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Effects of Hyperglycemia and Protein Kinase C on Connexin43 Expression in Cultured Rat Retinal Pigment Epithelial Cells

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Abstract. Previous results demonstrated that the intercellular communication mediated by gap junctions in retinal pigment epithelial (RPE) cells from the healthy Long Evans (LE) rat strain is higher than that from the dystrophic Royal College of Surgeons (RCS) rat strain. We examined connexin (Cx) expression in both cell types. At the mRNA level, a qualitatively similar expression pattern was found whereby Cx26, Cx32, Cx36, Cx43, Cx45 and Cx46 were all expressed. At the protein level, only Cx43 and Cx46 were detected. Expression of both isoforms was higher in LE-RPE as compared to RCS-RPE by a factor of 1.25 and 2 respectively. Phosphorylation of Cx43 was increased upon activation of protein kinase C (PKC) by 1 μM phorbol 12-myristate 13-acetate (PMA). The phosphorylation status was not changed in hyperglycemic conditions, but this treatment strongly decreased total Cx43 levels to about 75 and 40% (in LE-RPE and RCS-RPE cells respectively) of the control level in LE-RPE cells. This decrease could be overcome by PKC downregulation. These results demonstrate that PKC activation and hyperglycemic conditions have different effects on Cx43 and that PKC is involved in the metabolic pathway induced by hyperglycemic conditions.

Keywords: Gap junctions — Connexin — Phosphorylation — Protein kinase C — Hyperglycemic conditions — Retinal pigment epithelia

Introduction

We previously investigated by mechanical stimulation the intercellular Ca^{2+}-wave propagation in control Long Evans (LE-RPE) cells and in two experimental models, associated with a blood-retinal barrier breakdown at the RPE level (Vinores et al., 1995) and an abnormal proliferation of the RPE cells (Gartner & Henkind, 1982). The culturing of LE-RPE cells in a hyperglycemic medium was used as a model to study diabetes mellitus and RPE cells from the dystrophic Royal College of Surgeons strain (RCS-RPE) were used as a model for the study of retinitis pigmentosa. RPE cells play an important role in the pathogenesis of proliferative vitreoretinopathy in diabetic patients, by migrating through a retinal break and contributing to the formation of proliferative membranes. It is known that increased glucose concentrations can induce proliferation of RPE cells. It has been postulated that elevated glucose concentrations (≥14 mM glucose) can induce a decreased gap junctional intercellular communication (GJIC), which is associated with uncontrolled cell proliferation in various cell types, including RPE cells (Inoguchi et al., 1995; In't Veld, Pipeleers & Gepts, 1986; Knorr et al., 1993; Schiavinato et al., 1991). Retinitis pigmentosa is a progressive retinal degeneration that usually leads to blindness in later life. A blood-retinal barrier breakdown at the RPE level (Vinores et al., 1995) and proliferation of the RPE cells (Gartner & Henkind, 1982) have been reported in patients suffering from the disease. We have therefore investigated whether RCS-RPE cells also presented a decreased intercellular communication. It was demonstrated that the intercellular Ca^{2+}-wave propagation in LE-RPE cells could be inhibited at glucose concentrations above 14 mM and by activation of PKC with phorbol esters (PMA). The inhibitory effect of hyperglycemic conditions could be overcome by PKC downregulation. We also presented evidence that in RCS-RPE cells the Ca^{2+}-wave propagation rate was lower than in non-dystrophic LE-RPE cells. This inhibition was even more pronounced after stimulation of PKC by PMA or in high glucose solutions. Conversely, when the PKC activity was downregulated in RCS-RPE cells, the Ca^{2+}-wave

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propagation attained the same high level found for control LE-RPE cells (Stalmans & Himpens, 1997a, 1997b, 1998, 1999; Himpens et al., 1999).

We also demonstrated that the intercellular Ca^{2+}-wave propagation in RPE cells is mediated by gap junctions and that the effects described are due to modulation of the gap junctional intercellular communication (GJIC) between RPE cells (Stalmans & Himpens, 1997a, 1997b, 1998, 1999; Himpens et al., 1999).

Vertebrate gap junctions are composed of connexins, a family of highly related transmembrane-spanning proteins (Beyer, Paul & Goodenough, 1990). These connexins are identified by the molecular mass of their polypeptide chain (e.g. Cx26, Cx32, Cx43 . . .). The important role of these channels in intercellular communication has been demonstrated for various excitable and nonexcitable cells (Brink, Cronin & Ramanan, 1996; Simon & Goodenough, 1998; Naus et al., 1999; White & Paul, 1999; Yamasaki et al., 1999). Many correlations between GJIC and cellular processes, such as cellular growth control, cell differentiation, regulation of development, tissue homeostasis etc., are described (Beyer et al., 1990; Loewenstein & Rose, 1992; White, Bruzzone & Paul, 1995; Bruzzone, White & Paul, 1996; Goodenough, Goliger & Paul, 1996; Kumar & Gilula, 1996; Lo, 1999).

Since the effects of high glucose and PKC activation on the intercellular Ca^{2+}-wave propagation and also the differences between the two strains are due to gap junction modulation and, since GJIC can be regulated at the transcriptional, translational or post-translational levels of connexins (Loewenstein, 1988; Trosko & Ruch, 1998), we wanted to compare the effects of high glucose and of modulation of PKC activity on connexin isoform expression and/or connexin phosphorylation between the two RPE cell strains.

We therefore determined the connexin expression at the mRNA and at the protein level. Our results furthermore demonstrated that activation of PKC by phorbol esters increased Cx43 phosphorylation, while the presence of a high glucose concentration in the culture medium did not affect Cx43 phosphorylation, but decreased its expression level in both strains, and especially in RCS-RPE cells.

Materials and Methods

Cell Isolation and Cell Culture

The RPE cells were isolated from retinal tissue of 3- to 8-day old pigmented Long Evans rats (Charles River Laboratories, Boston, MA) or Royal College of Surgeons rats (RCS-Rdy-P+) rats (National Institutes of Health, Genetic Resource Section, Bethesda, MD) as previously described (Chang et al., 1991). Treatment of animals conformed to the National Research Council (NRC) Guide. Cells were plated in a 6-well dish at a density of approximately 1.3×10^4cells/cm^2, and grown in a low-glucose medium (DMEM containing 5 mM glucose) supplemented with 10% (v/v) FCS, 3.5 mM L-glutamine, 85 units/ml of penicillin and 85 μg/ml streptomycin and maintained at 37°C in a 5% CO_2-incubator. In part of the experiments, the cells were grown in a high-glucose medium (DMEM containing 25 mM glucose). Previous control experiments, where cells were grown in a medium supplemented with mannitol in order to obtain the same osmolarity as the medium containing 25 mM glucose, demonstrated that effects possibly due to a hyperglycemic condition are not caused by the increased osmolarity of the growth medium (Stalmans & Himpens, 1997b). Monolayers whereby individual cells made physical contact without cell overgrowth or dome formation were obtained after 5 days, and all experiments were performed in 3 to 7-day old cultures. The rat embryonic thoracic aorta smooth muscle cell line A7r5 was obtained from the American Type Culture Collection (CRL 1444; Rockville, MD) and ROS cells, a rat osteosarcoma cell line, were obtained from Dr. G. Rodan (Merck Research Laboratories, West Point, PA). Both cell lines were used for control experiments and cultured in the same conditions as the RPE cells.

