Effect of Increasing Glucose Concentrations and Protein Phosphorylation on Intercellular Communication in Cultured Rat Retinal Pigment Epithelial Cells

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Purpose. The intercellular communication between cultured rat retinal pigment epithelial (RPE) cells grown in increasing glucose concentrations or after modulation of the protein kinase C-induced protein phosphorylation was investigated by studying the conduction of the $[\text{Ca}^{2+}]_{i}$ wave elicited by mechanical stimulation and by analyzing the fluorescence recovery after photobleaching (FRAP).

Methods. Subconfluent monolayers of RPE cells isolated from neonatal Long Evans rats were cultured in growth medium with various glucose levels and analyzed using the fluorescent dye fluo-3 for measurements of intracellular $\text{Ca}^{2+}$ after mechanical stimulation and using 6-carboxyfluorescein diacetate to investigate the intercellular communication with FRAP.

Results. Mechanical stimulation in 5 or 12 mM glucose resulted in a $\text{Ca}^{2+}$ wave that spread centrifugally through the neighboring cells. An inhibition of the propagation of this wave, similar to that induced by halothane, could be observed in cells grown for 72 hours in 14-mM or higher concentrations of glucose. This inhibitory effect was not caused by a hypotonic effect, in that results of experiments on cells cultured in growth medium supplemented with mannitol instead of glucose did not differ from those of experiments in the control medium. Activation of protein kinase C by incubation of the cells for 30 minutes with phorbol 12-myristate 13-acetate (PMA) resulted in a strong inhibition of $[\text{Ca}^{2+}]_{i}$-wave propagation. This inhibition did not depend on the oxidizing effects of PMA because the addition of glaucine, a known antioxidant, did not prevent the inhibition. Cells grown for 72 hours in glucose-rich medium (25 or 50 mM) and in which all protein kinase C activity was downregulated by a previous 72-hour exposure to 1 $\mu$M PMA, did not display the inhibitory effect on the propagation of the $\text{Ca}^{2+}$ wave that is normally induced by this elevated glucose level. Stimulation or inhibition of protein kinase A activity by incubating RPE cells with Sp-cyclic adenosine monophosphate or Rp-cyclic adenosine monophosphate respectively, or inhibition of tyrosine kinase activity with herbimycin A did not alter the intercellular communication after mechanical stimulation. To determine whether the observed changes were caused by alterations in gap junction conductance (GJC), FRAP experiments were performed in control conditions, after a 30-minute incubation with PMA, and in cells cultured in 50 mM glucose in the presence and in the absence of 1 $\mu$M PMA. The measured GJC was consistent with the inhibitory effect on propagation of an intercellular $\text{Ca}^{2+}$ wave in all tested conditions.

Conclusions. In RPE cells, a glucose concentration of 14 mM (224 mg/dl) or higher inhibits $\text{Ca}^{2+}$-wave propagation and intercellular GJC. This effect may be mediated by protein kinase C activity. Invest Ophthalmol Vis Sci. 1997;38:1598-1609.

Retinal pigment epithelial cells (RPE cells) form a monolayer of cuboidal cells that separate the vascular choroidal system from the sensory retina. The blood–retinal barrier (BRB) is an extension of the blood–brain barrier and separates the internal environment of the eye from the vascular system. The internal side of the BRB is formed by the tight junctions between the vascular endothelial cells of the retinal vessels, whereas tight junctions between the RPE cells form the outer side of this barrier. Intercellular tight junctions prevent paracellular ion and water movement between the basal and the lateral cell sides of endothelium.
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port functions, mediated through gap junctions in edema. It has been suggested that leakage through graphic examination, which reveals leakage from the retinal vessels, and by the appearance of macular edema. It has been suggested that leakage through the intercellular tight junctions of RPE cells is involved in this process, but findings in other studies suggest that it is related to the impairment of the transcellular transport function.

Less is known about how the intercellular transport functions, mediated through gap junctions in RPE cells, are related to the development of diabetic retinopathy. Gap junctions are gated intercellular communications between cells that are permeable to such inorganic ions as Na⁺, K⁺, and Ca²⁺ and to such small molecules as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), and 1,4,5-inositol triphosphate. It has been demonstrated that cultured RPE cells can form tight junctions as well as gap junctions in vitro. However, to our knowledge, no study has been performed to measure the gap junction-mediated intercellular communication in RPE cells. The development of a technique for studying the gap junctions between the RPE cells by measuring the intercellular propagation of the Ca²⁺ waves offered the possibility of studying the properties of the gap junctions and thereby the modifications that might be accompanied by a disruption of the BRB.

