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LABORATORY INVESTIGATIONS

Adenosine-5′-triphosphate-induced Intracellular Calcium Changes through Gap-junctional Communication in the Corneal Epithelium

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Purpose: To investigate intercellular communication in the corneal epithelium, changes in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) induced by adenosine-5′-triphosphate (ATP) were analyzed.

Methods: Rabbit corneal epithelia were loaded with Ca^{2+} indicators and then stimulated by ATP (10 μM). $[Ca^{2+}]_i$ changes were monitored by means of conventional fluorescence and real-time confocal microscopy. The localization of connexin 50 (Cx50) was also immunohistochemically examined.

Results: ATP was found to induce $[Ca^{2+}]_i$ increase, which was often synchronized among adjacent cells. When wing cells were topically stimulated by treatment with ATP, the $[Ca^{2+}]_i$ increase spread to adjacent wing cells, but significant increase in $[Ca^{2+}]_i$ in the superficial and the basal cell layers was not observed. The propagation of the $[Ca^{2+}]_i$ change was suppressed in the presence of octanol (1 mM), a gap junction inhibitor. Immunohistochemical staining of Cx50 was strongly detected in the wing and basal cells, but faintly in the superficial cells.

Conclusions: There was intercellular communication accompanied by $[Ca^{2+}]_i$ changes in wing cell layers, but not in superficial or basal cell layers. The difference in $[Ca^{2+}]_i$ changes among the layers of the corneal epithelium suggests that the response of each cell layer plays a distinct role in proliferation and differentiation during recovery of the corneal epithelium. **Jpn J Ophthalmol 2002;46:479–487** © 2002 Japanese Ophthalmological Society

Key Words: Adenosine-5′-triphosphate, corneal epithelium, gap junction, intercellular communication, intracellular calcium.

Introduction

The cornea serves to protect the optical organ from the external environment. To maintain clear vision, it is important that the surface of the cornea remain smooth, so that light is uniformly refracted, and that defects can be resurfaced by an active repairing process. The corneal epithelium, in particular, has exceptional regenerative power when injured. Cell-to-cell communications play important roles in the maintenance of the stratified structure, as well as the regeneration of the corneal epithelium. A better understanding of intercellular communication would be predicted to shed light on the mechanism of proliferation, and the differentiation of epithelial cells.

Adenosine-5′-triphosphate (ATP), the principal high-energy intermediate compound in living cells, is also found in extracellular spaces. For example, nerve endings in most peripheral nervous systems have been found to secrete ATP, and neurotransmitters and injured cells are also known to leak ATP.[1] It has been reported that extracellular ATP induces a change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a variety of cells, including the corneal epithelium.[1–3] Changes in $[Ca^{2+}]_i$ via the Ca^{2+} channel of plasma membrane and/or release from the internal Ca^{2+}-store (eg, sarcoendoplasmic reticulum) play a role in intracellular signaling and the regulation of cellular activities.[4] Therefore, extracellular ATP is capable of acting as

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an extracellular messenger, and, thus, could stimulate the corneal epithelium at the time of injury.

We previously reported that ATP induces oscillatory fluctuations of $[Ca^{2+}]_i$ in the corneal epithelium, and that these oscillations were sometimes synchronized among adjacent cells in the mid-wing cell layer of the epithelium.[2] These findings suggest that the synchronization of oscillatory fluctuations of $[Ca^{2+}]_i$ might well be related to the gap junction, as has been indicated in other tissues,[5–7] and which is thought to be of fundamental importance in cell-to-cell coupling.[8] Intercellular communication coordinates the regulation of cellular growth, development, and differentiation.[9–11] The focus of the present study was to examine the issue of whether intercellular communication as the result of changes in $[Ca^{2+}]_i$ via gap junctions occurs in the corneal epithelium. To this end, corneal epithelium was treated with octanol, which is known to be a nonspecific inhibitor of gap junction permeability in a variety of cells.[7,12,13]

The stratified corneal epithelium is composed of a superficial cell layer, a wing cell layer in the mid region, and a basal cell layer, located on the stroma. It is possible that each cell layer plays a distinct role in the maintenance of structure and function in the corneal epithelium.[14] To investigate intercellular communication in each layer of the epithelium, we also determined $[Ca^{2+}]_i$ changes in epithelium that had been stimulated topically by ATP. This was accomplished by means of confocal microscopy, a technique that shows good spatial resolution. Gap junctions are comprised of connexins, an extended family of related polypeptides.[8] Hence, differences in $[Ca^{2+}]_i$ changes in layers with differences in the localization of connexin were examined.