RNA Isolation from LE- and RCS-RPE Cell Cultures and PCR Analysis

We have isolated poly $(A)^+$mRNA using the Micro-Fast Track™ kit (Invitrogen, San Diego, CA) and random-primed-first-strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Roche Diagnostics, Brussels, Belgium) essentially as described earlier (De Smedt et al., 1997). RT-PCR is used for the amplification of sequences from the different rat connexins (Cx26, Cx32, Cx36, Cx40, Cx43, Cx45, Cx46 and Cx50). Selection of specific primers was based on the published rat sequences for these different connexins (Paul, 1986; Beyer, Paul & Goodenough, 1987; Miller, Dahl & Werner, 1988; Zhang & Nicholson, 1989; Paul et al., 1991; Beyer et al., 1992; Hennemann et al., 1992a; Hennemann, Schwarz & Willecke, 1992b; Kanter, Saffitz & Beyer, 1992; Kanter, Saffitz & Beyer, 1994; Condorelli et al., 1998). To amplify Cx40, we used a common primer for simultaneous amplification of Cx40 and Cx43. The complete coding sequence of the rat Cx45 is still not known. The primers for the rat Cx45 sequence were constructed in a consensus region making use of the high identity for human and mouse Cx45 cDNA. In order to obtain an optimal match with the rat mRNA, mixtures of primers were used, with the diverging nucleotides located as far as possible from the primer's 3′-end. An overview of the primers and their coordinates with respect to the rat (r), human (h) and mouse (m) connexin sequences is given in Table I.

The amplification reaction mixture (50 μl) contained 0.4-2% of the first-strand reaction product and in addition 10 nmol of each dNTP, 25 pmol of the 5′ and 3′ primers, and 2.5 units of AmpliTaq® Gold DNA polymerase (Perkin Elmer, Foster City, CA) in the supplied buffer supplemented with 1 mM Mg^{2+}. The protocol included a first cycle consisting of 10 min of denaturation at 94°C, 1 min annealing at 55°C and 2 min extension at 72°C, followed by 30 cycles consisting of the same steps but with only 1 min of denaturation. In each case, poly $(A)^+$mRNA (not treated with reverse transcriptase) was used as a negative control for contaminating genomic DNA.

Evaluation of the expression of the different mRNAs was done by polyacrylamide gel electrophoresis. The RT-PCR amplification products and the corresponding negative controls were separated on a 10% polyacrylamide gel and visualized with Vistra Green™ (Amersham International, Bucks, United Kingdom). The subsequent fluorescent signal, detected with the Storm840 FluorImager (Molecular Dynamics, Sunnyvale, CA), was expressed in relative fluorescence units.

Table 1. PCR primers

Name	cDNA genbank number	Forward primer localization (nucleotides)	Reverse primer localization (nucleotides)
Cx26 (r)	X51615	310–336	767–793
Cx32 (r)	X04070	69–95	429–458
Cx36 (r)	Y16898	735–764	1330–1359
Cx40 (r)[a]	M83092	191–218	487–514
Cx43 (r)[a]	M19317	392–419	697–724
Cx43 (r)	M19317	275–304	1039–1067
Cx45 (m)[b]	X63100	453–479	1020–1048
Cx45 (h)[b]	U03493	2–23	564–592
Cx46 (r)	X57970	258–287	948–977
Cx50 (m)	M91243	559–584	977–1002

Forward and reverse primers selected for RT-PCR amplification of Cx26, Cx32, Cx36, Cx40 Cx43, Cx45, Cx46 and Cx50. The coordinates for localisation refer to the rat (r), mouse (m) and human (h) connexin sequences. The reverse primers are indicated as the reverse complement.

[a] Consensus primers for these two sequences were used for amplification of Cx40(r) and Cx43(r).

[b] Since the Cx45(r) sequence is not yet available, consensus primers based on the murine and human sequences were used for its amplification (*see* text for additional information).

ImageQuant NT4.2 software (Molecular Dynamics) allowed the volume quantification of each signal. The volume was defined as the integrated intensity of all the pixels in the spot, after subtraction of the background intensity and was proportional to the amount of DNA under investigation. Both in LE-RPE and RCS-RPE samples, the intensity of each calculated signal was normalized by comparison with the corresponding intensity of an internal standard, GAPDH (glyceraldehyde-phosphate-dehydrogenase).

Membrane Preparation and Protein Analysis

Cells were washed with PBS (without Ca^{2+} and Mg^{2+}), scraped in 1 ml of homogenization medium (in mM: 10 Tris-HCl, pH 7.4, 0.5 $MgCl_2$, 0.8 benzamidine, 0.2 PMSF; 1 μM leupeptin, 1 μM pepstatin A, 75 nM aprotinin) and subsequently homogenized by brief sonication (2 × 25 sec). A total microsomal fraction was precipitated by centrifugation (30 min, 136000 × g) and the pellet was resuspended in 40 μl of end medium (in mM: 20 Tris-HCl, pH 7.4, 300 sucrose, 0.8 benzamidine, 0.2 PMSF), frozen in liquid N_2 and stored at −85°C. Protein determination was according to Lowry et al. (1951).

Antibodies against Connexins

Monoclonal antibodies and affinity-purified rabbit polyclonal antibodies against Cx26 and Cx32 were purchased from Zymed (South San Francisco, CA). Affinity-purified rabbit anti-rat Cx46 antiserum was obtained from Biotrend (Köln, Germany). The polyclonal antibody against Cx36 was a generous gift from Barbara Teubner (Institut für Genetik, Univ. Bonn, Germany). A rabbit polyclonal antibody specific for Cx45 was kindly provided by Dr. T.H. Steinberg (Institute for Infectious Diseases, Washington University, St. Louis, USA). A polyclonal antibody against Cx43, similar to a previously described antibody (Musil, Beyer & Goodenough, 1990a), was raised in rabbits: a synthetic peptide, corresponding to amino acids 252-271 of the rat sequence was coupled to keyhole limpet hemocyanin. Two rabbits (SB135 and SB136) were repeatedly immunized with the coupled peptide and both developed an antibody specifically recognizing Cx43. In this study, the SB135 antiserum was used and control experiments indicated no cross-reaction with other connexin isoforms or any other protein (*data not shown*).

Immunoblotting of Connexins

Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Nonspecific binding sites on the membranes were blocked with PBS containing 0.1% Tween-20 and 5% nonfat dry milk. Blots were incubated with the specific anti-Cx antibody (anti-Cx26 and anti-Cx32: 1/1000; anti-Cx36: 1/500; anti-Cx43: 1/1500; anti-Cx45: 1/2000; anti-Cx46: 1/1000) for 2 hr at room temperature and subsequently washed 3 times in PBS + 0.1% Tween-20. Detection was performed using secondary antibodies coupled to alkaline phosphatase at a 1/8000 dilution and using Vistra™ ECF as substrate, according to the manufacturer's instructions (Amersham International, Bucks, United Kingdom). Dephosphorylation of the ECF substrate led to the formation of a stable fluorescent product. The subsequent fluorescent signal, detected with the Storm840 FluorImager, was expressed in relative fluorescence units. Volume quantification of those signals was performed as described above. For statistical analysis, the Student's t test was used.

