A Decreased Ca\textsuperscript{2+}-Wave Propagation Is Found among Cultured RPE Cells from Dystrophic RCS Rats

Peter Stalmans and Bernard Himpens

**PURPOSE.** The authors investigated intercellular communication among cultured rat retinal pigment epithelial (RPE) cells isolated from dystrophic Royal College of Surgeons (RCS) rats by studying the conduction of the \([\text{Ca}^{2+}]_c\), wave elicited by mechanical stimulation. The effect of protein phosphorylation was measured by modulating the protein kinase C (PKC), protein kinase A (PKA), and tyrosine kinase activity.

**METHODS.** Cultured RPE cells isolated from neonatal control Long-Evans (LE) and dystrophic RCS rats were analyzed using the fluorescent dye fluo-3 to measure the \([\text{Ca}^{2+}]_c\)-wave propagation on mechanical stimulation to investigate the intercellular communication.

**RESULTS.** Mechanical stimulation in LE-RPE cells resulted in a centrifugally spreading \([\text{Ca}^{2+}]_c\) wave through the neighboring cells. When a mechanical stimulus was applied on RCS-RPE cells, a significantly reduced \([\text{Ca}^{2+}]_c\)-response was found in the neighboring cells compared with that of control RPE cells. Activation of PKC almost completely blocked the mechanically induced \([\text{Ca}^{2+}]_c\) rise in the neighboring RCS-RPE cells. In contrast to LE-RPE cells, an activation of PKA also significantly decreased the \([\text{Ca}^{2+}]_c\)-wave propagation in RCS-RPE cells. Inhibition of PKA had no effect on the intercellular communication in LE- or RCS-RPE cells. In addition, when protein phosphatase activity or tyrosine kinase activity was inhibited, an increased \([\text{Ca}^{2+}]_c\) rise in the neighboring cells on mechanical stimulation was measured, reaching levels currently found for LE-RPE cells.

**CONCLUSIONS.** In dystrophic RCS-RPE cells, a decreased intercellular \([\text{Ca}^{2+}]_c\)-wave propagation is found. This intercellular communication can be mediated by protein phosphorylation. (*Invest Ophthalmol Vis Sci.* 1998;39:1493-1502)
conductance (GJC) is associated with uncontrolled cell growth in various cell types.\textsuperscript{20-22} We previously suggested that a decreased GJC among RPE cells, modulated by protein kinase C (PKC), could be associated with the blood retinal breakdown that occurs in diabetes, and that increased RPE cell proliferation might result in the formation of proliferative vitreoretinopathy.\textsuperscript{8}

Because a blood retinal barrier breakdown and an increased proliferation of RPE cells also occur in retinitis pigmentosa,\textsuperscript{23} it is possible that a decreased intercellular communication would induce RPE cell proliferation in this disease.

Although cultured RPE cells lose some properties of the RPE cells in situ, they remain useful for the investigation of their fundamental properties.\textsuperscript{24} We have therefore used primary cultured RPE cells to investigate the intracellular Ca\textsuperscript{2+} wave propagation in these cells. The mechanism of intracellular and intercellular Ca\textsuperscript{2+} mobilization induced by mechanical stimulation in RCS-RPE cells and control LE-RPE cells was compared. We found that the mechanism of Ca\textsuperscript{2+} mobilization after mechanical stimulation was identical with that found in the control LE-RPE cells. However, the intercellular communication among RCS-RPE cells was significantly suppressed and is modulated by PKC, protein kinase A (PKA), and tyrosine kinase activation.

\section*{Materials and Methods}

\subsection*{Preparation of Retinal Pigment Epithelial Cell Cultures}

The RPE cells were isolated from the eyes of 3- to 8-day-old pigmented Long-Evans rats (Charles River Laboratories, Boston, MA) or RCS rats (RCS-Rdy-P\textsuperscript{7}; National Institutes of Health, Genetic Resource Section, Bethesda, MD) as previously described.\textsuperscript{6,8,25} Treatment of animals conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Unless otherwise specified, cells were plated in four- or eight-chamber coverglass (Lab-Tek Chambered; Nunc, Naperville, IL) at a density of approximately 1.5 \times 10^5 cells/cm\textsuperscript{2} and grown at 37°C in RPMI 1640 (containing 12 mM glucose) supplemented with 20% fetal calf serum and 1% penicillin-streptomycin.

