleotide was being added there was an immediate elevation in $[Ca^{2+}]_i$. Furthermore, 2.5 μM ATP and 2.5 μM UTP caused similar increases in average fluorescence (104% and 132%, respectively). These results suggest that propagation of the Ca^{2+} wave could occur through activation of the purinergic receptor signaling pathway. There are two major classes of purinergic receptors (Harden et al., 1995), and because only pretreatment of unwounded epithelial cells with thapsigargin inhibited the Ca^{2+} response to ATP, we conclude that these cells express the P2Y receptor subtype (Fig. 9C). Furthermore, experiments that were conducted using the phospholipase C inhibitor U73122 (50 μM) resulted in an injury-induced Ca^{2+} wave similar to that observed for thapsigargin-treated cells after injury, where the Ca^{2+} wave did not propagate and only cells immediately adjacent to the wound edge displayed an increase in $[Ca^{2+}]_i$ (Fig. 9D, Fig. 6C). Together these data suggest that mechanical injury causes release of ATP and activation of P2Y purinergic receptors.

DISCUSSION

Proper closure of an epithelial wound requires neighboring cells to be aware of the injury. Intercellular communication is therefore required to coordinate cellular activities in the vicinity of the injury to produce the changes responsible for wound repair, such as migration and proliferation. Ca^{2+}-mediated signal transduction is often employed to translate extracellular stimuli into these functional cellular processes (Berridge and Dupont, 1994). In this study we investigated the Ca^{2+} response following mechanical injury in a human corneal epithelial cell line by focusing on the causes and sources of the $[Ca^{2+}]_i$ elevation, as well as on the mechanism of signal propagation to neighboring cells. We observed Ca^{2+} waves similar to those described for a number of other cell types. However, although many of these previous reports involved gentle stimulation or mechanical stretching of a single cell without causing membrane damage (Charles et al., 1991; Demer et al., 1993; Sigurdson et al., 1993; Boitano et al., 1994; Enomoto et al., 1994; Sanderson et al., 1994), this paper

describes the Ca^{2+} response to 'gross injury' that involves removal or clearing of a region of cells (Hinman et al., 1997; Sammak et al., 1997).

Our goal was to evaluate Ca^{2+} signaling resulting from mechanical injury to determine how Ca^{2+} wave propagation is mediated. Creating a circular wound 200 to 400 μm in diameter resulted in an immediate transient intercellular Ca^{2+} wave that traveled away from the injury site. The source of Ca^{2+} was determined to be both extracellular and intracellular (ER) stores. Specifically, when cells were incubated in Ca^{2+}-free HEPES-buffered saline the increase in $[Ca^{2+}]_i$ at the wound edge depended on extracellular Ca^{2+} potentially entering through leaky membranes created by physical injury or by the opening of stretch-activated Ca^{2+} channels in the plasma membrane. However, the presence of the Ca^{2+} wave under these extracellular Ca^{2+}-free conditions suggests that wave propagation relies on intracellular Ca^{2+} stores and that the propagation is not dependent on $[Ca^{2+}]_i$ changes that occur in cells at the immediate wound edge. The fact that the mechanically stimulated cells obtain extracellular Ca^{2+} whereas the wave relies on intracellular Ca^{2+} is consistent with theories that the injury response is a two-phase process (Boitano et al., 1994; Sanderson et al., 1994; Hansen et al., 1995; Frame and de Feijter, 1997; Venance et al., 1997).

One potential mechanism for wave propagation is via diffusion of an intracellular mediator through gap junction complexes. This mechanism occurs in a variety of cell types, including articular chondrocytes (D'Andrea and Vittur, 1996), airway epithelial cells (Hansen et al., 1993; Boitano et al., 1994), glial cells (Charles et al., 1992; Venance et al., 1997), smooth muscle cells (Christ et al., 1992; Young et al., 1996) and endothelial cells (Drumheller and Hubbell, 1991). There is some controversy as to whether Ca^{2+} or another messenger, such as IP_3, passes through gap junctions to create the wave (Boitano et al., 1992; Sanderson et al., 1994; D'Andrea and Vittur, 1997; Jorgensen et al., 1997; Grandolfo et al., 1998). However, evidence for a second mechanism emerged when it was demonstrated that waves could cross acellular regions and that fluid flow could affect propagation of the wave (Enomoto et al., 1992; Hassinger et al., 1996; Sammak et al., 1997; Guthrie et al., 1999). Thus the injury could mediate a signal that induced diffusion of an extracellular messenger released from the damaged cells. Interestingly, propagation via this mechanism was discovered in some cells that lacked gap junctions (Frame and de Feijter, 1997). Furthermore, there is evidence in some culture systems that both mechanisms co-exist, simultaneously and independently (Enomoto et al., 1994; Frame and de Feijter, 1997; Sammak et al., 1997; Braet et al., 2001).

Our results suggest that the Ca^{2+} wave in HCE-Ts or primary epithelial cells does not require gap junctions but depends on a diffusable extracellular factor. This conclusion is based on experiments we performed using gap junction inhibitors, FRAP analysis and wave propagation across an acellular region (Fig. 8). Even incubation in EGTA for 30 minutes, which resulted in rounding-up of the cells and loss of cell-cell contact, did not inhibit wave propagation. Although functioning gap junctions are known to be present in the corneal epithelium in vivo (Dong et al., 1994; Williams and Watsky, 1997), several endogenous and exogenous factors, such as low or high $[Ca^{2+}]_i$, may modulate intercellular communication through gap junctions in cultured cells (Goodenough et al., 1996; Jansen et al., 1996; Frame and de Feijter, 1997).