Alkaline Phosphatase Treatment

Total microsomes were suspended in 5 ml of buffer (in mM: 100 Tris-HCl, pH 7.4, 40 NaCl, 1 $MgCl_2$, 0.8 benzamidine, 0.2 PMSF, 1 μM leupeptin, 1 μM pepstatin A, 75 nM aprotinin). After centrifugation (30 min, 136000 × g), the pellet was resuspended in 40 μl buffer supplemented with 0.1% SDS. Molecular biology grade calf alkaline phosphatase (160 U; Roche Diagnostics, Brussels, Belgium) was added to 20 μl of the microsomes. Equal amounts of H_2O were added to the remaining 20 μl of the microsomes. After incubating the samples for 10 min at room temperature, the reaction was stopped by addition of loading buffer to a final concentration of 3% SDS, 115 mM sucrose, 65 mM Tris-HCl (pH 6.8) and 1% β-mercaptoethanol and boiling the samples for 5 min at 95°C. Finally, the samples were submitted to SDS-polyacrylamide gel electrophoresis and immunoblotted as described above.

Results

Connexin Expression in LE-RPE and in RCS-RPE Primary Cultures

We first made a qualitative comparison of the expression of connexin mRNAs in LE-RPE and RCS-RPE cells, to investigate possible differences in connexin isoform expression between the two strains (Fig. 1). We screened the LE-RPE and the RCS-RPE mRNAs using specific RT-PCR primers and looked for the most ubiquitous connexins i.e. Cx26, Cx32, Cx36, Cx40, Cx43, Cx45, Cx46 and Cx50. Cx30, Cx30.3, Cx31, Cx31.1 and Cx37 were not investigated since Cx30, Cx30.3, Cx31 and Cx31.1 were not expressed in the mouse retina and since Cx37 was only detected within blood vessels of the gan-

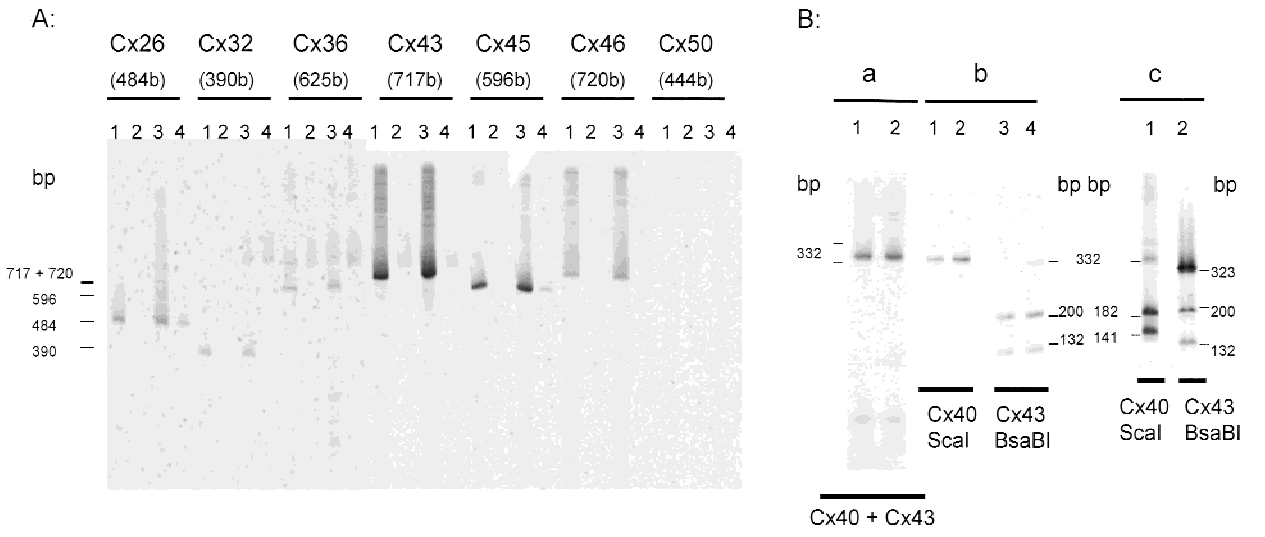

Fig. 1. Screening of different connexin isoforms in LE-RPE and RCS-RPE primary cultures. (*A*) Poly $(A)^+$mRNA was reverse transcribed and RT-PCR-amplified as described in the Materials and Methods. LE-RPE and RCS-RPE cells (lanes *1* & *3* respectively) were screened and the amplified connexin isoforms are indicated (Cx26, Cx32, Cx36, Cx43, Cx45, Cx46 and Cx50). Poly $(A)^+$mRNA, not treated with reverse transcriptase, was used as a negative control in each case (lanes *2* & *4* respectively). The bars indicate the length (in bp) of the PCR products. (*B*) Reverse transcription and RT-PCR were performed with the poly $(A)^+$mRNA isolated from LE-RPE or RCS-RPE primary cultures (*a* and *b*) and from A7r5 cells (*c*) as described in Materials and Methods. A common primer was used for optimal amplification of Cx40 and Cx43 (*a*). The two amplified products were discriminated using restriction enzymes with a specific cleavage site in the diverging part of the sequence. (*b*). The first pair of lanes (*1, 2*) shows that ScaI, which specifically cuts Cx40, had no effect. The second pair of lanes (*3, 4*) shows the effect of BsaBI, which specifically cuts Cx43. Lanes *1, 3* refer to normal low glucose conditions for LE-RPE and lanes *2, 4,* for RCS-RPE. (*c*). Lanes *1* and *2* represent the effect of ScaI and of BsaBI, respectively, for A7r5 cells. The bars indicate the length (in bp) of the PCR products and of the restriction fragments. In lane *C2,* the 323 bp band represents the uncut Cx40 product.

glion cell layer, suggesting endothelial expression (Güldenagel et al., 2000). In each case poly $(A)^+$mRNA without reverse transcriptase was used as a negative control in LE-RPE (Fig. 1*A*, lanes *2*) and in RCS-RPE cells (Fig. 1*A*, lanes *4*). To amplify Cx40, we used a common primer for simultaneous amplification of Cx40 and Cx43 (Fig. 1*B* (*a*)).