Materials and Methods

Experiments were conducted according to the guidelines of the ethics committee for animal treatment of Iwate Medical University, Morioka, Japan, and the ARVO statement on the care and use of animals in vision research.

Preparation of Corneal Epithelium

This procedure was performed as described in a previous report.[2] Briefly, an epithelial sheet was obtained by dispase digestion from the cornea of an adult male rabbit (JW; weighing 2.0–2.5 kg). The epithelial sheet was then gently dissected free of the stroma and disrupted by pipetting in HEPES-buffered Ringer's solution (HR). Experiments were performed within 12 hours after tissue isolation.

The standard HR (pH 7.4, adjusted with NaOH) contained 118 mM NaOH, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.13 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM D-glucose, 2.0 mM sodium glutamate, 10 mM HEPES, MEM amino acid solution (Gibco, Grand Island, NY, USA) and 0.2% bovine serum albumin (Sigma, St. Louis, MO, USA). HR was oxygenated, before use.

Loading of Ca^{2+}-sensitive Dyes

Spatiotemporal changes in the $[Ca^{2+}]_i$ of the corneal epithelium were determined by ratiometrical measurements using the fluorescent dyes, Fura-2 and Indo-1 (Dojindo Laboratories, Kumamoto), which are Ca^{2+} indicators.[2,3,15] The excitation and emission spectra of both Fura-2 and Indo-1 are shifted on binding to Ca^{2+}. These spectral shifts make it possible to use them in the measurement of Ca^{2+} concentrations. The quantitation of $[Ca^{2+}]_i$ via ratiometric measurement is essentially not affected by differences in dye distribution or photo-bleaching.

Epithelial specimens were placed on glass cover slides coated with Cell-Tak (a nontoxic adhesive reagent; Collaborative Research, Bedford, MA, USA). The slides were placed in a perfusion chamber. Acetoxymethyl esters (Fura-2/AM and Indo-1/AM) and a detergent (cremophor EL; Nakalai tesque, Kyoto) facilitated dye-loading. The specimens were loaded with 1 μM Fura-2/AM or 10 μM Indo-1/AM with 0.02% cremophor EL by incubation in the above described HR for 45 minutes at room temperature (20°–25°C). Specimens that were loaded with Ca^{2+} indicators were then perfused with the oxygenated HR at room temperature for observation of $[Ca^{2+}]_i$ changes.

ATP-induced $[Ca^{2+}]_i$ Change in the Epithelium with Fura-2/AM

The samples loaded with Fura-2 were then stimulated by replacing the standard HR with HR containing ATP (10 μM). $[Ca^{2+}]_i$ changes in the corneal epithelium loaded with Fura-2/AM were observed by ratiometry, in conjunction with a dual wavelength excitation technique.[2,16] Briefly, $[Ca^{2+}]_i$ changes in a Fura-2-loaded specimen were monitored using a conventional inverted microscope (IX 70 with an U Apo 40/340, N.A. 0.90 objective; Olympus, Tokyo) and a digital image processor (Argus-50/CA; Hamamatsu Photonics, Hamamatsu). Time courses for $[Ca^{2+}]_i$ in certain areas were analyzed based on consecutive digital ratio images. Areas of about 4 μm^2 of the

image were identified, and $[Ca^{2+}]_i$ was plotted as a function of time for each of these sites. However, it was difficult to distinguish individual cell profiles, because of the fluorescent flare of the image.

ATP-induced $[Ca^{2+}]_i$ Change of the Epithelium with Indo-1/AM

The specimens that were loaded with Indo-1 were topically stimulated by ATP (10 μM: minimum concentration to induce evident response of the cell). A microinjector (Eppendorf 5254; Hamburg, Germany) with a manipulator (Eppendorf 5191) was used for this procedure.

Specimens were slightly dome-shaped, and the superficial and basal cell layers showed convex and concave profiles, respectively (Figure 1). The superficial cell layer largely covered the dome-shaped specimens. The periphery of the fluorescent images mainly represented the superficial cell layer when the focal plane was adjusted to the wing cell layer of the epithelial sheet.[2,3] Some wing cells were localized at the peripheral regions. The previous studies showed that the intercellular communication was mainly thought to be in the wing cell layer.[2,3] Therefore we aimed to confirm the propagation of $[Ca^{2+}]_i$ changes from wing cells to wing cells and/or to other cell layers. For this purpose, the injector tip was positioned near a wing cell, which appeared as a round profile in a fluorescent image. The basal layer was not directly exposed to the perfusion solution. Therefore we could not position the injector tip near basal cells and could not confirm cell-to-cell communication in basal cell layers.