It has been demonstrated that an elevated glucose level can induce alterations in the number and in the function of intercellular gap junctions in endothelial cells, pancreatic islet cells, nerve cells, and proximal tubule cells. It was therefore of interest to investigate whether intercellular communication in RPE cells could also be altered by increased glucose concentration as occurs in diabetes. To mimic the effect of hyperglycemia on RPE cells in vivo, we used the experimental model of cultured RPE cells in vitro in a medium with increasing glucose concentration (5, 12, 14, 16, 18, 20, 25, and 50 mM). Aspecific effects caused by the hyperosmolarity of this solution have been excluded by performing similar experiments in media of the same osmolarity as a 25- or 50-mM glucose concentration using the sugar mannitol, which cannot be metabolized.

Furthermore, RPE cells play an important role in the pathogenesis of proliferative vitreoretinopathy by migrating through a retinal break and contributing to the formation of the proliferative membranes. It is known that decreased intercellular gap junction conductance (GJC) is associated with uncontrolled cell growth in various cell types. Increased glucose concentrations can induce proliferation of RPE cells. It is therefore possible that a decreased intercellular communication would induce proliferation of RPE cells and that this change would contribute to the development of proliferative vitreoretinopathy. In spite of marked improvements in the medical therapy for diabetes, this pathology remains one of the major causes of severe loss of vision and of blindness.

Although cultured RPE cells lose some properties of the in situ RPE cells, they remain useful for the investigation of their fundamental properties. We have therefore used cultured RPE cells to investigate the action of glucose-rich conditions on the functions of these cells and eventually to contribute to the understanding of the breakdown of the BRB and the initiation of proliferative vitreoretinopathy that occurs in diabetic patients. To determine whether the observed Ca²⁺ signaling was indeed mediated through intercellular gap junctions, experiments were performed measuring the fluorescence recovery after photobleaching (FRAP). Because it is known that GJC is dependent on the phosphorylation activity by protein kinase C (PKC), we investigated the effects of drugs that modify the activities of these enzymes. Knowing that the effect of an increased glucose concentration may be related to an increased PKC activity, we also investigated whether a downregulation of PKC could reverse the effects of the glucose-rich medium.

The purpose of this study was to investigate the effect of increased glucose concentration on the propagation of Ca²⁺ waves and the GJC between RPE cells and to obtain evidence for the contribution of protein phosphorylation by various protein kinases to this process.

**MATERIALS AND METHODS**

**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) as previously described. Treatment of animals conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Cells were plated in 4- or 8-chamber Lab-Tek Chambered Coverglass (Nunc, Naperville, IL) at a density of approximately 1.5 × 10⁴/cm² and were grown at 37°C in RPMI 1640, containing 20% fetal calf serum with 1% penicillin–streptomycin, or in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum with 1% penicillin–streptomycin.
The RPE cells present a polygonal shape with an average cellular surface of about 340 μm². 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 cultures. Observation under light microscope did not reveal any difference between RPE cultures grown in RPMI 1640 and those grown in DMEM.

Materials

Materials were obtained as follows: dispase (Boehringer Mannheim, Mannheim, Germany); trypsin (Difco, Detroit, MI); RPMI 1640 (with 12 mM glucose), DMEM (with 5 mM glucose), HBSS (Hanks’ balanced salt solution), and penicillin-streptomycin (Gibco BRL, Life Technologies; Destelbergen, Belgium); fetal calf serum (Boehringer Ingelheim Bioline, OR); glucose and mannitol (Merck, Darmstadt, Germany); fetal calf serum (Boehringer Ingelheim Bremen, Germany); halothane (Zeneca, Destelbergen, Belgium); dispase (Boehringer Ingelheim Bremen, Belgium); 6-carboxyfluorescein diacetate, fluorescein diacetate, and okadaic acid (Molecular Probes, Eugene, OR); glucose and mannitol (Merck, Darmstadt, Germany); ethanolamine (Zeneca, Destelbergen, Belgium); PMA and glaucine (Sigma–Aldrich, Bornem, Belgium); herbimycin A (Kamiya Biomedical, Thousand Oaks, CA); and Sp-cAMP and Rp-cAMP (Biologic Life Science Institute, Bremen, Germany). For experiments in 140 mM K⁺, Na⁺ was replaced by K⁺. All solutions were buffered at pH 7.3. Increasing glucose concentrations of 12 to 25 mM were obtained by adding glucose to the DMEM, which contains 5 mM glucose. The 50 mM medium was obtained by adding 38 mM mannitol to RPMI 1640, which contains 12 mM glucose. The hypertonic mannitol solution was obtained by adding 20 mM mannitol to DMEM or by adding 38 mM mannitol to RPMI 1640 to achieve equiosmolar conditions in mediums with 25 or 50 mM glucose, respectively.

Fluorescence Measurements

All experiments were performed several times on cells from different culture wells and at different days of culture. The n value represents the number of investigated cells, and all experimental data are average values obtained from identical experiments.