In both strains (Fig. 1*A:* LE-RPE cells, lanes *1* and RCS-RPE cells, lanes *3*) we observed the presence of Cx26, Cx32, Cx36, Cx43, Cx45 and Cx46 mRNA. Cx50 could not be detected even after additional amplification cycles. The Cx36 signals were obtained after amplification for 34 cycles instead of for 30 cycles for the other connexins. For Cx26 and Cx45, there was a weak signal in the negative control indicating a slight contamination by genomic DNA in RCS-RPE cells (Fig. 1*A*, lanes *4*). As the values for the negative controls (without reverse transcriptase) were 7 times (Cx26) or 33 times (Cx45) lower than the values for the corresponding RT-PCR samples, it can be concluded that both Cx26 and Cx45 mRNA are also expressed in those cells. For Cx40, we had to discriminate with specific restriction enzymes between Cx40 and Cx43 because the two amplification products have approximately the same length (Fig. 1*B* (*b*)). When BsaBI, an enzyme specific for Cx43, was used, the expected restriction fragments of 200 and 132 nucleotides were revealed. When ScaI, an enzyme specific for Cx40, was added, no restriction fragments were detected (Fig. 1*B* (*b*)), indicating that Cx40 is not expressed in RPE cells. To validate this screening method we applied the same procedure on the smooth muscle cell line A7r5, a cell line known to express Cx40 (Beyer et al., 1992). As can be seen in Fig. 1*B* (*c*), the method revealed in A7r5 cells both Cx40 restriction fragments of 182 and 141 bp (lane *1*) in addition to the Cx43 restriction fragments of 200 and 132 bp (lane *2*).

In order to find out whether the expression levels of the connexin mRNAs in LE- and RCS-RPE cells in the control condition are different, the intensity of each signal was normalized to that of GAPDH, used as an internal standard. In each of three experiments, we observed that Cx43 and Cx45 were significantly more expressed in LE-RPE than in RCS-RPE cells (*data not shown*).

Connexin Isoforms in LE-RPE and RCS-RPE under Hyperglycemic Conditions

The expression of connexin isoforms in LE-RPE and RCS-RPE cells, cultured in low (5 mM) glucose medium, in low glucose medium with PKC activation or after PKC downregulation, cultured in high (25 mM) glucose medium and in high glucose medium with PKC downregulation, were compared. PKC activation was induced

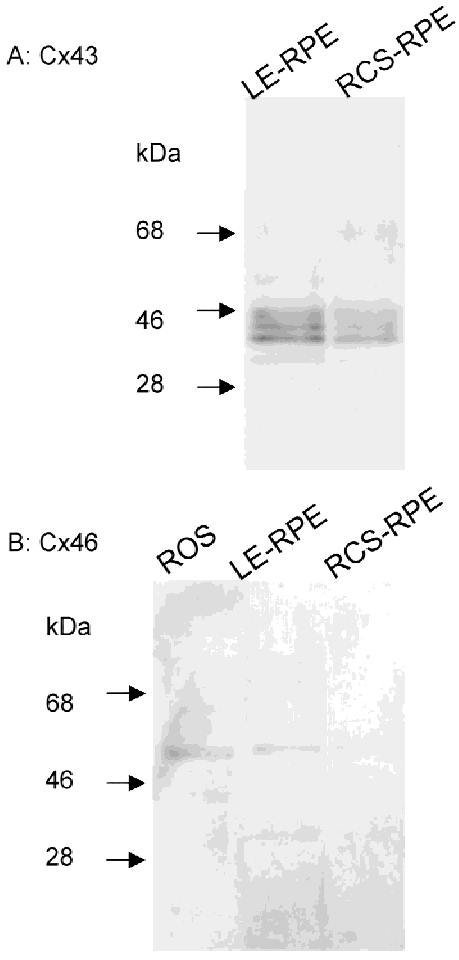

Fig. 2. Detection of Cx43 and Cx46 at the protein level in LE-RPE and RCS-RPE primary cultures. Equal amounts (10 μg) of total microsomes were separated on a 10% SDS-polyacrylamide gel and transferred to Immobilon-P. Both Cx43 (*A*) and Cx46 (*B*) were detected with the respective isoform-specific antibody. Total microsomes prepared from ROS cells were used as a positive control for Cx46. The arrows indicate the positions of the molecular weight markers.

by treatment of the cultured cells for 30 min with 1 μM PMA before RNA preparation, while PKC downregulation was achieved by a 72-hr treatment with PMA, since Nishikawa et al. (1995) have demonstrated that downregulation of PKC can be obtained by a prolonged exposure to 1 μM TPA. We analyzed in those conditions both Cx43 and Cx45, which had the highest mRNA expression level. Both Cx43 and Cx45 mRNAs were expressed after PKC modulation in both normal and hyperglycemic conditions. Similar results were obtained for Cx26, Cx32 and Cx46. Data were not shown since no differences in connexin isoform expression between either primary culture could be demonstrated. We can thus conclude that high-glucose conditions and PKC modulation did not affect the mRNA expression pattern of the connexin isoforms in RPE cells.

Detection of Connexins at the Protein Level

It was important to verify the presence of the various connexin isoforms at the protein level. Immunoblot analysis with an antibody specific for the Cx43 isoform revealed the presence of Cx43 in RPE cells (Fig. 2*A*). Moreover, the antibody recognized different protein species of Cx43 that represent different phosphorylation states of the protein (*see* below). Cx46 was detected as a weak single band with a M_r of about 50 kDa in RPE cells, as well as in ROS cells, an osteosarcoma cell line used as a positive control for Cx46 (Fig. 2*B*). No additional phosphorylated Cx46 species were detected in either RPE or ROS cells. Three independent immunoblotting experiments using a specific antibody against Cx45 (Steinberg et al., 1994; van Veen, van Rijen & Jongsma, 2000) could not demonstrate the presence of Cx45 in LE- and RCS-RPE cells. This indicated that Cx45 is not present at the protein level in RPE cells or is only present at levels below the detection limit of the antibody. Cx26, Cx32 and Cx36 were also undetectable at the protein level, although we could detect Cx26 and Cx32 in a mouse liver lysate which was used as a positive control (*data not shown*). The lack of detection of Cx26, Cx32 and Cx36 at the protein level correlated with the low level of mRNA for these isoforms (Fig. 1).

Detection of the Phosphorylation Level of Cx43

Western blot analysis revealed the presence of different Cx43 protein species, which represented the nonphosphorylated form (NP) and the phosphorylated forms of Cx43 (P1, P2, Px) migrating in the 40–46 kDa range (*data not shown*). The P1, P2 and the Px form refer to post-translational addition of phosphate, which is accompanied with a shift in the apparent molecular weight (Musil et al., 1990b). Cx43 is first phosphorylated to the P1 form and then to the slower migrating P2 form. The Px form is a hyperphosphorylation state, which can be induced in various cell types by PKC activation, and which is accompanied by a decreased GJIC. It should be noted that the P1/P2/Px nomenclature does not indicate the number of phosphate residues attached to each form since the stoichiometry of phosphorylation is unknown (Musil et al., 1990b). In control conditions, the ratio of [P2 + Px]/[NP + P1] in LE-RPE cells was 0.94 ± 0.14 ($n = 12$), a value not significantly different from the level measured in RCS-RPE cells (0.80 ± 0.08; $n = 12$).