Stimulation fluid (less than 0.1 μL) containing ATP was ejected at 1,000 hpa. In a preliminary observation, the diffusion of the fluid was restricted to an oval region (about 10 × 30 μm in axis). The size of the epithelial cells was about 10–20 μm in diameter. Therefore, the ATP containing HR stimulated only a few cells in the immediate vicinity of the injector tip.

A confocal ultraviolet (UV)-laser scanning microscope (RCM/Ab; Nikon, Tokyo) was used to visualize $[Ca^{2+}]_i$ changes in individual epithelial cells in the specimens that had been loaded with Indo-1/AM.

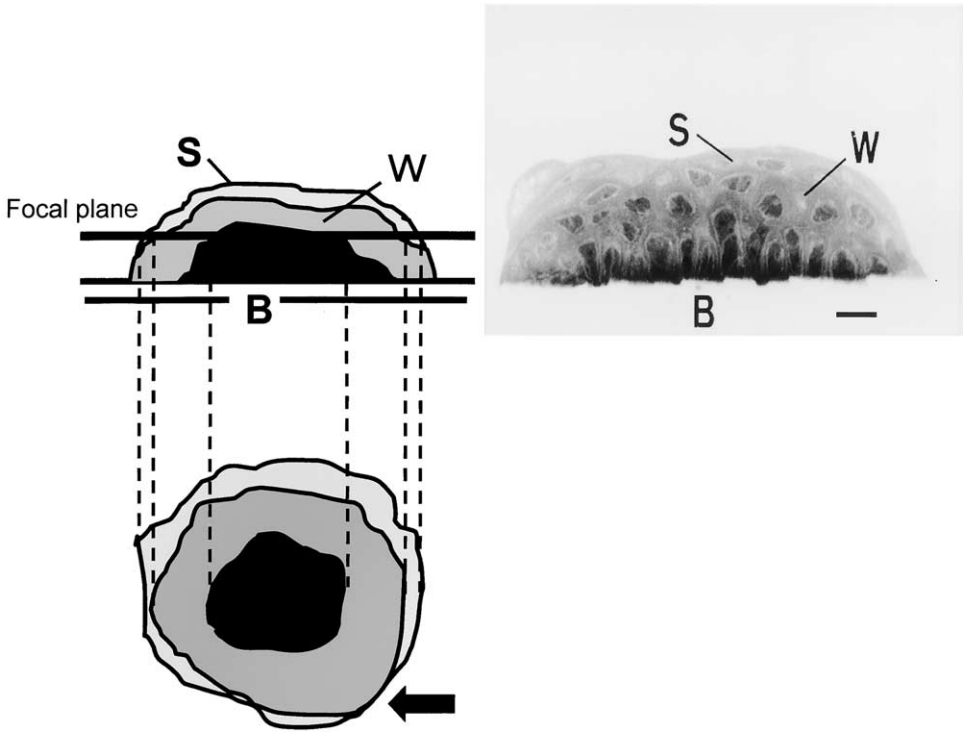

Figure 1. Light micrograph and diagram of the epithelial sheet from rabbit cornea for $[Ca^{2+}]_i$ measurement. Epon semithin sections were stained by toluidine blue. The sheet is composed of superficial (S), wing (W), and basal (B) cell layers. The diagram illustrates the relationship between a transverse profile and a fluorescent image of the specimen. Peripheral, mid, and central zones of the fluorescent image represent superficial, wing, and basal cell layers, respectively. We used the specimen in which wing cells were partly at the periphery, when the wing cell layer of the specimen was stimulated topically by adenosine-5′-triphosphate (thick arrow). Bar = 10 μm.