Cells were incubated for 30 minutes at 37°C in 20 μM fluo-3 AM for [Ca²⁺], measurements or in 20 μM 6-carboxyfluorescein diacetate for FRAP experiments. These dyes were dissolved in HBSS containing (1.3 mM Ca²⁺) 0.4% 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 to each well.

[Ca²⁺] Measurements

The fluo-3 fluorescence was measured with the Meridian Insight confocal microscope (Okemos, MI), based on an Olympus IMT2 inverted microscope (Olympus, Tokyo, Japan), with a D-plan APO 100X (NA1.25) or an S-plan APO 60X (NA1.4) oil-immersion objective. A light-optical control image was obtained with a second charge-coupled device camera attached to the side of the microscope. The dye was excited at 488 nm (532 Argon Ion Laser; Coherent, Palo Alto, CA) and fluorescent light was collected at 530 nm. The fluorescent light was amplified using an image intensifier (Dage MTI, Michigan, MI) and was collected by a cooled CCD camera (Meridian). The obtained video image was taped on a high quality S–VHS video tape (Sony SVO 9620, Sony, Tokyo, Japan), equipped with a digital noise reduction board and a RS232 computer interface. From the video recording, the recorded images can be analyzed at any desired frequency, as described previously.13

Fluo-3 was chosen as a Ca²⁺ indicator because of its large optical signal, which allowed a very good signal-to-noise ratio in a single frame. The lack of excitation or excitation spectral shift of fluo-3 on Ca²⁺ binding makes it difficult to calibrate fluorescence signals in precise, absolute values of free [Ca²⁺]. Therefore, all reported fluorescence measurements (expressed in units) are normalized values relative to basal fluorescence intensities, after background correction. All experiments were repeated several times. Averaged normalized values, standard deviation, standard error, and Student’s t-test values (two-tailed, two-sample unequal variance) were calculated using Excel 7.0 in Windows NT 4.0 (Microsoft, Redmond, WA). Statistical significance was set at a level of P<0.05. To avoid differences in recorded data caused by different laser intensities, different fluorescent dye batches, and other variables, experimental data were always compared with that collected in control experiments performed on the same day on cells isolated from the same animals and grown in identical conditions.

Measurements by Fluorescence Recovery After Photobleaching

The FRAP technique is a technique widely used to study GJC in various cell types.37 In this technique, a high-power laser beam is used to destroy photochemically the fluorescent-dye selectivity in an individual cell of a monolayer, whereafter the reappearance of the fluorescent dye diffusing through the gap junctions from the neighboring cells into the bleached cell is monitored.37-39 The fluorescence of the cells was measured with the Meridian Ultima confocal microscope, also based on an Olympus IMT2 inverted microscope, with an S-plan APO 60X (NA1.4) oil-immersion objective. A light-optical control image was obtained with a charge-coupled device camera attached to the microscope. The dye was excited at 488 nm (Enterprise Laser, Coherent) and fluorescent light was collected by a photomultiplier after passage through a 570-nm...
cutoff filter. Image size was typically 768 x 512 points with an 8-bit pixel depth. Before bleaching, cells were marked by drawing a polygon over each cell, and a prebleach image was scanned. Bleaching was performed by applying 10 to 25 laser shots (depending on the cell size) of 100 ms duration on a central cell with an intensity 20 times that of the scan strength. Immediately thereafter, 12 images were collected at 20-second intervals. The fluorescence value in each polygon was then calculated by adding all pixel fluorescence values (8 bits per pixel). Inasmuch as all cells showed bleaching because of the scanning light, the fluorescence values were corrected toward a peripheral cell, showing a typical declining intensity in fluorescence as a function of time. The recovery of fluorescence in the bleached cell was calculated at 1-minute intervals and compared with recovery in the prebleach scan.

**Mechanical Stimulation**

The mechanical stimulation of single RPE cells was obtained, without damaging the cell, by inducing a short-lasting deformation of the cell with a glass micropipette (tip diameter <1 μm) mounted on a vertical microinjection system (IMT2-SYF; Narishige, Tokyo, Japan).