To confirm that the Cx43 bands with slower electrophoretic mobilities indeed represented phosphorylated products, dephosphorylation experiments were performed (*data not shown*). Treatment of total microsomes from LE-RPE cells and RCS-RPE cells with alkaline phosphatase converted Cx43-P1, Cx43-P2 and Cx43-Px forms into the Cx43-NP form. A quantitative recovery

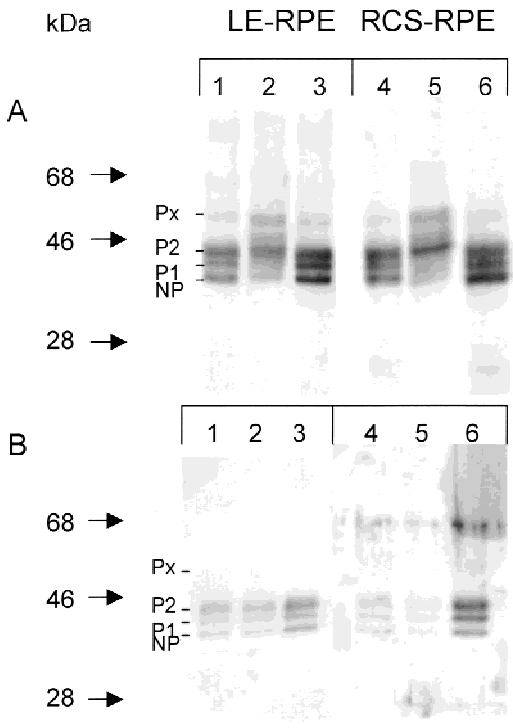

Fig. 3. The effect of PMA on the state of phosphorylation of Cx43 is time-dependent. Immunoblot of Cx43 in total microsomes obtained from LE-RPE (lane *1–3*) and RCS-RPE (lane *4–6*): (*A*) control cultures (lanes *1* and *4*) and cultures treated with 1 μM PMA for 30 min, which results in a PKC activation (lanes *2* and *5*), or for 72 hr, which results in a PKC downregulation (lanes *3* and *6*). (*B*) control cultures (lanes *1* and *4*), cells grown in hyperglycemic conditions (25 mM) (lanes *2* and *5*) and cells grown in high glucose medium with all PKC activity downregulated (lanes *3* and *6*). The positions of the nonphosphorylated (NP) and phosphorylated (P1, P2, Px) forms of Cx43 are indicated. The arrows indicate the positions of molecular weight markers.

of Cx43 immunoreactivity at the electrophoretic mobility corresponding to the NP-form was induced. Control experiments with water instead of alkaline phosphatase did not lead to such a conversion, indicating that the increase in migration rate of the Cx43 bands was indeed due to dephosphorylation catalyzed by alkaline phosphatase.

Effect of PMA and High Glucose on Cx43 Phosphorylation

In view of the proposed effect of protein kinases in general, and of PKC in particular, on intercellular communication we investigated the possible occurrence of changes in the levels of Cx43 and Cx46 phosphorylation under different experimental conditions. To investigate the effect of direct PKC activation on Cx43 phosphorylation, we treated both LE-RPE and RCS-RPE cells with 1 μM PMA for 30 minutes. We observed a change in the electrophoretic migration of Cx43 bands (Fig. 3*A*). Direct PKC activation induced a significant increase of the ratio [P2 + Px]/[NP + P1] as compared with control cells in both cell strains. This increase resulted from a rise in [P2 + Px] with a concomitant reduction in [NP + P1]. The ratio in LE-RPE and RCS-RPE cells increased to a value of, respectively, 3.21 ± 0.28 ($n = 4$; $P < 0.05$ vs 0.94 ± 0.14 in LE-RPE control cells) and 2.51 ± 0.31 ($n = 5$; $P < 0.01$ vs 0.80 ± 0.08 in RCS-RPE control cells) indicating an increase in P2 and Px formation at the expense of the NP and P1 forms. In both cell lines PKC downregulation by 72 hr PMA application did not result in a significant difference in phosphorylation levels as compared to control conditions (Fig. 3*A*). From these experiments we conclude that direct activation of PKC by short-term treatment with phorbol esters induced an increased phosphorylation of Cx43.

We did not find a significant effect of culturing the RPE cells in the presence of high glucose concentrations (25 mM) on the [P2 + Px]/[NP + P1] ratio in LE- or RCS-RPE cells (1.06 ± 0.14; $n = 9$ in LE-RPE cells and 0.75 ± 0.10, $n = 4$ in RCS-RPE cells), as compared to culturing the cells in normal glucose (5 mM) medium (0.94 ± 0.14, $n = 12$ in LE-RPE cells and 0.80 ± 0.08, $n = 12$ in RCS-RPE cells). Downregulation of PKC in high glucose medium did also not affect the [P2 + Px]/[NP + P1] ratio in LE-RPE cells (Fig. 3*B*). In none of the experimental conditions used, including PKC activation or culture in high glucose medium, phosphorylated forms of Cx46 could be detected (*data not shown*).

The Cellular Level of Cx43 and Cx46 in RPE Cells

As a further step, the total level of Cx43 cellular protein in RPE cells was measured. Therefore, total microsomal proteins were separated on a 10% SDS-polyacrylamide gel and transferred to Immobilon-P. The amount of Cx43 was determined using the anti-Cx43 antibody, taking all molecular weight species into account. There was a small, but significant ($P < 0.1$) difference in Cx43 level between LE-RPE and RCS-RPE control cells (Table 2, *A*), the latter having a 20% lower Cx43 level.

The cellular level of Cx46 was determined with the anti-Cx46 antibody. Cx46 was expressed at about 2 times higher levels in LE-RPE cells than in RCS-RPE cells ($P < 0.05$), whereas ROS cells expressed levels which were more than twice the levels found in RCS-RPE cells (Table 2, *B*).

Effect of PMA and of High Glucose on the Cellular Level of Cx43 and Cx46

As could be expected, there was no effect of PKC activation (30 minutes incubation with 1 μM PMA) on the level of Cx43 as compared to the level in normal low

Table 2. Total Cx43 and Cx46 cellular level in LE-RPE and RCS-RPE cells

A	Cx43 (ratio)				B	Cx46	
Treatment	LE-RPE	(*n*)	RCS-RPE	(*n*)	Cells	Ratio	(*n*)
Control (low glucose)	1	(12)	0.80 ± 0.14^{a}	(12)	ROS/LE-RPE	1.89 ± 0.18^{b}	(4)
30 min PMA	0.99 ± 0.18	(4)	0.86 ± 0.35	(6)	ROS/RCS-RPE	2.88 ± 0.33^{c}	(4)
72 hr PMA	1.60 ± 0.32^{a}	(5)	1.90 ± 0.34^{a}	(10)			
hglc	0.78 ± 0.06^{c}	(10)	0.42 ± 0.37^{b}	(6)	RCS-RPE/LE-RPE	0.58 ± 0.27^{b}	(8)
hglc + 72 hr PMA	2.60 ± 0.64^{a}	(7)	5.12 ± 0.45^{b}	(3)			

A. Analysis and comparison of the Cx43 levels in LE- and RCS-RPE cells in normal low glucose conditions, after activation by phorbol esters (30 min PMA), PKC downregulation (72 hr PMA), growth in 25 mM glucose (hglc) or PKC downregulation in high glucose (hglc + 72 hr PMA). Analysis was performed on 10 μg protein samples after SDS-PAGE, electrotransfer and immunodetection. The observed Cx43 density was normalized to the LE-RPE cell control low glucose density (ratio). **B.** Analysis of the total cellular level of Cx46 in ROS, LE-RPE and RCS-RPE cells cultured in the normal low glucose condition. For statistical analysis, the Student's t test was used ([a], $P < 0.1$; [b], $P < 0.05$; [c], $P < 0.01$). Data are means ± SEM; *n* is the number of assays.