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Details of the use of confocal microscopy for $[Ca^{2+}]_i$ measurement have been reported previously.[3,17,18] Briefly, the specimens were irradiated with a UV beam (351 nm) generated by an argon ion laser for excitation. The fluorescence emission was guided through a water-immersion objective (Nikon C Apo 40×, N.A. 1.15) to a pinhole diaphragm. A dichroic mirror split the emission at 440 nm, and emissions below 440 nm and above 440 nm were directed into separate photomultipliers. Real time images (15–30 frames/s) were stored in a high-speed hard drive in RCM/Ab, and a ratio image from each pair was then computed, as the fluorescence intensity less that 440 nm to that greater than 440 nm. A higher ratio is indicative of a higher $[Ca^{2+}]_i$. Ratio images were displayed in pseudocolor with 256 colors; with red representing a high $[Ca^{2+}]_i$ and blue and green a low $[Ca^{2+}]_i$.

Three-dimensional image analyses were attempted for $[Ca^{2+}]_i$ changes. In order to observe consecutive changes in $[Ca^{2+}]_i$ in different layers of the corneal epithelium, the stage of the microscope was periodically moved along the z-axis (10–20 μm in depth). We adjusted the uppermost focal plane to the surface of the epithelial cell layer and the lowermost to the basal cell layer, but an image of any given plane contained various cell types, because the specimens were deformed, as described below. Areas comprising about 4 μm^2 of an image were identified, and $[Ca^{2+}]_i$ changes in these regions were recorded.

Photo-bleaching as the result of laser beam scanning was relatively rapid. Within a few minutes of scanning, the fluorescence was too weak to be detected by the photo-multipliers, in contrast to digital imaging using Fura-2/AM, as described above. In the case of imaging using confocal microscopy, $[Ca^{2+}]_i$ changes in epithelial tissue must be examined under defined conditions for a short period, at the expense of image sharpness.

Immunohistochemistry for Light Microscopy

The rabbit corneas were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and cryosections of the corneas (about 15 μm thick) were prepared with a microtome. The sections were incubated for 3 days at 4°C with a primary mouse monoclonal antibody to connexin 50 (Cx50; Zymed, San Francisco, CA, USA) at a dilution of 1:50. After rinsing with PBS, they were then processed using the Streptavidine-Biotin method (SAB-PO kit; Nichirei, Tokyo). Immunoreactivity was identified by 3,3-diaminobenzidine tetrahydrochloride staining.

Results

Structure of the Specimen of Corneal Epithelium

$[Ca^{2+}]_i$ levels in the corneal epithelium that retained essential structure indicative of superficial, mid-wing, and basal cell layers were measured (Figure 1). The viability and structural integrity of the specimens processed in this manner have been confirmed previously.[2] Briefly, to confirm the viability of an epithelial sheet, some specimens were incubated in HR containing 1 μM ethidium homodimers-1 (EthD-1; a fluorescent dye that binds to DNA). As a result, few cells of the specimens emitted fluorescence after the incubation in EthD-1. This suggested that the epithelial sheets preserved the transport mechanism of the cell membrane. Electron microscopy revealed normal ultrastructure in the specimens.

ATP-induced $[Ca^{2+}]_i$ Changes in Corneal Epithelium

Overall $[Ca^{2+}]_i$ changes in the corneal epithelium were observed by a digital imaging method using conventional fluorescence microscopy, and the results were consistent with our previous study.[2] Briefly, extracellular ATP (10 μM) caused a biphasic response of $[Ca^{2+}]_i$, with the first phase consisting of a rapid increase in $[Ca^{2+}]_i$ (spike phase) and the second consisting of a sustained $[Ca^{2+}]_i$ increase (plateau phase) with oscillatory fluctuation (Figure 2). The $[Ca^{2+}]_i$ fluctuations in different areas were sometimes synchronized. However, a time course of $[Ca^{2+}]_i$ change did not always represent single cell response, because the fluorescent flare from cells in the out-of-focus plane overlay the mid wing cell layer.

Propagation of ATP-induced $[Ca^{2+}]_i$ Changes in Wing Cell Layer

Optical slicing by confocal microscopy eliminated fluorescent flare. Hence, each cell profile was clear in a confocal image. Flat cells that were located in the peripheral region of the image were superficial cells, and the oval/round cells were wing cells. Basal cells were also round, and located in the central region of the image. The extent of dye loading of basal cells was often too faint to analyze, and only bright cells were used for quantitation.