In primary culture, the RPE cells presented a polygonal shape with an average cellular surface of about 340 \mu m\textsuperscript{2}.\textsuperscript{5} Subconfluent monolayers, whereby individual cells made physical contact without cell overgrowth or dome formation, were obtained after 3 days, and all experiments were performed in 3- to 7-day-old primary cultures.

\subsection*{Materials}

Dispase was obtained from Boehringer Mannheim (Mannheim, Germany). Trypsin was obtained from DIFCO (Detroit, MI). Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640, Hanks’ balanced salt solution (HBSS), and penicillin-streptomycin were obtained from Life Technologies (Eugene, OR). Fetal calf serum was obtained from Boehringer Ingelheim BioWhittaker (Verviers, Belgium). Glucose was obtained from Merck (Darmstadt, Germany). Fetal calf serum was obtained from Boehringer Ingelheim BioWhittaker (Verviers, Belgium). Fluoro-3 AM, Pluronic, and okadaic acid were obtained from Molecular Probes (Eugene, OR). Phorbol 12-myristate 13-acetate (PMA), Thapsigargin, Gd\textsubscript{2}O\textsubscript{3}, and verapamil were obtained from Sigma-Aldrich (Bornem, Belgium). Herbimycin A was from Kamiya Biomedical (Thousand Oaks, CA). Sp-adenosine 3',5'-monophosphorothioate (Sp-cAMP) and Rp-adenosine 3',5'-monophosphorothioate (Rp-cAMP) were from Biolog Life Science Institute (Bremen, Germany). All solutions were buffered at pH 7.3. Gadolinium was prepared by dissolving 50 mg/ml Gd\textsubscript{2}O\textsubscript{3} in heated 6 M HCl as stock solution. Subsequently, this stock solution was diluted as necessary and buffered to reach the physiological pH level.

\subsection*{Fluorescence Measurements}

All experiments were performed multiple times on cells from different culture wells, and at different days of culture. The \( n \) value represents the number of investigated cells.

For \([Ca^{2+}]_i\) measurements, cells were incubated for 30 minutes at 37°C in the presence of 20 \mu M fluo-3 acetoxyethyl ester (fluo-3 AM), dissolved in HBSS (1.3 mM Ca\textsuperscript{2+}) containing 0.4% of Pluronic to enhance the solubility of the ester form of the dye. After loading with the fluorescent dye, each well was rinsed twice with HBSS before adding the final volume of HBSS in each well.

The fluo-3 fluorescence was measured with a confocal microscope (Insight; Meridian, Okemos, MI), based on an inverted microscope (model IMT2; Olympus, Tokyo, Japan), with a D-plan APO 100X (NA1.25) or S-plan APO 60X (NA1.4) oil-immersion objective. A light-optical control image was obtained with a second charge-coupled device (CCD) camera attached to the side of the microscope. The dye was excited at 488 nm using an argon ion laser (model 532; Coherent, Palo Alto, CA), and fluorescent light was collected at 530 nm. The fluorescence light was amplified using an image intensifier (Dage MTI, Michigan, MI) and collected by a cooled-CCD camera (Meridian). The video image was recorded on high-quality S-VHS videotape (model SVO 9620; Sony, Tokyo, Japan), equipped with a digital noise-reduction board and an RS232 computer interface. From the video recorder, the recorded images can be analyzed at any desired frequency using self-developed software as described previously.\textsuperscript{6,8}

The fluo-3 fluorescent dye was chosen as the Ca\textsuperscript{2+} indicator because of its large optical signal, which allows a good signal-to-noise ratio in a single frame. However, the lack of emission or excitation spectral shift of fluo-3 on Ca\textsuperscript{2+} binding makes it difficult to calibrate fluorescence signals in terms of absolute values of free [Ca\textsuperscript{2+}]\textsubscript{i}. Therefore, all reported fluorescence measurements (expressed in arbitrary units) are normalized values relative to the basal fluorescence intensities, after background correction. The averaged normalized fluorescence value in neighboring cells was computed only from the responsive cells, indicating that the fluorescence increased by 50% or more after applying the mechanical stimulus. Averaged normalized values, standard error, and \( t \)-test values (two-tailed, two-sample unequal variance) were calculated using Excel 97 in Windows NT 4.0 (Microsoft, Redmond, WA) on a Pentium-based personal computer (HB-Systems, Kessel-Lö, Belgium). To avoid differences in experimental data resulting from different laser intensities or different fluorescent dye batches, experimental data were always compared with control experiments performed on the same day on cells isolated from the same animals.
Mechanical Stimulation