glucose conditions for both LE-RPE and RCS-RPE cells (Fig. 3*A*; Table 2, *A*). Upon downregulation of all PKC activity (72 hr incubation with 1 μM PMA), the Cx43 level of both cell strains showed a significant increase (1.6-fold and 1.9-fold, for LE-RPE and RCS-RPE cells respectively) in comparison to the level in LE-RPE cells at normal glucose concentrations. The Cx43 level after PKC downregulation was similar in LE-RPE and RCS-RPE cells. Incubation of the cells at high glucose concentration, however, caused a significant decrease of the Cx43 level to about 78 and 42% of the control low glucose Cx43 value in LE-RPE cells (Fig. 3*B*; Table 2, *A*). Remarkably, the observed decreases in the high glucose medium could be prevented by PKC downregulation; in the latter condition the Cx43 level showed a significant increase in comparison with the level in normal low glucose condition (2.60-fold and 5.12-fold for LE-RPE and RCS-RPE cells respectively). We conclude that high glucose reduces the total cellular Cx43 level but that this effect is counteracted and reversed by PKC downregulation. The hyperglycemic effect was more pronounced in RCS-RPE cells, suggesting an additional activation of PKC by high glucose.

The low intensities of the observed Cx46 signals in the control condition, in high glucose and after PMA treatment, precluded a determination of eventual significant differences between the cell types.

Discussion

Connexins are encoded by a gene family, the members of which are differentially expressed in various types of tissues (Dermietzel & Spray, 1993; Goodenough et al., 1996; White et al., 1995). We determined the presence of Cx26, Cx32, Cx43, Cx45 and Cx46 at the mRNA level in LE- as well as in RCS-RPE cells. The same connexin isoforms were expressed in both strains cultured in normal low glucose solutions as well as in hyperglycemic conditions or after PKC modulation. Cx40 and Cx50 were not expressed in RPE cells.

At the protein level, we could only detect Cx43 and Cx46 in both strains. The fact that Cx26, Cx32, Cx36 and Cx45 were detected in RPE cells at the mRNA level but not at the protein level, suggests a low expression level of those four isoforms, below the detection limit of our antibodies.

The best characterized, widely expressed connexin is Cx43, found in heart (Beyer et al., 1987) and many other tissues (Beyer et al., 1989; Dermietzel & Spray, 1993) as well as in a number of established cell lines (Dermietzel & Spray, 1993; Musil et al., 1990b). Mutations in Cx43 have been reported to underlie various cardiac malformations in viscero-atrial heteroataxia syndromes (White & Paul, 1999). Cx43 is also an important connexin in epithelia (Beyer et al., 1989; Dermietzel & Spray, 1993). In RPE cells, we detected the Cx43 protein both by immunoblotting and immunochemistry. By immunochemistry, Cx43 was detected in the plasma membrane as well as intracellularly. The plasma membrane localization makes it conceivable that Cx43 plays an important role in the GJIC between RPE cells.

Cx43 is a phosphoprotein; in basal conditions, Cx43 is present in RPE cells in a nonphosphorylated (NP) and three phosphorylated (P1, P2, Px) forms. Alkaline phosphatase treatment converted all phosphorylated forms into the nonphosphorylated form in RPE cells, as was also observed in other cell types (Laird, Puranam & Revel, 1991; Musil et al., 1990b).

In spite of the different behaviour of the intercellular Ca^{2+}-wave during mechanical stimulation (Himpens et al., 1999), we found no difference of the basal [P2 + Px]/[NP + P1] ratio between the LE-RPE and RCS-RPE cell strains.

Treatment with phorbol esters increased the Cx43 phosphorylation level as deduced from the increase in

the [P2 + Px]/[NP + P1] ratio. Our data are in agreement with findings reported earlier in Madin-Darby canine kidney cells (Berthoud et al., 1992), in rat liver epithelial cells (Asamoto et al., 1991; Berthoud et al., 1993) and in Novikoff hepatoma cells (Lampe, 1994). PKC-dependent phoshorylation of Cx43 converts the NP and P1 form to the P2 and Px form. This effect was found in both strains and could be prevented by PKC downregulation. Since PKC downregulation did not result in a complete dephosphorylation of Cx43, the basal Cx43 phosphorylation is probably caused by other protein kinase activity or by a PMA-insensitive PKC isoenzyme. Our findings of an increased Cx43 phosphorylation by PKC activation together with the finding of decreased intercellular communication in this condition is in agreement with Saez and coworkers (Saez et al., 1993). They gave indications that activation of PKC by phorbol ester tumor promotors (like PMA) generally resulted in a reduction in GJIC accompanied by phosphorylation of Cx43. Serine-368 was identified as a major phosphorylation site that is phosphorylated by PKC in vitro and in vivo in cells stimulated with PMA (Lau et al., 1996).

High glucose solutions reduced the GJIC in RPE (Himpens et al., 1999), endothelial (Inoguchi et al., 1995) and smooth muscle (Kuroki et al., 1998) cells. In smooth muscle cells this is associated with a rise in Cx43 phosphorylation which could be reversed by PKC inhibition with staurosporin or calphostin C (Kuroki et al., 1998). In RPE cells, the effect of high glucose on the GJIC could also be prevented by PKC downregulation (Stalmans & Himpens, 1997b) and we therefore hypothesized that PKC activation could be involved. We found, however, no evidence for a rise of the [P2 + Px]/[NP + P1] ratio upon growth in high glucose solutions. This suggests that the pathway used by high glucose is more complex than the short-term activation of PKC by PMA. Analysis of the specific role of different PKC isoforms, such as PKCα and PKCβ, that may play an important role in intercellular Ca^{2+}-signalling (Ishii et al., 1996), is one possibility to resolve this question.

Finally, the cellular level of Cx43 was compared in the different experimental conditions because it has already been described that a difference in GJIC can be due to a change in the connexin level (Bager et al., 1994; Oh, Grupen & Murray, 1991). There is only a small difference in Cx43 level between the LE-RPE and RCS-RPE cells. This difference was abolished after downregulation of all PKC activity, which suggests a higher basal PKC activity in RCS-RPE cells as compared with LE-RPE cells. After direct PKC activation with phorbol esters, no change in Cx43 level was observed. We found, however, that the total cellular Cx43 level was significantly reduced after growth in high glucose medium to about 78 and 42% of the Cx43 concentration in LE-RPE cells. The decrease by high glucose could be prevented and the Cx43 level was even significantly increased after PKC downregulation in the high glucose medium, which is an additional indication that PKC may be involved in some parts of the metabolic pathway induced by high glucose. This decrease in the cellular Cx43 level was more pronounced in RCS-RPE cells than in LE-RPE cells, which indicated an additional activation of PKC by high glucose concentrations. These data may indicate that, in addition to the gap junctional permeability, also the number of functional connections between the cells may play an important role in GJIC in RPE cells. This is in agreement with the reported rise of GJIC in C6 glioma cells, transfected with Cx43 (Charles et al., 1992). Further experiments are required to find out whether the decrease of Cx43 concentration observed in high-glucose medium is located at the level of the plasma membrane or intracellularly or both.