To stimulate wing cells directly, we selected tissue specimens in which the wing-cell layer was exposed to the perfusion fluid, in an optical sliced image. when ATP (10 μM) containing HR was ejected from the tip, the $[Ca^{2+}]_i$ of wing cells near the ejector tip was found to be increased, and the increase was then observed to

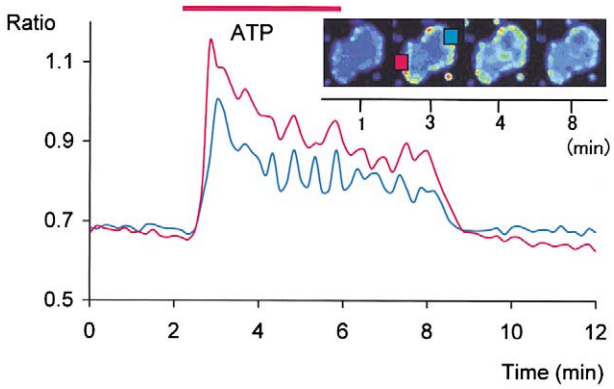

Figure 2. Pseudocolor images and time course of adenosine-5′-triphosphate (ATP)-induced $[Ca^{2+}]_i$ changes in the rabbit corneal epithelium under conventional fluorescence microscopy. Focal plane was adjusted at the mid wing cell layer. Peripheral cells were superficial and mid wing cells. At the resting stage, the $[Ca^{2+}]_i$ level was maintained at a low level. When ATP (10 μM) was added to the perfusion solution, $[Ca^{2+}]_i$ was found to increase. Red and blue lines of the graph represent $[Ca^{2+}]_i$ of certain areas (about 4 μm^2; shown by red and blue squares in the digital image). The ATP-induced Ca^{2+} response was biphasic: initial phase showing an acute increase, and the second plateau phase maintaining a high $[Ca^{2+}]_i$ level. The oscillatory fluctuations in the plateau phase were sometimes synchronized among the adjacent regions in the wing cell layer.

propagate successively to adjacent cells (n = 10), ultimately to numerous wing cells of the specimens (Figure 3). The $[Ca^{2+}]_i$ changes in two cells that were located at positions distant from one another were synchronized; the start of the initial phase and the decline pattern of the plateau phase were the same for most cells examined (n = 10) (Figure 4).

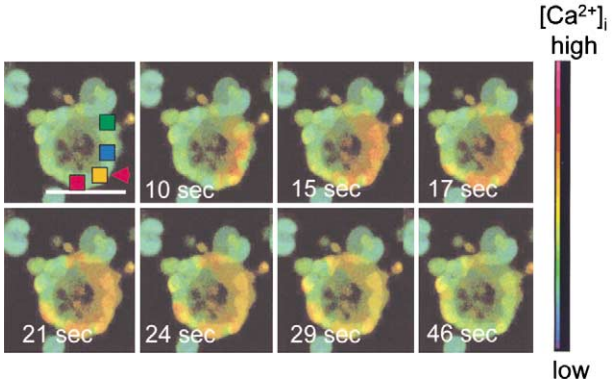

Figure 3. Pseudocolor images of adenosine-5′-triphosphate (ATP)-induced $[Ca^{2+}]_i$ changes in the rabbit corneal epithelium by real-time confocal microscopy. Each cell profile is evident. The focal plane was adjusted at the wing cell layer. Wing cells of the specimen were stimulated topically by ATP (red arrow). Bar = 100 μm. Times after topical application of ATP are depicted in the images.

Propagation of $[Ca^{2+}]_i$ Changes from Wing Cells to Other Cell Layers

To observe the propagation of ATP-induced $[Ca^{2+}]_i$ changes from the wing cell layer to the surface and basal cell layers, the fluorescent images of different focal planes were acquired three dimensionally. Although the stepping motor that moves the specimen stage along the Z-axis is too slow to acquire real-time images, the minimum time interval (2 frames/s) for image acquisition in each plane was sufficient to allow observation of changes in $[Ca^{2+}]_i$ of the epithelial cells.

After topical stimulation on a wing cell by ATP, the $[Ca^{2+}]_i$ increase spread to other wing cells, but the increases in $[Ca^{2+}]_i$ of superficial or basal cell layers (n = 10) were faint (Figures 5A, B, C). Thus the intercellular propagation of $[Ca^{2+}]_i$ changes within wing cell layers occurred, but the propagation of changes to other cell layers was limited.