A short-lasting deformation was induced in single RPE cells with a glass micropipette (tip diameter <1 μm) mounted on a vertical micro-injection system (model IMT2-SYF; Narishige, Tokyo, Japan) without damaging the cell.6,8

RESULTS

Control Conditions: LE Rats

As previously described,6,8 mechanical stimulation of a single LE-RPE cell in the presence of external Ca2+ induced a spreading rise of the [Ca2+]. The [Ca2+]i rise started at the point of the mechanical stimulus and attained an amplitude of 7.6 ± 0.5 (n = 16). The Ca2+ wave spread over the mechanically stimulated cell and was conducted with some delay to the surrounding two to three cell layers (up to ±100 μm from the stimulated cells) (Fig. 1A).

The amplitude of the [Ca2+]i transient declined as a function of the distance of the surrounding cell from the mechanically stimulated cell (Fig. 1G). The amplitude in the first layer of neighboring cells was 5.3 ± 0.2 (n = 167). In LE-RPE cells, 91% of the neighboring cells showed a significant [Ca2+]i rise (fluorescence rise >1.5). The propagation of this [Ca2+]i wave to the neighboring cells could be reversibly blocked by a 6 mM concentration of the gap-junction blocker halothane, depolarizing the cells in a medium containing 140 mM K+, or by increasing the glucose concentration above 14 mM.6,8

Dystrophic Retinal Pigment Epithelial Cells: RCS Rats

When an individual cell of a cultured monolayer of RCS-RPE cells was mechanically stimulated, the resultant [Ca2+]i rise in the mechanically stimulated cell (7.4 ± 0.6, n = 16) was not different from that in LE-RPE cells (P = 0.43). However, the amplitude of the [Ca2+]i rise in the neighboring cells was significantly lower (~49%) than in LE-RPE cells (P = 0.0013) (Table 1, Fig. 2), and did not spread further beyond this first layer (Fig. 1B). Only 72% of the neighboring cells responded with a detectable [Ca2+]i rise, as compared with 91% response in LE-RPE cells.

The recovery of the fluorescence to baseline level in the mechanically stimulated cell was not different from control conditions. However, the return of [Ca2+]i to basal levels in the neighboring cells occurred faster than in LE-RPE cells. Baseline fluorescence levels were typically attained after less than 1 minute, whereas in LE-RPE cells it required 2.5 minutes.6

Effect of Altering the Plasmalemmal Ca2+ Influx

Influence of Extracellular Ca2+.

In keeping with previous findings for LE-RPE cells,6 a mechanical stimulus applied to RCS-RPE cells in the absence of external Ca2+ ([Ca2+]o), using a medium without Ca2+ and containing 2 mM EGTA, elicited no [Ca2+]i rise in the mechanically stimulated or neighboring cells (Table 1), despite functional intracellular [Ca2+]i stores. Therefore, the [Ca2+]i rise in the mechanically stimulated cells induced by a mechanical stimulus depends on Ca2+ influx as the initial messenger.

Effect of Blocking the Voltage-Dependent Ca2+ Influx.

In a previous study of LE-RPE cells, we found that blocking the L-type voltage-dependent plasmalemmal Ca2+ channels with verapamil did not alter the Ca2+ rise in the mechanically stimulated cells in Ca2+-containing solutions.6 Likewise, preincubation of RCS-RPE cells for 30 minutes in the presence of 10 μM verapamil did not affect the [Ca2+]i rise in the mechanically stimulated cell (P = 0.478) or neighboring cells (P = 0.378), although the number of responsive neighboring cells was lower than that of control RCS-RPE cells (Table 1, Fig. 2).

Effect of Blocking the Stretch-Sensitive Ca2+ Influx.