Cx46 is the second connexin detected in RPE cells by immunoblotting. Cx46 expression has been typically associated with plasma membrane gap junction channels in lens (Paul et al., 1991). In total lens protein extracts, two forms of Cx46 were found; a phosphorylated form with a M_r of 68 kDa and a nonphosphorylated form with a M_r of 53 kDa (Koval et al., 1997) and the ability to produce the 68 kDa form is correlated with the ability to form gap junctions containing Cx46. Koval et al. (1997) also illustrated that primary rat osteoblastic cells and two osteosarcoma cell lines (ROS and UMR cells) only produce the 53 kDa (nonphosphorylated) form of Cx46 and that this connexin, in contrast with Cx43 and Cx45 in those cells, was largely retained as a monomer in a trans-Golgi compartment. This study suggests that oligomerization may be required for connexin transport from the trans-Golgi cistern to the cell surface. Cx46 is detected in RPE cells as a single band with a M_r of about 50 kDa, even after PKC activation or culturing in high glucose solutions. Therefore, Cx46 in RPE cells could be retained in the trans-Golgi cistern as a monomer. In the latter case, Cx46 won't take part in forming functional gap junction channels in RPE cells.

In summary, we have found that the changes in Cx43 phosphorylation or in Cx43 cellular level, in both LE-RPE and RCS-RPE cells, paralleled the earlier observations of the effects on intercellular Ca^{2+}-wave propagation. The difference in the intercellular Ca^{2+}-wave propagation between LE- and RCS-RPE cells is reflected in quantitative differences in the Cx43 level in the two cell types.

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References

Asamoto, M., Oyamada, M., el Aoumari, A., Gros, D., Yamasaki, H. 1991. Molecular mechanisms of TPA-mediated inhibition of gap-junctional intercellular communication: evidence for action on the assembly or function but not the expression of connexin 43 in rat liver epithelial cells. *Mol. Carcinog.* **4:**322–327

Bager, Y., Kenne, K., Krutovskikh, V., Mesnil, M., Traub, O., Warngard, L. 1999. Alteration in expression of gap junction proteins in rat liver after treatment with the tumour promoter 3,4,5,3′,4′-pentachlorobiphenyl. *Carcinogenesis* **15:**2439–2443

Berthoud, V.M., Ledbetter, M.L., Hertzberg, E.L., Saez, J.C. 1992. Connexin43 in MDCK cells: regulation by a tumor-promoting phorbol ester and Ca^{2+}. *Eur. J. Cell. Biol.* **57:**40–50

Berthoud, V.M., Rook, M.B., Traub, O., Hertzberg, E.L., Saez, J.C. 1993. On the mechanisms of cell uncoupling induced by a tumor promoter phorbol ester in clone 9 cells, a rat liver epithelial cell line. *Eur. J. Cell. Biol.* **62:**384–396

Beyer, E.C., Kistler, J., Paul, D.L., Goodenough, D.A. 1989. Antisera directed against connexin43 peptides react with a 43-kD protein localized to gap junctions in myocardium and other tissues. *J. Cell Biol.* **108:**595–605

Beyer, E.C., Paul, D.L., Goodenough, D.A. 1987. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* **105:**2621–2629

Beyer, E.C., Paul, D.L., Goodenough, D.A. 1990. Connexin family of gap junction proteins. *J. Membrane Biol.* **116:**187–194

Beyer, E.C., Reed, K.E., Westphale, E.M., Kanter, H.L., Larson, D.M. 1992. Molecular cloning and expression of rat connexin40, a gap junction protein expressed in vascular smooth muscle. *J. Membrane Biol.* **127:**69–76

Brink, P.R., Cronin, K., Ramanan, S.V. 1996. Gap junctions in excitable cells. *J. Bioenerg. Biomembr.* **28:**351–358

Bruzzone, R., White, T.W., Paul, D.L. 1996. Connections with connexins: the molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238:**1–27

Chang, C.W., Roque, R.S., Defoe, D.M., Caldwell, R.B. 1991. An improved method for isolation and culture of pigment epithelial cells from rat retina. *Curr. Eye Res.* **10:**1081–1086

Charles, A.C., Naus, C.C., Zhu, D., Kidder, G.M., Dirksen, E.R., Sanderson, M.J. 1992. Intercellular calcium signaling via gap junctions in glioma cells. *J. Cell Biol.* **118:**195–201

Condorelli, D.F., Parenti, R., Spinella, F., Trovato Salinaro, A., Belluardo, N., Cardile, V., Cicirata, F. 1998. Cloning of a new gap junction gene (Cx36) highly expressed in mammalian brain neurons. *Eur. J. Neurosci.* **10:**1202–1208

De Smedt, H., Missiaen, L., Parys, J.B., Henning, R.H., Sienaert, I., Vanlingen, S., Gijsens, A., Himpens, B., Casteels, R. 1997. Isoform diversity of the inositol trisphosphate receptor in cell types of mouse origin. *Biochem. J.* **322:**575–583

Dermietzel, R., Spray, D.C. 1993. Gap junctions in the brain: where, what type, how many and why? *Trends Neurosci.* **16:**186–192

Gartner, S., Henkind, P. 1982. Pathology of retinitis pigmentosa. *Ophthalmology* **89:**1425–1432

Goodenough, D.A., Goliger, J.A., Paul, D.L. 1996. Connexins, connexons, and intercellular communication. *Annu. Rev. Biochem.* **65:**475–502

Güldenagel, M., Sohl, G., Plum, A., Traub, O., Teubner, B., Weiler, R., Willecke, K. 2000. Expression patterns of connexin genes in mouse retina. *J. Comp. Neurol.* **425:**193–201

Hennemann, H., Kozjek, G., Dahl, E., Nicholson, B., Willecke, K. 1992a. Molecular cloning of mouse connexins26 and -32: similar genomic organization but distinct promoter sequences of two gap junction genes. *Eur. J. Cell. Biol.* **58:**81–89

Hennemann, H., Schwarz, H.J., Willecke, K. 1992b. Characterization of gap junction genes expressed in F9 embryonic carcinoma cells: molecular cloning of mouse connexin31 and -45 cDNAs. *Eur. J. Cell. Biol.* **57:**51–58

Himpens, B., Stalmans, P., Gomez, P., Malfait, M., Vereecke, J. 1999. Intra- and intercellular Ca^{2+} signaling in retinal pigment epithelial cells during mechanical stimulation. *FASEB J.* **13:**S63–S68

Inoguchi, T., Ueda, F., Umeda, F., Yamashita, T., Nawata, H. 1995. Inhibition of intercellular communication via gap junction in cultured aortic endothelial cells by elevated glucose and phorbol ester. *Biochem. Biophys. Res. Commun.* **208:**492–497

In't Veld, P.A., Pipeleers, D.G., Gepts, W. 1986. Glucose alters configuration of gap junctions between pancreatic islet cells. *Am. J. Physiol.* **251:**C191–C196

Ishii, H., Jirousek, M.R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S.E., Kern, T.S., Ballas, L.M., Heath, W.F., Stramm, L.E., Feener, E.P., King, G.L. 1996. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science* **272:**728–731