**RESULTS**

**Mechanical Stimulation**

**Control Conditions.** As previously described, mechanical stimulation of a single RPE cell in the presence of external Ca²⁺ induced a spreading rise of the [Ca²⁺];. The increase in [Ca²⁺]; started at the point of the mechanical stimulus, spread over the mechanically stimulated (MS) cell and was conducted, with some delay, to the neighboring 2 to 3 cell layers surrounding this MS cell (up to ±120 μm from the stimulated cells). The amplitude of the [Ca²⁺]; transient declined as a function of the distance of the neighboring (NB) cell from the MS cell (Fig. 1A). The propagation of this [Ca²⁺]; wave to the NB cells could be reversibly blocked by 6 mM of the gap junction blocker halothane or by depolarizing the cells in a medium containing 140 mM K⁺. ¹³

**Effect of Increased Glucose.** In all previously described experiments, the cells were grown in a culturing medium containing 12 mM glucose. To investigate the effect of various glucose concentrations on RPE cells, the cells were plated and grown for 72 hours in a culturing medium containing 5, 12, 14, 16, 18, 20, 25, or 50 mM glucose. Mechanical stimulation of the cells grown in all tested glucose concentrations induced a [Ca²⁺]; increase that was not different from that seen in the control experiments (Table 1, Fig. 2). Growing the cells in concentrations of 5 or 12 mM glucose did not significantly modify the increase in [Ca²⁺]; in the NB cells (P = 0.01). However, the ensuing rise in [Ca²⁺]; in the NB cells was not only reduced but also occurred less frequently when glucose was increased to a concentration of 14 mM or higher (Table 1). Counting the neighboring cells that showed a significant rise in [Ca²⁺]; (normalized increase >1.5),
TABLE 1. Averaged Rise of the Normalized Fluorescence Value in Retinal Pigment Epithelial Cultures After Mechanical Stimulation Under Various Experimental Conditions

<table>
<thead>
<tr>
<th></th>
<th>Mechanically Stimulated Cells</th>
<th>Neighboring Cells</th>
<th>% of Responsive Neighboring Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean  SE  n</td>
<td>Mean  SE  n</td>
<td></td>
</tr>
<tr>
<td>Glc 5 mM</td>
<td>8.6  0.3  24</td>
<td>5.7  0.2  159</td>
<td>97</td>
</tr>
<tr>
<td>Glc 12 mM</td>
<td>8.6  0.3  45</td>
<td>5.5  0.2  313</td>
<td>89</td>
</tr>
<tr>
<td>Glc 14 mM</td>
<td>9.3  0.3  11</td>
<td>4.7*  0.2  68</td>
<td>87</td>
</tr>
<tr>
<td>Glc 16 mM</td>
<td>9.3  0.7  11</td>
<td>2.5*  0.2  76</td>
<td>71</td>
</tr>
<tr>
<td>Glc 18 mM</td>
<td>9.0  0.5  11</td>
<td>2.2*  0.1  74</td>
<td>74</td>
</tr>
<tr>
<td>Glc 20 mM</td>
<td>8.8  0.3  23</td>
<td>2.1  0.1  114</td>
<td>74</td>
</tr>
<tr>
<td>Glc 25 mM</td>
<td>8.5  0.4  15</td>
<td>2.5  0.2  87</td>
<td>66</td>
</tr>
<tr>
<td>Glc 50 mM</td>
<td>7.8  0.3  20</td>
<td>1.8*  0.1  136</td>
<td>35</td>
</tr>
<tr>
<td>Glc 50 mM after 36 hours</td>
<td>7.6  0.8  5</td>
<td>3.9*  0.3  42</td>
<td>80</td>
</tr>
<tr>
<td>Glc 5 mM and mannitol 20 mM</td>
<td>10.3  0.7  11</td>
<td>6.8  0.4  70</td>
<td>100</td>
</tr>
<tr>
<td>Glc 12 mM and mannitol 38 mM</td>
<td>7.4  0.5  10</td>
<td>5.1  0.5  42</td>
<td>100</td>
</tr>
<tr>
<td>PMA 30' in 5 mM Glc</td>
<td>7.9  0.6  13</td>
<td>2.1*  0.2  75</td>
<td>10</td>
</tr>
<tr>
<td>PMA 30' in 12 mM Glc</td>
<td>7.3  0.7  14</td>
<td>1.8*  0.1  113</td>
<td>21</td>
</tr>
<tr>
<td>PMA 30' in 25 mM Glc</td>
<td>8.7  0.7  10</td>
<td>2.0*  0.2  63</td>
<td>25</td>
</tr>
<tr>
<td>PMA 30' in 50 mM Glc</td>
<td>8.0  0.8  8</td>
<td>1.9*  0.1  61</td>
<td>28</td>
</tr>
<tr>
<td>PMA and glucoside</td>
<td>6.8  0.5  9</td>
<td>1.5*  NA  58</td>
<td>2</td>
</tr>
<tr>
<td>Glc 12 mM and PMA 72 hours</td>
<td>9.9  1.2  11</td>
<td>6.9  0.5  60</td>
<td>97</td>
</tr>
<tr>
<td>Glc 25 mM and PMA 72 hours</td>
<td>7.8  0.4  10</td>
<td>5.9  0.3  37</td>
<td>92</td>
</tr>
<tr>
<td>Glc 50 mM and PMA 72 hours</td>
<td>8.0  0.5  11</td>
<td>6.0  0.3  55</td>
<td>81</td>
</tr>
<tr>
<td>Glc 50 mM and PMA &gt;36 hours</td>
<td>7.5  0.8  9</td>
<td>4.3  0.3  49</td>
<td>75</td>
</tr>
<tr>
<td>Sp-cAMP</td>
<td>9.5  0.5  26</td>
<td>4.5  0.3  192</td>
<td>96</td>
</tr>
<tr>
<td>Rp-cAMP</td>
<td>8.1  0.7  15</td>
<td>4.8  0.3  27</td>
<td>92</td>
</tr>
<tr>
<td>Herbinycin A</td>
<td>6.6  0.4  5</td>
<td>4.2  0.5  34</td>
<td>82</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>10.6  0.3  12</td>
<td>6.2  0.4  72</td>
<td>94</td>
</tr>
</tbody>
</table>