When the stretch-sensitive Ca2+-influx channels were blocked by 10 mM gadolinium, we also found a decreased [Ca2+]i rise on mechanical stimulation in the mechanically stimulated cells (~46%, P = 0.00689) and the neighboring cells (~40%, P = 0.000164), but no change in the number of responsive neighboring cells (Table 1, Fig. 2).

Effect of Blocking the Voltage-Dependent Ca2+ Stores.

When PKC was downregulated in LE-RPE cells by incubation with thapsigargin, which depletes all intracellular Ca2+ stores by blocking the endoplasmatic Ca2+-ATPase activity, decreased the [Ca2+]i rise in the mechanically stimulated and neighboring cells on mechanical stimulation.5 When this experiment was repeated in RCS-RPE cells, we found a similarly decreased [Ca2+]i rise in the mechanically stimulated cells (P < 10−5) and in the neighboring cells (P < 10−5) and a decreased number of responsive neighboring cells on mechanical stimulation (Table 1, Fig. 2).

When gadolinium was added to the Ca2+-containing incubation medium after depletion of the intracellular Ca2+ stores with thapsigargin, we could not elicit any [Ca2+]i rise in the mechanically stimulated or neighboring cells using a mechanical stimulus, either in RCS or in LE-RPE cells (Table 1, Fig. 2).

We can therefore conclude that in RCS-RPE cells a mechanical stimulus elicits a Ca2+ influx, followed by a release of Ca2+ from the intracellular Ca2+ stores.

Effect of Phosphorylation on the Intracellular and Intercellular Ca2+ Waves

Protein Kinase C.

The activation of PKC in LE-RPE cells inhibited intracellular [Ca2+]i, wave propagation and decreased GJC.6 To investigate the effect of PKC on GJC in RCS-RPE cells, the PKC activity was stimulated by incubating the cells for 30 minutes in a medium supplemented with 1 μM PMA before mechanostimulation.8 This treatment did not affect the [Ca2+]i rise elicited by mechanical stimulation in the mechanically stimulated cell (P = 0.4744; Table 1, Figs. 1D, 1J, 3). However, only 5% of the neighboring cells presented a significant [Ca2+]i rise (Table 1, Figs. 1D, 1J, 3). As a result of the limited number of responsive neighboring cells, neither an average Ca2+ rise nor a statistical significance was calculated.

When PKC was downregulated in LE-RPE cells by incu-
FIGURE 1. A centrifugally propagating calcium wave elicited by a mechanical stimulus. The top images (A through F) show a time series of fluorescent images of groups of retinal pigment epithelial (RPE) cells loaded with fluo-3 in different conditions. All images were colored using a pseudocolor scale to enhance visibility: Bluish colors represent lower \([\text{Ca}^{2+}]\); reddish colors represent higher \([\text{Ca}^{2+}]\). The color scale at the top left represents the fluorescence intensity induced by the \([\text{Ca}^{2+}]\) concentration expressed in units of fluorescence intensity. The white arrow indicates the cell that will be mechanically stimulated (MS). The upper left image in each series (A through F) shows the fluorescence intensities in resting condition immediately before stimulation. Every consecutive image (left to right, top to bottom) is taken at a time interval of 0.5 seconds. The bottom graphs (G through L) represent the normalized fluo-3 fluorescence in the MS (red lines) and in the neighboring (NB) cells that are directly connected to the MS cell (green lines) as a function of time in various conditions. At the arrow, the mechanical stimulus was applied. When a mechanical stimulus is applied on a central Long-Evans (LE)-RPE cell (A), the calcium rises in the stimulated cell (G, red line).
bation for 72 hours with 1 μM PMA, a classical procedure to eliminate all PKC activity, we found no effect on either the [Ca\textsuperscript{2+}]\text{c} rise in the mechanically stimulated cells or the intercellular communication.\textsuperscript{8}

Similarly, downregulation of PKC in RCS-RPE cells did not affect the [Ca\textsuperscript{2+}]\text{c} rise in the mechanically stimulated cells (P = 0.961; Table 1, Figs. 1C, II, 3). In contrast, 86% of the downregulated neighboring cells responded with an increase in [Ca\textsuperscript{2+}]\text{c}, and the average amplitude of the response was 2.3-fold higher (P < 10\textsuperscript{-10}; Table 1, Fig. 3). The latter values are comparable to those found for control LE-RPE cells (P = 0.243; Table 1, Fig. 3). This indicates that PKC activity is significantly enhanced in RCS-RPE cells. Inhibition of this PKC activity restored the intercellular communication to levels found in LE-RPE cells.