Kanter, H.L., Saffitz, J.E., Beyer, E.C. 1992. Cardiac myocytes express multiple gap junction proteins. *Circ. Res.* **70:**438–444

Kanter, H.L., Saffitz, J.E., Beyer, E.C. 1994. Molecular cloning of two human cardiac gap junction proteins, connexin40 and connexin45. *J. Mol. Cell. Cardiol.* **26:**861–888

Knorr, H.L., Linde-Behringer, M., Gossler, B., Mayer, U.M. 1993. Human retinal pigment epithelium in vitro: influence of low oxygen tension, glucose and insulin. *Ophthalmic Res.* **25:**226–234

Koval, M., Harley, J.E., Hick, E., Steinberg, T.H. 1997. Connexin46 is retained as monomers in a trans-Golgi compartment of osteoblastic cells. *J. Cell Biol.* **137:**847–857

Kumar, N.M., Gilula, N.B. 1996. The gap junction communication channel. *Cell* **84:**381–388

Kuroki, T., Inoguchi, T., Umeda, F., Ueda, F., Nawata, H. 1998. High glucose induces alteration of gap junction permeability and phosphorylation of connexin-43 in cultured aortic smooth muscle cells. *Diabetes* **47:**931–936

Laird, D.W., Puranam, K.L., Revel, J.P. 1991. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem. J.* **273:**67–72

Lampe, P.D. 1994. Analyzing phorbol ester effects on gap junctional communication: a dramatic inhibition of assembly. *J. Cell Biol.* **127:**1895–1905

Lau, A.F., Kurata, W.E., Kanemitsu, M.Y., Loo, L.W., Warn-Cramer, B.J., Eckhart, W., Lampe, P.D. 1996. Regulation of connexin43 function by activated tyrosine protein kinases. *J. Bioenerg. Biomembr.* **28:**359–368

Lo, C.W. 1999. Genes, gene knockouts, and mutations in the analysis of gap junctions. *Dev. Genet.* **24:**1–4

Loewenstein, W.R. 1988. Genetic regulation of cell-to-cell communication. *Braz. J. Med. Biol. Res.* **21:**1213–1223

Loewenstein, W.R., Rose, B. 1992. The cell-cell channel in the control of growth. *Semin. Cell Biol.* **3:**59–79

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.F. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.

Miller, T., Dahl, G., Werner, R. 1988. Structure of a gap junction gene: rat connexin-32. *Biosci. Rep.* **8:**455–464

Musil, L.S., Beyer, E.C., Goodenough, D.A. 1990. Expression of the gap junction protein connexin43 in embryonic chick lens: molecu-

lar cloning, ultrastructural localization, and post- translational phosphorylation. *J. Membrane Biol.* **116:**163–175

Musil, L.S., Cunningham, B.A., Edelman, G.M., Goodenough, D.A. 1990. Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell. Biol.* **111:**2077–2088

Naus, C.C., Bani-Yaghoub, M., Rushlow, W., Bechberger, J.F. 1999. Consequences of impaired gap junctional communication in glial cells. *Adv. Exp. Med. Biol.* **468:**373–381

Nishikawa, K., Yamamoto, S., Nagumo, H., Maruyama, K., Kato, R. 1995. The presence of phorbol ester responsive and nonresponsive forms of the zeta isozyme of protein kinase C in mouse epidermal cells. *Cell Signal* **7:**491–504

Oh, S.Y., Grupen, C.G., Murray, A.W. 1991. Phorbol ester induces phosphorylation and down-regulation of connexin 43 in WB cells. *Biochim. Biophys. Acta* **1094:**243–245

Paul, D.L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.* **103:**123–134

Paul, D.L., Ebihara, L., Takemoto, L.J., Swenson, K.I., Goodenough, D.A. 1991. Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of Xenopus oocytes. *J. Cell Biol.* **115:**1077–1089

Saez, J.C., Berthoud, V.M., Moreno, A.P., Spray, D.C. 1993. Gap junctions. Multiplicity of controls in differentiated and undifferentiated cells and possible functional implications. *Adv. Second Messenger Phosphoprotein Res.* **27:**163–198

Schiavinato, A., Morandin, A.R., Guidolin, D., Lini, D., Nunzi, M.G., Fiori, M.G. 1991. Perineurium of sciatic nerve in normal and diabetic rodents: freeze- fracture study of intercellular junctional complexes. *J. Neurocytol.* **20:**459–470

Simon, A.M., Goodenough, D.A. 1998. Diverse functions of vertebrate gap junctions. *Trends Cell Biol.* **8:**477–483

Stalmans, P., Himpens, B. 1997a. Confocal imaging of Ca^{2+} signaling in cultured rat retinal pigment epithelial cells during mechanical and pharmacologic stimulation. *Invest. Ophthalmol. Vis. Sci.* **38:**176–187

Stalmans, P., Himpens, B. 1997b. Effect of increasing glucose concentrations and protein phosphorylation on intercellular communication in cultured rat retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **38:**1598–1609

Stalmans, P., Himpens, B. 1998. A decreased Ca^{2+}-wave propagation is found among cultured RPE cells from dystrophic RCS rats. *Invest. Ophthalmol. Vis. Sci.* **39:**1493–1502

Stalmans, P., Himpens, B. 1999. Properties of intra- and intercellular Ca^{2+} -wave propagation elicited by mechanical stimulation in cultured RPE cells. *Cell Calcium* **25:**391–399

Steinberg, T.H., Civitelli, R., Geist, S.T., Robertson, A.J., Hick, E., Veenstra, R.D., Wang, H.Z., Warlow, P.M., Westphale, E.M., Laing, J.G. 1994. Connexin43 and connexin45 form gap junctions with different molecular permeabilities in osteoblastic cells. *Embo J.* **13:**744–750

Trosko, J.E., Ruch, R.J. 1998. Cell-cell communication in carcinogenesis. *Front. Biosci.* **3:**D208–236

van Veen, T.A., van Rijen, H.V., Jongsma, H.J. 2000. Electrical conductance of mouse connexin45 gap junction channels is modulated by phosphorylation. *Cardiovasc. Res.* **46:**496–510

Vinores, S.A., Kuchle, M., Derevjanik, N.L., Henderer, J.D., Mahlow, J., Green, W.R., Campochiaro, P.A. 1995. Blood-retinal barrier breakdown in retinitis pigmentosa: light and electron microscopic immunolocalization. *Histol. Histopathol.* **10:**913–923

White, T.W., Bruzzone, R., Paul, D.L. 1995. The connexin family of intercellular channel forming proteins. *Kidney Int.* **48:**1148–1157

White, T.W., Paul, D.L. 1999. Genetic diseases and gene knockouts reveal diverse connexin functions. *Annu. Rev. Physiol.* **61:**283–310

Yamasaki, H., Omori, Y., Krutovskikh, V., Zhu, W., Mironov, N., Yamakage, K., Mesnil, M. 1999. Connexins in tumour suppression and cancer therapy. *Novartis Found. Symp.* **219:**241–254

Zhang, J.T., Nicholson, B.J. 1989. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. *J. Cell Biol.* **109:**3391–3401