* Value different from the control experiments in NB cells.
PMA = phorbol 12-myristate 13-acetate; cAMP = cyclic adenosine monophosphate.

FIGURE 2. Effect of increasing glucose on the intercellular progression of a Ca 2+ wave progression during mechanical stimulation. At all tested glucose concentrations (5, 12, 14, 16, 18, 20, 25, and 50 mM), the amplitude of the increase in calcium in the mechanically stimulated cells (○, left scale) was not affected. However, when the glucose concentration in the culturing medium was increased to 14 mM or more, a reduced response in the neighboring cells (□, left scale) was observed. The percentage of responsive neighboring cells (x, right scale) gradually decreased for increasing glucose concentrations in the culturing medium.
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FIGURE 3. Changes of \([Ca^{2+}]_i\) concentrations, as estimated from the normalized fluo-3 fluorescence in mechanically stimulated and in neighboring cells during mechanical stimulation. The change of the fluo-3 fluorescence induced by mechanical stimulation is shown for the mechanically stimulated (MS; open columns) and the neighboring cells (NB; shaded columns). The percentage of neighboring cells in which an increase in \([Ca^{2+}]_i\) is observed is given by the black columns (right scale). (A) The following experimental conditions are represented: control experiments in 5 mM glucose (control Glc 5), cells cultured for 72 hours in 25 mM glucose (HGlc 25), cells cultured in 5 mM glucose plus 20 mM mannitol (mannitol 20), cells in a 30-minute incubation with 1 \(\mu\)M phorbol 12-myristate 13-acetate (PMA) in 5 mM glucose, and cells cultured for 72 hours in 25 mM glucose and with protein kinase C downregulated by PMA (HGlc 25 + PKC downreg). (B) The following experimental conditions are represented: control experiments in 12 mM glucose (control Glc 12), cells cultured for 72 hours in 50 mM glucose (HGlc 50), cells cultured in 12 mM glucose plus 38 mM mannitol (mannitol 38), cells in a 30-minute incubation with 1 \(\mu\)M PMA and 50 mM glaucine (PMA + glaucine), cells cultured for 72 hours in 50 mM glucose and with PKC downregulated by PMA (HGlc 50 + PKC downreg), and cells cultured for 72 hours in 12 mM glucose and with PKC downregulated by PMA (Glc 12 + PKC downreg). (C) The following experimental conditions are represented: the average of all control experiments (control), 6 mM halothane (halothane), a 30-minute incubation with Sp-cAMP (Sp-cAMP), a 30-minute incubation with Rp-cAMP (Rp-cAMP), a 30-minute incubation with 1 \(\mu\)M herbimycin A (herbimycin A), and a 30-minute incubation with 1 \(\mu\)M okadaic acid (Okadaic Acid). The amplitude of the normalized fluo-3 fluorescence in the mechanically stimulated cells in each of the experimental conditions did not differ from that in the control experiments. Both a decrease of this fluorescence compared with that in the control experiments (*) and a decreased percentage of responsive cells were found in the neighboring cells in the presence of 6 mM halothane; in cells cultured for 72 hours in 25 or 50 mM glucose (HGlc); and after 30-minute incubation with 1 \(\mu\)M PMA, with or without 50 mM glaucine.

revealed a decreasing percentage as a function of increasing glucose concentration (Figs. 2, 3A, 3B).

In some experiments the RPE cells were initially exposed for 3 days to 12 mM glucose and thereafter for 36 hours were exposed only to a 50-mM glucose solution. Again, the number of responsive NB cells decreased significantly, and the amplitude of the rise in \([Ca^{2+}]_i\), also decreased (\(n = 42\); Table 1).