Because we demonstrated that hyperglycemic conditions increased the PKC activity in LE-RPE cells,\textsuperscript{9} we have investigated whether changes in the glucose concentration of the medium could affect the reduced intercellular communication in RCS-RPE. All above-mentioned experiments were performed on cells cultured in RPMI medium containing 12 mM glucose. When RCS-RPE cells were cultured in DMEM medium containing 5 mM glucose, the responses in the mechanically stimulated cells (P = 0.733) and the neighboring cells (P = 0.507) were unchanged (Table 1, Fig. 3), in keeping with our previous results for LE-RPE cells.\textsuperscript{8}

When glucose in the DMEM medium was increased to 25 mM for 72 hours, mechanical stimulation did not affect the Ca\textsuperscript{2+} rise in the mechanically stimulated cells (P = 0.961; Table 1, Fig. 3). However, the [Ca\textsuperscript{2+}]\text{c} rise in the neighboring

![Figure 1](https://example.com/figure1.png)

(Continued) The [Ca\textsuperscript{2+}]\text{c} also rises in the NB cells in contact with the MS cell, and after a time delay in the more peripheral cell layers surrounding the MS cell (A). The NB cells in direct contact with the MS cell presented a variable [Ca\textsuperscript{2+}]\text{c} rise (G, green lines). When a similar experiment is performed on Royal College of Surgeons (RCS)-RPE cells (B, H), a similar Ca\textsuperscript{2+} rise is observed in the MS cell (H, red line), but only a limited response can be seen in the NB cells (H, green lines). After downregulation of all protein kinase C (PKC) activity in RCS-RPE cells (C, I), a mechanical stimulation induces a similar Ca\textsuperscript{2+} rise in the MS cell (I, red line). In the NB cells, however, an increased Ca\textsuperscript{2+} rise can be observed (I, green lines). When PKC activity is increased in RCS-RPE cells (D, J), almost no Ca\textsuperscript{2+} increase can be found in the NB cells on mechanical stimulation (J, green lines), whereas the Ca\textsuperscript{2+} rise in the MS cell remains unaltered (J, red line). To a lesser extent, increasing the protein kinase A (PKA) activity in RCS-RPE cells (E, K) also decreased the intercellular Ca\textsuperscript{2+} wave propagation in the NB cells (K, green lines), while the Ca\textsuperscript{2+} increase in the MS cell remains unaltered (K, red line). When the PKA activity is increased in RCS-RPE cells after downregulation of all PKC activity (F, L), only a limited inhibition of the intercellular Ca\textsuperscript{2+}-wave propagation can be observed in the NB cells (L, green lines), while the mechanically induced Ca\textsuperscript{2+} rise in the MS cell remains unchanged (L, red line).
cells was further blunted \((P < 10^{-4})\) and the number of responsive cells (39%) was much lower than in RCS-RPE cells grown in 5 mM or 12 mM glucose (Table 1, Fig. 3). The observed effects of \(\beta\)-cAMP in cells that had been preincubated for 72 hours with various concentrations of okadaic acid, a potent inhibitor of the major Ser/Thr-protein phosphatases.\(^{39}\) We observed previously that 1 \(\mu\)M okadaic acid did not decrease the \([Ca^{2+}]_c\) rise in the mechanically stimulated or neighboring LE-RPE cells.\(^{26}\) However, incubation of RCS-RPE cells in the presence of 1 \(\mu\)M herbinycin A increased the amplitude of the \([Ca^{2+}]_c\) rise in neighboring cells after mechanical stimulation by 57% \((P < 10^{-4};\) Table 1, Fig. 3). The observed effects of \(\beta\)-cAMP indicate that PKA activation under basal conditions is not enhanced, whereas PKC is. We were interested to find out whether the effect of increasing PKA activity could be more prominent after downregulation of the PKC activity. We therefore increased the PKA activity with \(\beta\)-cAMP in cells that had been preincubated for 72 hours in the presence of 1 \(\mu\)M PMA (Table 1, Figs. IF, IL, 3). After downregulation of PKC, activation of PKA had little effect on the \([Ca^{2+}]_c\) rise in the mechanically stimulated cells \((P = 0.71)\), but it decreased greatly the intercellular communication: the response rate in neighboring cells fell from 80% to 48%, and the \([Ca^{2+}]_c\) rise in the responsive neighboring cells decreased by 59% \((P = 0.0068)\). This indicates that PKA can inhibit the intercellular communication in RCS-RPE cells independently of PKC activation.