Effects of Octanol

To determine whether the gap junction was involved in the synchronization and propagation of $[Ca^{2+}]_i$, specimens were pretreated with octanol (1 mM). By fluorescence microscopy, it was shown that octanol inhibited the plateau phase of the ATP-induced $[Ca^{2+}]_i$ increase, but not the spike phase (data not shown). Confocal mi-

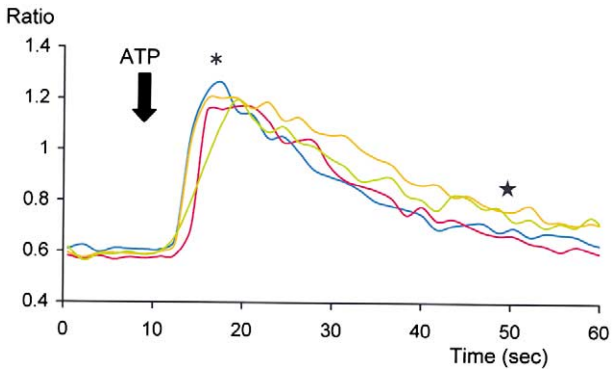

Figure 4. Time course for $[Ca^{2+}]_i$ changes in the wing cell layer by topical adenosine-5′-triphosphate (ATP)-stimulation under confocal microscopy. Four colored lines represent $[Ca^{2+}]_i$ changes of certain areas (about 4 μm^2; shown in colored squares in Figure 3). The specimens were stimulated topically by ATP (arrow). ATP-induced $[Ca^{2+}]_i$ changes were biphasic, the same as observed by fluorescence microscopy. Synchronization at the initial phase (*) and the plateau phase (★) of the four cells is evident, although they were located at distant positions.

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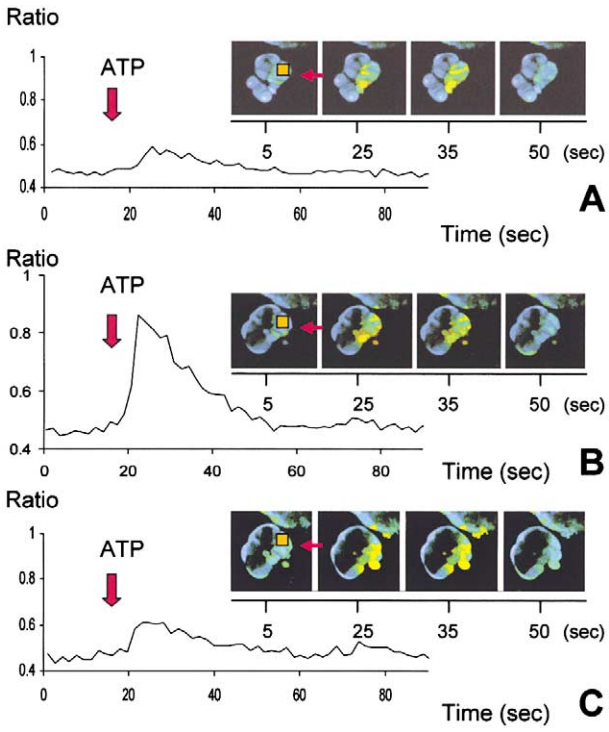

Figure 5. Time course of $[Ca^{2+}]_i$ changes in different layers of the corneal epithelium. Wing cells were topically stimulated by adenosine-5′-triphosphate (ATP; 10 μM). (**A**) $[Ca^{2+}]_i$ changes in the superficial cell layer [about 10 μm above (**B**)] was not prominent. (**B**) $[Ca^{2+}]_i$ changes in the wing cell layer propagated to adjacent wing cells. (**C**) $[Ca^{2+}]_i$ changes in the basal cell layer [about 10 μm under (**B**)] were not extensive.

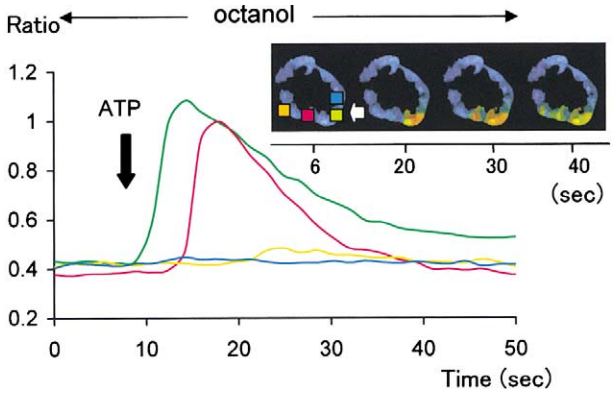

Figure 6. Time course for adenosine-5′-triphosphate (ATP)-induced $[Ca^{2+}]_i$ changes under confocal microscopy, showing the effect of gap junction inhibitor. Four colored lines represent $[Ca^{2+}]_i$ changes of certain areas in colored squares (about 4 μm^2). The specimens were stimulated topically by ATP (arrow). Octanol (1 mM) inhibited the synchronization of $[Ca^{2+}]_i$ changes of the initial phase and the plateau phase, even though the four observed cells were adjacent. No oscillatory fluctuation was observed.