The identification of the extracellular messenger is currently being investigated. A number of soluble factors produced by cells mobilize Ca^{2+}, among them nucleotides, glutamate, acetylcholine and growth factors. ATP causes Ca^{2+} waves in glial cells (Centemeri et al., 1997; Guthrie et al., 1999), mast cells (Osipchuk and Cahalan, 1992), osteoblasts (Jorgensen et al., 1997), chondrocytes (D'Andrea and Vittur, 1996), airway epithelial cells (Hansen et al., 1993) and mammary epithelial cells (Enomoto et al., 1994). Corneal epithelial cells used in this study responded to both ATP and UTP, and our experiments and those of others using apyrase suggested ATP as a potential candidate (Guthrie et al., 1999). ATP can function as an extracellular messenger for a variety of cellular processes in both excitable and nonexcitable cells (Burnstock, 1997; Neary et al., 1999). By binding the P2Y purinergic receptor, ATP initiates an intracellular signaling cascade that begins with the synthesis of IP_3 by the action of phospholipase C and then release of Ca^{2+} from intracellular storage sites (Harden et al., 1995; North and Barnard, 1997). This IP_3-mediated mechanism is consistent with our results showing that thapsigargin inhibits propagation of the Ca^{2+} wave. In addition, our results suggest that an extracellular messenger is released upon cell damage. But in cases where cell damage does not occur, active secretion of the messenger must occur. This has important implications for understanding whether the wave is generated by release of the messenger from a single point source or by a regenerative mechanism in which there is sequential secretion of the messenger from cells along the path of the Ca^{2+} wave (Osipchuk and Cahalan, 1992; Sneyd et al., 1994; Frame and de Feijter, 1997; Guthrie et al., 1999).

It will also be important to coordinate the knowledge obtained from these studies with the information that growth factors such as EGF play a significant role in epithelial wound repair (Leibowitz et al., 1990; Moulin, 1995; Steed, 1998). Addition of EGF to control HCE-Ts results in elevation of $[Ca^{2+}]_i$ as the EGF diffuses across the field, but this response is not immediate (Fig. 3) and may take 20 to 40 seconds to detect. This delay may be due to the fact that receptor dimerization is required for EGF-receptor activation, resulting in the release of intracellular Ca^{2+} stores through a phospholipase C/IP_3-mediated mechanism. In contrast, we demonstrated that rapid propagation of the injury-induced Ca^{2+} wave is independent of the activation of EGF receptors by wounding cells after they were incubated in tyrphostin AG1478 (Fig. 5B).

Although activation of the EGF receptor is not required for wave propagation, there is a synergistic relationship between the addition of EGF and the amplitude of the Ca^{2+} wave when the wound is made during EGF-induced $[Ca^{2+}]_i$ elevation. The fact that the Ca^{2+} response to EGF cannot be mimicked by other growth factors that bind tyrosine kinase receptors, such as PDGF-BB, suggests some specificity. Furthermore, lacritin, a novel secretion enhancing factor isolated from the lacrimal gland, also induces a distinct wave form in corneal epithelial cells that is not a typical target wave (Sanghi et al., 2001). This is important when one considers that the corneal epithelium is bathed in tear fluid, rich in a variety of soluble factors, many of which become elevated following corneal injury (Sheardown and Cheng, 1996; Vesaluoma et al., 1997a;

Vesaluoma et al., 1997b). Other studies in our laboratory have concluded that addition of exogenous EGF results in faster wound closure as well as in an increase in integrin β4 receptor in cultured corneal epithelial cells (Song et al., 2001). We believe that this result provides evidence that enhancement of the injury-induced early Ca^{2+} response by the presence of EGF may have important longer term effects on wound repair. This may be especially important for certain pathologies, such as diabetes, in which cells are shown to be less responsive to growth factors (Embil et al., 2000; Ladin, 2000).

Finally, it is important to consider the biological significance of an elevation in $[Ca^{2+}]_i$ that travels as a wave. There are numerous cytosolic proteins that alter their activity after binding to Ca^{2+}. In addition to cell type and surrounding environment, the timing, duration, frequency and amplitude of Ca^{2+} oscillations most probably play a role in determining which specific signaling pathways are activated following injury. Signaling pathways that lead to changes in expression, localization or activity of proteins involved in cellular adhesion, migratory ability and proliferation are candidates to be investigated. Studies have shown that Ca^{2+} may regulate motility (Brundage et al., 1991; Gilbert et al., 1994), proliferation (Byron and Villereal, 1989; Wahl and Gruenstein, 1993; Means, 1994), differentiation, and secretion (Marks and Maxfield, 1990). We have also shown that initial contact of corneal epithelial cells with a substrate is accompanied by Ca^{2+} oscillations (Trinkaus-Randall et al., 2000), and these results are supported by the more recent adhesion studies of Juliano (Short et al., 2000). The data presented and discussed here suggest a potentially significant role for the Ca^{2+} wave in coordinating the cellular processes important for wound repair.

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