**Tyrosine Kinase.** To investigate the influence of tyrosine kinase activity on GJC,\(^{34}\) we studied the effect of mechanical stimulation was investigated after incubation for 30 minutes in the presence of 1 \(\mu\)M herbinycin A, an inhibitor of tyrosine kinase activity.\(^{55-58}\) We previously found that the inhibitor did not affect the investigated parameters in LE-RPE cells.\(^{8}\) However, incubation of RCS-RPE cells in the presence of 1 \(\mu\)M herbinycin A increased the amplitude of the \([Ca^{2+}]_c\) rise in neighboring cells after mechanical stimulation by 57% \((P < 10^{-4};\) Table 1, Fig. 3). Also the number of responsive neighboring cells was increased to the level observed in control LE-RPE cells. The response of the mechanically stimulated cells was not modified by herbinycin A \((P = 0.941;\) Table 1, Fig. 3).

**Okadaic Acid.** A possible role of Ser/Thr-protein phosphatase activity on GJC\(^{36}\) was studied by incubating cells for 30 minutes with various concentrations of okadaic acid, a potent inhibitor of the major Ser/Thr-protein phosphatases.\(^{39}\) We observed previously that 1 \(\mu\)M okadaic acid did not decrease the \([Ca^{2+}]_c\) rise in mechanically stimulated or neighboring LE-RPE cells.
Decreased Ca$^{2+}$-Wave Propagation among Dystrophic RCS-RPE Cells

**Figure 3.** Alterations in intercellular [Ca$^{2+}$]$_j$ wave progression by measuring the normalized fluo-3 fluorescence in mechanically stimulated (MS) cells and in neighboring (NB) cells during mechanical stimulation. The change of the fluo-3 fluorescence induced by mechanical stimulation is shown for the MS cells (open columns) and the NB cells (shaded columns). The percentage of NB cells in which a [Ca$^{2+}$]$_j$ rise is observed is given by the black columns (right scale). The following experimental conditions are represented: control experiments in Royal College of Surgeons (RCS)-retinal pigment epithelial (RPE) cells (Control RCS); after activation of PKC activity using 1 μM phorbol 12-myristate 13-acetate (PMA 30' RCS); after downregulation of all PKC activity in RCS-RPE cells (PKC Downreg RCS); after culturing the cells in 25 mM glucose (25 mM Glucose RCS) and in 5 mM glucose (5 mM Glucose RCS); after inhibition of PKA activity using 1 μM Rp-cAMP (Rp-cAMP RCS); after inhibition of PKA activity when PKC activity is downregulated (PKC Downreg + Rp-cAMP RCS); after inhibition of tyrosine kinase activity using 1 μM herbimycin A (Herbimycin A RCS); and after inhibition of protein phosphatase activity using different concentrations of okadaic acid: 10 μM (Okadaic Acid 10 μM RCS), 1 μM (Okadaic Acid 1 μM RCS), and 0.1 μM (Okadaic Acid 0.1 μM RCS). Compared with control RCS-RPE cells, a decreased Ca$^{2+}$ rise in the NB cells was found after growing the cells in 25 mM glucose, after PKC activation using PMA, and after incubation with Rp-cAMP, with or without previous downregulation of PKC. Compared with control RCS-RPE cells, an increased Ca$^{2+}$ rise in the NB cells on mechanical stimulation was found after downregulation of PKC activity, after incubation with herbimycin A, or in the presence of a supramaximal concentration of 10 μM okadaic acid.