croscopy revealed that octanol suppressed the propagation of an ATP-induced $[Ca^{2+}]_i$ increase from stimulated cells, although a few cells responded (Figure 6), resulting from diffusion of the ATP-solution, as described in Materials and Methods. No synchronization of $[Ca^{2+}]_i$ changes was observed (n = 10). Oscillatory fluctuations also disappeared.

Detection of Cx50

Cx50 immunostaining was observed in the three layers. However, the staining of the superficial cell layer was faint. Wing cells showed the strongest level of staining at the cell boundaries in all layers of the corneal epithelium (Figure 7).

Discussion

The present study demonstrates that the gap junction might well be related to the propagation of the $[Ca^{2+}]_i$ increase in the corneal epithelium, as stimulated by extracellular ATP. In addition, a difference in $[Ca^{2+}]_i$ changes in the different layers of the epithelium was clearly detected.

Possibility of Intercellular Communication in ATP-induced $[Ca^{2+}]_i$ Increase

Extracellular ATP could affect intracellular signaling linked to $[Ca^{2+}]_i$ changes in the rabbit corneal

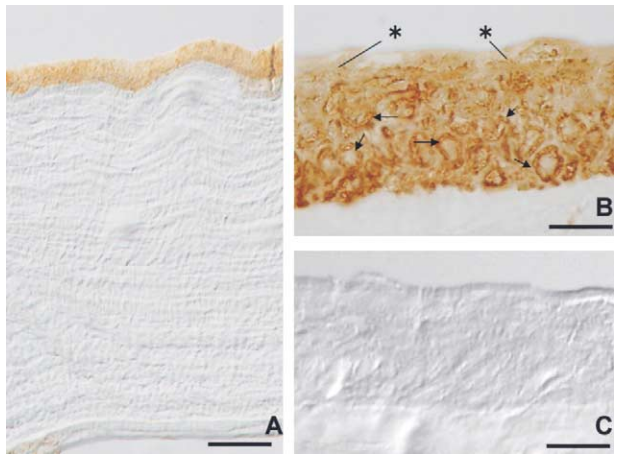

Figure 7. Immunohistochemical findings for Connexin 50 (Cx50). (**A**) Low magnification view of immunostaining for Cx50 in normal rabbit cornea. Bar = 100 μm. (**B**) High magnification of the area in (**A**) demonstrating Cx50 immunoreactivity in the corneal epithelium. Note the immunostaining of cell boundary of wing cells (arrows). Staining of the superficial cell layer was faint (*). Bar = 10 μm. (**C**) High magnification view of a negative control, which was not reacted with the primary monoclonal Cx50 antibody. Bar = 10 μm.

epithelium, which may possess P2Y receptors.[2] It is well known that the activation of P2Y receptors, which are coupled to G-proteins, elicits the production of inositol 1, 4, 5-triphosphate (IP3) and the subsequent mobilization of Ca^{2+} from intracellular stores, eg, the sarcoendoplasmic reticulum.[19] In previous studies,[2,3] we concluded that the ATP-induced initial spike of corneal epithelium is caused by the mobilization of Ca^{2+}, and that the subsequent plateau phase is the result of Ca^{2+} influx, ie, capacitive calcium entry.[20,21] In addition, synchronized oscillatory fluctuations of $[Ca^{2+}]_i$ are characteristic in the epithelium.[2,3]

A growing body of evidence exists to suggest that individual oscillatory fluctuations are often synchronized in various cells.[22–27] Ca^{2+} signaling can be transmitted through the communicating junctions (namely, gap junctions) to adjacent cells.[2,3] The synchronization of $[Ca^{2+}]_i$ changes in the corneal epithelium suggests that ATP from injured cells induces $[Ca^{2+}]_i$ changes in corneal cells and these changes can be propagated to neighbor cells via the gap junctions. This view was also supported by the propagation of $[Ca^{2+}]_i$ changes in the present study.