To exclude the possibility that the observed effects of high glucose on the intercellular conduction would be caused by the increased osmolarity of the growth medium, cells were cultured in a medium containing 5 mM glucose and supplemented with 20 mM mannitol, or in a medium containing 12 mM glucose supplemented with 38 mM mannitol. These culturing media have the same osmolarity as those containing 25 mM and 50 mM glucose, respectively. On subjecting these cells to the same procedure of mechanical stimulation, both the MS and the NB cells showed an increase in \([Ca^{2+}]_i\), that was not statistically different from that observed in cells grown in the standard medium (\(P > 0.2\); Table 1, Figs. 3A, 3B).

Effect of Phosphorylation-Protein Kinase. To fulfill the purpose of the study to investigate the effect of PKC on GJC,14,51,32 cells were incubated for 30 minutes in a medium containing 5 or 12 mM glucose supplemented with 1 \(\mu\)M of PMA. This short exposure to PMA resulted in an activation of PKC, but it did not affect the increase in \([Ca^{2+}]_i\) elicited by mechanical stimulation (Figs. 3A, 3B) in the MS cell. However, only 10% and 21% of the NB cells (Fig. 1B) displayed a significant \([Ca^{2+}]_i\) increase (\(n = 75\) for the 5 mM glucose medium and \(n = 115\) for the 12 mM glucose medium, respectively). Moreover, this increase was significantly lower (\(P < 0.0001\) in both conditions) than that in the respective control conditions (Table 1, Fig. 2). The addition of 50 mM of the antioxidant glaucine\(^{40,41}\) did not modify the effect of PKC on the conduction of \(Ca^{2+}\) waves in a 12-mM glucose medium (\(n = 58\)) (Table 1, Figs. 3A, 3B).
To determine whether an additional effect of PMA could be obtained at still higher glucose concentrations, cells were incubated for 30 minutes in media containing 25 and 50 mM glucose supplemented with 1 μM PMA. No further significant decrease in the (Ca^{2+}) signal could be observed in either the MS cells (P > 0.4 and P > 0.8, respectively) or in the NB cells (P > 0.7 and P > 0.3, respectively; Table 1).

**Interaction of Glucose-Rich Medium and Protein Kinase C.** Downregulation of PKC can be obtained by prolonged exposure to 1 μM PMA. For these experiments cells were grown for 72 hours after plating in a medium containing 20, 25, or 50 mM glucose and 1 μM PMA. These cultured cells displayed the same increase in [Ca^{2+}] in both the MS and NB cells as was displayed in the respective control conditions for the matching glucose concentrations (Table 1, Figs. 3A, 3B), indicating that the inhibitory effect of the glucose-rich medium on the intercellular conduction did not occur if the PKC activity was downregulated.

Cells were also grown for 72 hours in RPMI 1640 with 12 mM glucose in the presence of 1 μM PMA to investigate the effect of a downregulation of PKC in a glucose concentration that does not decrease the intercellular Ca^{2+} signaling. The [Ca^{2+}], response after mechanical stimulation was not significantly altered in the MS cells (P > 0.7) or in the NB cells (P > 0.5) compared with the response in the control experiments (Table 1, Fig. 3B).

**Protein Kinase A.** The effect of a modulation of the PKA activity on GJCs was investigated by incubating the cells for 30 minutes in the presence of 1 μM Sp-cAMP to increase PKA activity or of 1 μM Rp-cAMP to decrease PKA activity. These modulations of the PKA activity did not affect the [Ca^{2+}] increase after mechanical stimulation, either in the MS cells (Sp-cAMP, n = 26; Rp-cAMP, n = 15) or in the NB cells (Sp-cAMP, n = 192; Rp-cAMP, n = 27; Table 1, Fig. 3C).

**Tyrosine Kinase.** To determine the effect of tyrosine kinase activity on GJCs, the effect of mechanical stimulation was investigated after incubation for 30 minutes in the presence of 1 μM herbimycin A, an inhibitor of tyrosine kinase activity. Again the increases in [Ca^{2+}], were neither affected in the MS (n = 5) or in the NB cells (n = 34; Table 1, Fig. 3C).

**Okadaic Acid.** A possible role of the activity of serine-threonine protein phosphatase on GJCs was studied by adding 1 μM of the potent protein phosphatase inhibitor okadaic acid for 30 minutes. It was found that okadaic acid did not modify the amplitude of the increase [Ca^{2+}], after mechanical stimulation or the number of responsive cells (n = 34; Table 1, Fig. 3C).