Cells on mechanical stimulation$^{8}$

RCS-RPE cells were incubated in the presence of okadaic acid (Table 1, Fig. 3), which did not affect the Ca$^{2+}$ rise in the mechanically stimulated or neighboring cells at a concentration of 0.1 (mechanically stimulated: $P = 0.984$; neighboring: $P = 0.09$) or 1 μM (mechanically stimulated: $P = 0.943$; neighboring: $P = 0.72$). However, when the RCS-RPE cells had been incubated with an unusually high concentration (10 μM) of okadaic acid, a mechanical stimulus elicited a higher [Ca$^{2+}$]$_j$ rise in the neighboring cells (+65%, $P < 10^{-6}$). Also, the number of responsive neighboring cells increased to 83%. However, at this high concentration, okadaic acid is known to
have nonspecific side-effects, such as breaking down the cytoskeleton.  

**DISCUSSION**

**Mechanism of the Mechanically Induced Ca$^{2+}$-Wave Propagation**

We have previously demonstrated that mechanical stimulation of an individual cell in a cultured monolayer induces an intracellular [Ca$^{2+}$], rise and a propagating [Ca$^{2+}$] wave through the mechanically stimulated and neighboring cells. It was also shown that the intercellular Ca$^{2+}$-wave propagation is dependent on GJCs. We suggested that the mechanism of the Ca$^{2+}$-rise in the mechanically stimulated cell is Ca$^{2+}$ influx, followed by Ca$^{2+}$ release from the intracellular Ca$^{2+}$ stores. This [Ca$^{2+}$] rise probably induces the diffusion through the intercellular gap junctions of a second messenger that elicits a [Ca$^{2+}$] rise in the neighboring cells.

The dependence of the mechanically induced [Ca$^{2+}$] rise on Ca$^{2+}$ influx was also demonstrated in RCS-RPE cells by performing the mechanical stimulus in a Ca$^{2+}$-free solution, whereby no [Ca$^{2+}$] rise could be elicited. The use of the Ca$^{2+}$ antagonist verapamil showed that voltage-dependent, L-type plasmalemmal Ca$^{2+}$ channels are not involved in the initial Ca$^{2+}$ influx. However, when stretch-sensitive Ca$^{2+}$-influx channels were blocked using gadolinium, a decreased [Ca$^{2+}$] rise was found in the mechanically stimulated cells and the neighboring cells, indicating that non-voltage-dependent, stretch-sensitive channels are involved in the mechanically induced Ca$^{2+}$ influx.

RCS-RPE cells behaved like LE-RPE cells in that depletion of the intracellular Ca$^{2+}$ stores using thapsigargin reduced the [Ca$^{2+}$] rise in the mechanically stimulated cells. The intracellular [Ca$^{2+}$] rise can then only occur by Ca$^{2+}$ influx and is not followed by a [Ca$^{2+}$] release. This supports our hypothesis that the initial Ca$^{2+}$ influx through the stretch-sensitive plasmalemmal channels triggers the Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores. When a mechanical stimulus was applied in Ca$^{2+}$-containing medium to RCS-RPE cells that had previously been Ca$^{2+}$-depleted with thapsigargin, and that were subsequently incubated with gadolinium, we could not detect a [Ca$^{2+}$] rise anymore in the mechanically stimulated cells or the neighboring cells. This effect of combined inhibition of Ca$^{2+}$ influx and [Ca$^{2+}$] release confirms the dependence of the mechanically induced [Ca$^{2+}$] rise on both mechanisms. In this respect RCS-RPE cells behaved identically to LE-RPE cells.

**Intercellular Communication in RCS-Retinal Pigment Epithelial Cells: Effect of Protein Phosphorylation**

In comparison with LE-RPE cells, RCS-RPE cells displayed a decreased intercellular [Ca$^{2+}$]-wave propagation after mechanical stimulation. We showed previously, using the fluorescence recovery after photobleaching technique, that this intercellular Ca$^{2+}$ wave is gap junction mediated. It suggests that RCS-RPE cells have a decreased GJC compared with nondystrophic LE-RPE cells.

Gap junctions form intercellular communication channels in many cell types. Inorganic ions and some small molecules such as cAMP and inositol-triphosphate move through the channels by passive diffusion. Some long-chain alcohols like halothane and octanol have been found to inhibit GJC, as we also reported for halothane in LE-RPE cells.