Heterogeneity in Intercellular Communications Among Different Layers

Three-dimensional confocal microscopy, over a time course, showed that the ATP-induced $[Ca^{2+}]_i$ changes were developed in the wing cells, but not propagated to superficial and to basal cells (Figures 5A–C). When the corneal epithelium is damaged, it is likely that the cells of the wing cell layer would transmit information rapidly to adjacent cells and thus encourage wound healing. On the other hand, the cells of the superficial cell layer might not require meaningful intracellular communication, because they will soon be shed. The significance of the decreased propagation of $[Ca^{2+}]_i$ changes among basal cells remains an enigma. Immature cells may not have the ability to communicate by showing $[Ca^{2+}]_i$ changes, as discussed below.

Involvement of Gap Junctions

It has been reported that octanol affects jap junctional communication as well as Ca^{2+} influx and Ca^{2+} mobilization from Ca^{2+} stores.[7,28,29] The Ca^{2+} mobilization may be involved in intercellular propagation.[30] In the present study, octanol affected the plateau phase, the propagation and the synchronization of ATP-induced $[Ca^{2+}]_i$ changes, but not the initial increase of stimulated cells per se. The initial phase is caused by the Ca^{2+} mobilization, but not by Ca^{2+} influx.[2,3] Therefore, we concluded that the inhibitory effect of octanol on the synchronization and propagation can be the result of suppression in gap junctional communication. Inhibition of Ca^{2+} influx participates in the suppression of the plateau phase.

Localization of Connexins

Gap junctions that are composed of different connexins may have unique properties with respect to permeability, voltage, and pH dependence. Two connexins, Cx43 and Cx50, have been reported in the rat, bovine, and rabbit corneal epithelium.[31–34] Epithelial Cx43 is localized exclusively in the basal cells, whereas Cx50 appears to be present in all corneal epithelial cells.[30–32] Wing cells showed evidence of intercellular communications in the present study, although they lack Cx43.[32–34] Therefore, it is possible that Cx50 plays a role in intercellular communication.

Consistent with our Ca^{2+} imaging data, Matic et al[33] reported that Cx50 is present predominantly in the suprabasal wing layers, minimally in flattened superficial cell layers, and only with weak staining in the basal layer, as evidenced by an immunohistochemical study. We confirmed the immunohistological staining pattern of Cx50 in the present study. In addition, it has been reported that the reduced Cx50 levels in the more superficial rabbit cells is consistent with the reported paucity of ultrastructural junctional profiles in these cells.[35] Therefore, differences in the localization of Cx50 among layers could cause a difference in the changes in $[Ca^{2+}]_i$ in these layers.

Although Cx50 is present in basal cells,[32–34] intercellular propagation of $[Ca^{2+}]_i$ change was faint in these cells. Interestingly, limbic basal cells, which are proliferative stem cells, lack Cx50 in the chicken, human, mouse, and rabbit,[36] suggesting that intercellular communications through gap junctions consisting of Cx50 are underdeveloped in younger immature corneal epithelial cells. The Cx50 observed in the basal cells could be immature, and not be able to establish intercellular communications accompanied by $[Ca^{2+}]_i$ changes. It cannot be denied, however, that other $[Ca^{2+}]_i$-independent intercellular communications exist, and Cx43 could be involved in such processes. Otherwise, information from an injured area would not be able to reach proliferative stem cells.

Conclusion

The present data indicates that the gap junction, which consists of Cx50, is related to the ATP-induced intracellular communication in wing cells of the cor-

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neal epithelium. Although, in practice, the molecule that transmits Ca^{2+} signaling through the gap junction has not yet been determined, IP3 or other chemical mediators represent potential candidates.[30] In any case, ATP from one injured cell of the corneal epithelium stimulates adjacent cells, and $[Ca^{2+}]_i$ changes extend to neighboring cells in the wing cell layer. We hypothesize that, after the signals completely reach the wing cell layer, the response of the cells to regenerate, proliferate, and differentiate would start, but the putative $[Ca^{2+}]_i$-independent intercellular signal mechanism remains to be examined.

On the other hand, a prolonged $[Ca^{2+}]_i$ increase would be toxic to cells, and persistent ATP stimulation may induce cell death. ATP-analogs and/or antagonists are used on corneal injuries to facilitate wound healing or to inhibit the inflammatory process. However, prior to the clinical usage of ATP, the heterogeneity of ATP-induced $[Ca^{2+}]_i$ changes in different cell layers must be understood, because each cell layer plays a distinct role in proliferation and differentiation.

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