**Spatiotemporal Changes After Mechanical Stimulation.** In the previously described experiments it was demonstrated that exposure to glucose-rich solutions or modulation of the phosphorylation level did not significantly alter the changes in [Ca^{2+}], in the MS cells. To find out whether changes also occurred at the subcellular level under the various conditions, the normalized fluorescence was determined in nuclear and cytosolic areas in the MS and NB cells by drawing polygons in these two compartments. During mechanical stimulation the increase in Ca^{2+} concentration in the cell’s nucleus ([Ca^{2+}]) was higher than that in the cytosol (n = 31), suggesting the presence of a nucleocytoplasmic Ca^{2+} gradient, as already reported in other cell types. Also in the NB cells a nucleocytoplasmic Ca^{2+} increase and gradient were present (n = 45; Fig. 4). A similar pattern of increases in Ca^{2+} in the cell nucleus and cytosol occurred in MS cells cultured in 50 mM glucose (n = 18) with activated (n = 14) or downregulated PKC (n = 15). Those observations are in agreement with our previous findings. The changes in nucleic and cytosolic concentrations of Ca^{2+} in NB cells were depressed in cultures with 50 mM glucose (n = 12) and in cultures with 12 mM glucose and PKC activation (n = 12; Fig. 4). After downregulation, PKC cultures exposed to 50 mM glucose expressed Ca^{2+} transients in their NB cells, which were only slightly lower than those displayed in control conditions, confirming results at the cellular level (Fig. 4).

**Fluorescence Recovery After Photobleaching**

Analysis by the FRAP method was used as an alternative means for investigating the open state of the gap junctions and for confirming whether a modification of the intercellular GJC was responsible for the observed changes in increase of [Ca^{2+}], after mechanical stimulation. The percentage of reappearance of the fluorescence of 6-carboxyfluorescein diacetate in the bleached cell was compared for the different experimental procedures (Fig. 5). The values obtained with FRAP after a 10-minute incubation with 6 mM halothane or a 30-minute incubation with 1 μM PMA were significantly decreased compared with values recorded in the control experiments (P < 0.0005). Also, cells grown for 72 hours in a medium containing 50 mM glucose presented a significantly decreased value with 1 minute of FRAP (P < 0.005; Figs. 5, 6). However, cells grown for 72 hours in the glucose-rich medium and with PKC activity downregulated by prolonged exposure to 1 μM PMA expressed a value after 1 minute of FRAP that was not significantly different (P > 0.15) from that expressed in the control cells (Figs. 5, 6).

**DISCUSSION**

Gap junctions form intercellular communication channels in a variety of cell types. In contrast with the ion channels in their plasmalemma, they are relatively nonspecific in their permeability. Movement of the
Intercellular Communication in Retinal Pigment Epithelial Cells

Mechanically Stimulated Cells

Neighboring Cells

FIGURE 4. Calcium increase in nucleus and cytosol. The measured relative increase in fluorescence after mechanical stimulation is displayed in mechanically stimulated cells (left) and in neighboring cells (right). The increase in fluorescence in control conditions (12 mM glucose) is represented by the open columns, in cells cultured in 50 mM glucose concentration by the gray columns, and in cells exposed for 30 minutes to 1 µM phorbol 12-myristate 13-acetate by the black columns. The hatched columns represent the data obtained after growing the cells in 50 mM glucose with downregulated protein kinase C. In all tested conditions, the calcium increase in the cytoplasm was less than that in the nucleus.

FIGURE 5. Fluorescence recovery values after photobleaching. The values after 1 minute of fluorescence recovery are given for the control experiments (Control), in cells exposed to 6 mM halothane (Halothane), in cells incubated for 30 minutes with 1 µM phorbol 12-myristate 13-acetate (PMA 30'), in cells cultured for 72 hours in 50 mM glucose (HGlc), and in those cultured for 72 hours in 50 mM glucose in the presence of 1 µM PMA (HGlc&PMA). The measurements reveal a significant decrease (*) induced by 6 mM halothane, by a 30-minute incubation with 1 µM PMA, and by culture for 72 hours in 50 mM glucose. If the cells were cultured for 72 hours in 50 mM glucose in the presence of 1 µM PMA, the inhibitory effect of 50 mM glucose did not occur.

inorganic ions and also of a number of small molecules, including cAMP and 1,4,5-inositol triphosphate, occurs through the channels by passive diffusion. Some long-chained alcanols like halothane and octanol inhibit GJC, as in the results we previously reported with halothane in our cultured RPE cells. Applying the FRAP technique has confirmed this observation. It is known that GJC is dependent on voltage changes, as we have demonstrated in RPE cells using K⁺-rich medium.

Gap junctions are formed by more than 10 different types of channel proteins called connexins (Cx). These proteins are identified by a number corresponding to their molecular weight in kDa. 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 recently that RPE cells also express Cx43 in vitro (unpublished data, Malfait and De Smedt, Laboratory of Physiology, KULeuven, Belgium 1996).