Gap junctions are formed by more than ten different types of channel proteins called connexins (Cx). These proteins are identified by a number corresponding to their molecular mass in kilodaltons. The best known connexins are Cx26, Cx32, and Cx43. Most cell types seem to express Cx43, phosphorylation of which modifies the GJC. It was demonstrated that cultured LE-RPE cells also express Cx43. Different protein kinases such as PKA, PKC, and tyrosine kinase can alter the GJC in various cell types by modifying the configuration of the connexin subunits or by inhibiting the aggregation of connexin 43 protein.

We demonstrated previously that PKC, either directly by the phorbol ester PMA, which activates mainly PKC, or indirectly using elevated glucose concentrations, inhibits the gap-junction-mediated intercellular communication among LE-RPE cells.

In RCS-RPE cells the [Ca$^{2+}$] wave progression was already decreased in comparison with nondystrophic LE-RPE cells. This inhibition was even more pronounced by stimulation of PKC by PMA and by a high-glucose concentration. Conversely, when the PKC activity was downregulated in RCS-RPE cells, the [Ca$^{2+}$] wave propagation attained the high level found for control LE-RPE cells. This suggests that RCS-RPE cells have most likely a higher basal PKC activity in comparison with LE-RPE cells. Because phorbol esters may induce some nonspecific phosphorylations, further investigation, for example, using more isoenzyme-specific phorbol esters, transfection of PKC isoenzymes, or analysis of the tissue distribution of PKC isoenzymes will allow us to define the PKC isoenzyme(s) involved.

It has been demonstrated that PKA induces phosphorylation of connexins. Activation of PKA increases GJC in, for example, hepatocytes and increases the expression of the Cx43 gene but decreases GJC in other cell types, such as neurons and corneal epithelial cells. Basal PKA activity is low in RCS-RPE cells as shown by the absence of effect of an inhibition of PKA. In our study, activation of PKA in RCS-RPE cells using $S_{cAMP}$ resulted in a decreased propagation of the [Ca$^{2+}$] wave induced by mechanical stimulation. This contrasts to previous findings in LE-RPE cells, where no effect of $S_{cAMP}$ was observed.

When the intercellular communication had been facilitated by downregulation of PKC, we observed that activation of PKA resulted in a particularly important suppression of the GJC. Obviously, this effect of PKA cannot be explained by activation of PKC. It may indicate that one or more isoforms of connexin can be phosphorylated to a larger extent by PKA if they have not been phosphorylated by PKC.

Although the phosphorylation of gap junctions is reversed by protein phosphatases, most protein-phosphatase inhibitors did not decrease GJC. Likewise, we did not detect any effect of the protein-phosphatase inhibitor, okadaic acid, at concentrations up to 1 nM, in LE-RPE cells and in RCS-RPE cells. Only a supramaximal concentration of 10 nM okadaic acid produced an increased propagation of the [Ca$^{2+}$] wave in RCS-RPE cells. Because it is known that okadaic acid at this concentration disrupts the cytoskeleton, which plays an important role in the intercellular communication, we believe that this effect is not caused by the activity of the substance as a protein-phosphatase inhibitor.
In the present study, the inhibition of tyrosine kinase activity by herbimycin A resulted in an increased \( \left[ \text{Ca}^{2+} \right]_i \) rise in the neighboring cells, in contrast to previous negative results with LE-RPE cells. Because a decreased tyrosine kinase activity increases GJC among cultured RPE cells, it can be suggested that RCS-RPE cells have an increased basal tyrosine kinase activity, which inhibits GJC among the cells.

In conclusion, a mechanical stimulus induces in RCS-RPE cells a \( \left[ \text{Ca}^{2+} \right]_i \) increase in the stimulated cell, which is dependent on \( \text{Ca}^{2+} \) influx and \( \left[ \text{Ca}^{2+} \right]_i \) release. The subsequent \( \left[ \text{Ca}^{2+} \right]_i \)-wave propagation is significantly lower in RCS-RPE cells as compared with LE-RPE cells. This effect is most likely caused by an increased PKC activity. However, the intercellular communication among RCS-RPE cells is also more sensitive to PKA and tyrosine kinase activity, in comparison with LE-RPE cells.

References