Different protein kinases (PKA, PKC, and tyrosine kinase) can induce different functional changes of the GJC. Although PKA increased GJC, this effect seems to be related to an increased expression of the Cx43 gene. In the current study using Sp-cAMP or Rp-cAMP we could not observe in cultures of RPE cells any short-term effect of the modulation of PKA activity on the propagation of the [Ca²⁺] wave induced by mechanical stimulation.
Activation of PKC inhibits the GJC in many cell types by modifying the assembly of connexins at the plasma membrane level or by inhibiting the aggregation of Cx43 subunits. However, it was found that inhibition of dephosphorylation by most protein phosphatase inhibitors did not decrease GJC. Nor did we find an effect of okadaic acid on the propagation of the Ca²⁺ wave in RPE cells, as has been previously described in hamster embryo cells. This finding could indicate that cultured RPE cells in the basal state have low PKC activity. This hypothesis is also supported by finding a normal propagation of the Ca²⁺ wave in cells cultured at a normal glucose concentration after PKC downregulation.

In the current study, we have observed that the [Ca²⁺], wave progression is appreciably inhibited by increasing the PKC activity with a 30-minute exposure to PMA. The experiments using FRAP have confirmed this decrease in GJC and again suggest an important role of PKC activity on the GJC in cultured RPE cells. Both PKA and PKC are serine-threonine kinases and although it has been suggested that they might affect the GJC indirectly by activating PKC, results reported in more recent publications demonstrate that these kinases directly phosphorylate Cx43. This is a possible mechanism of action for various growth factors, including epidermal growth factor, platelet-derived growth factor, and basic fibroblast growth factor, whose receptors are transmembrane proteins with tyrosine kinase activity. However in our study, the inhibition of tyrosine kinase activity by herbimycin A did not result in a decreased [Ca²⁺] wave in the NB or MS cells, indicating that a baseline tyrosine kinase activity is not required for a functional GJC in cultured RPE cells.

It was of interest to investigate whether the gap junction conductance of cultured RPE cells is also modified...
by experimental conditions with increased glucose concentration, as occurs in diabetes.\textsuperscript{14,20–28} A decreased GJC has already been described in cultured aortic endothelial cells exposed to a high-glucose medium,\textsuperscript{14} suggesting that this change could also occur in the endothelial cells forming the internal part of the BRB. Our results indicate that the transmission of the \([Ca^{2+}]\), wave elicited by mechanical stimulation in RPE cells was also inhibited by a medium containing a glucose concentration of 14 mM (224 mg/dl) or higher. These glucose concentrations are commonly observed in poorly treated diabetes. The glucose concentrations did not affect the increase in \([Ca^{2+}]\) in the MS cells, but the amplitude of the \([Ca^{2+}]\) increase in the NB cells and the number of communicating neighboring cells declined as a function of the glucose concentration. The decreased GJC under these conditions has also been demonstrated using the FRAP technique.

Knowing that higher glucose levels can increase PKC activity,\textsuperscript{14,67–69} we investigated whether this mechanism could explain the observed changes in GJC of RPE cells. Adding PMA to a growth medium containing either 25 or 50 mM glucose for 72 hours resulted in the normal propagation of a \([Ca^{2+}]\) wave from the MS cell to NB cells and normal values of GJC, measured with FRAP. These results can be explained by the downregulation of the PKC activity by the prolonged exposure of the cells to PMA and are compatible with the hypothesis that a high glucose level could increase PKC activity, which in turn would decrease GJC, probably by phosphorylation of Cx43.

Decreased intercellular gap junction conductance is known to induce uncontrolled cell growth in various cell types,\textsuperscript{27,28} a phenomenon that has also been described in RPE cells exposed to increased glucose concentrations.\textsuperscript{24}

A proliferation of RPE cells plays an important role in the pathogenesis of proliferative vitreoretinopathy\textsuperscript{25,26}, and it is possible that increased glycemia, as occurs in poorly controlled diabetes, may decrease the GJC whereby the proliferation of RPE cells would be stimulated.

After determining the spatiotemporal changes in RPE cells, we observed that in the MS cells and in the NB cells the increase in nuclear calcium was significantly higher than that in the cytoplasm and that this nuclear–cytoplasmic ratio was not altered either by 50 mM glucose, by PMA, or by 50 mM glucose after PKC downregulation.

In conclusion, the transmission of a \([Ca^{2+}]\) wave and the GJC in cultured RPE cells can be inhibited by elevated glucose levels, possibly by increasing the PKC activity. Stimulation of PKA activity, decreasing PKA or tyrosine kinase activity, or inhibiting protein phosphatase activity did not alter the intercellular transmission of a \([Ca^{2+}]\) wave induced by mechanical stimulation.

**Key Words**
calcium wave, confocal imaging, diabetes, FRAP, gap junctions, glucose, intercellular communication, phosphorylation, retinal pigment epithelial cells

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