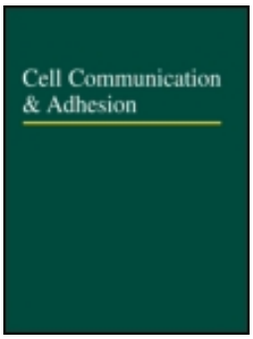

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Impaired Gap Junction Formation and Intercellular Calcium Signaling in Urinary Bladder Cancer Cells can be Improved by Gö6976

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Purpose: Calcium wave propagation and connexin 26, 32 and 43 expression were studied in normal and malignant urothelial cells.

Materials and Methods: Human urothelial cell cultures were established from tissue biopsies obtained from three healthy control persons and compared to human transitional cell carcinoma (TCC) cell line 5637. Fluo-3 was used to study intercellular calcium signaling in urothelial cells. The cells were stimulated mechanically in the presence of inhibitors of gap-junctional or ATP-mediated communication to determine which pathways are operative in intercellular calcium signaling. In addition, Gö6976 was used to determine the effects of PKC α and βI inhibition on intercellular calcium signaling.

Results: In normal urothelial cells, the primary pathway for intercellular calcium mediated cell signaling was gap junctional intercellular communication (GJIC), but the paracrine ATP-mediated signaling was also operative. In 5637 TCC cells, GJIC and ATP-mediated signaling routes were altered when compared to normal urothelial cells. More specifically, inhibition of GJIC resulted in a complete block of intercellular calcium signaling, while inhibition of ATP-mediated signaling decreased signal transduction in 5637 TCC cells. The results of the present study also demonstrated that connexin 26 was the most abundant gap junction plaque protein in cultured normal human urothelial cells and that it did not form gap junction plaques in 5637 TCC cell culture. Treatment with Gö6976 induced gap junction plaque formation by connexin 26 in 5637 TCC cells. In addition, the exposure to Gö6976 enhanced intercellular calcium mediated signaling in 5637 TCC cells, but not in normal cells.

Conclusions: The results of the present study suggest that gap junctions play a major role in intercellular calcium signaling in urothelial cells. In addition, intercellular calcium signaling is altered in urinary bladder carcinoma cells, and it can be improved by PKC α and βI inhibition.

(Supplementary materials are available for this article. Go to the publisher's online edition of Cell Communication and Adhesion for the following free supplemental resources; Movie files of Fig. 2 normal Gö6976−, normal Gö6976+, TCC Gö6976−, TCC Gö6976+ and image of Supplementary Figure 1).

Keywords fluo-3, protein kinase C, urothelium, epithelial cells, cell communication, calcein

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INTRODUCTION

Urinary bladder transitional cell carcinoma (TCC) is one of the most common malignancies in western countries. Despite intensive research, the pathogenesis of TCC is still largely unknown. While research focuses mainly on genetic factors contributing to TCC carcinogenesis, molecular alterations leading to changes in microenvironment and/or intercellular communication of tumor cell populations are often neglected. Thus, our aim was to study intercellular calcium mediated communication between TCC cells. The present study shows alterations in calcium mediated cell-cell communication in TCC, suggests a mechanism behind these alterations, and provides a tool to increase intercellular communication.

Calcium signal transduction operates through two different pathways in urothelium: 1) gap junctional intercellular communication (GJIC) and 2) extracellular, ATP-mediated, communication (1). Gap junctions consist of connexin proteins (Cx) which form channels between cells allowing the passage of intercellular signal molecules such as Ca^{2+} -ion, inositol-1,4,5-triphosphate (IP_3) and cyclic AMP (2). The most important molecule for calcium wave transmittance is thought to be IP_3 which can activate the IP_3 receptor, a calcium release channel of the internal stores, and launch a global calcium signal inside a cell (3). However, in mammary epithelial cells the extracellular soluble ligands such as ATP also play a role in transmitting the calcium wave (4). The extracellular pathway includes ATP secretion which activates purinergic receptors on neighboring cells and subsequently triggers the increase of IP_3, diacylglyserol (DAG) and intracellular calcium. These two communication routes can transmit intercellular calcium signals, known as calcium waves, in response to various stimuli.

The steady-state levels of Cx26 mRNA have been shown to be decreased in human bladder cancer cells when compared to normal urothelial cells (5). In addition, GJIC is shown to be decreased in grade 3 bladder cancer cell culture compared to clonal, normally growing, HCV-29 cell culture as estimated by dye-coupling assay studies (6). One immunohistological study of human bladder tumors has also shown that the major connexin protein, Cx26 does not form gap junction plaques but localizes diffusely inside tumor cells (7). To our knowledge, alterations to, or the significance of, the extracellular signaling route have not been reported in urothelial cells.

Ca^{2+} and DAG are essential for the activation of classical protein kinase C (PKC) isoenzymes (α, βI, βII and γ). The role of phospholipase C (PLC) in PKC activation is to generate DAG and IP_3, which in turn leads to the release of calcium from intracellular stores and to the activation of classical PKCs (8).

PKC isoenzymes have also an important function as phosphorylators of selected connexin proteins and inhibitors of GJIC in various cell types (9–14). For example PKCγ causes reduction in cell surface Cx43 in lens epithelial cells (15), and PKCα, β and δ have been found to be critical in inhibition of GJIC in various fibroblast cell lines (16). In addition 12-O-tetradecanoyphorbol-13-acetate (TPA), an activator of PKC, has been shown to decrease dye transfer in the Cx26 coupled human hepatoma SKHepl cell line suggesting PKC's role in Cx26 mediated gap junction regulation (17). In contrast, another study demonstrated TPA treatment to increase Cx26 constructed functional coupling of gap junctions in a post-translational manner in neuroblastoma cells (18). The possibility of Cx26 regulation by post-transational manner has also been demonstrated by a cell-free system and that regulation requires specific sequence in the N-terminal end (19). In addition it has been demonstrated that a basement membrane protein, laminin-322, increases dye coupling in a Cx26 specific manner. However, the study reveals the discrepancy that increase in dye coupling is not necessarily connected with improved IP_3 transmitted calcium waves (20).

Based on studies with rat urinary bladder cells it has been suggested that connexin proteins may function as tumor suppressors by improving calcium mediated signal transduction and growth regulation also in bladder cancer (21, 22). In addition, transfection of human urinary bladder carcinoma cells with Cx26 adenovirus vector has resulted in down-regulation of

proliferation, increased apoptosis, and reduced tumor formation (23).

Inhibition of PKCα and βI isoenzymes with Gö6976 increases cell junction formation, decreases migration and invasion in human bladder cancer cells (24), and prevents cell proliferation (25). Furthermore, PKCα activity has been shown to regulate the formation of desmosomes in epithelial cells in a calcium dependent manner.

In the present study we characterize the route of calcium waves in human urothelial cells and demonstrate that Gö6976 improves the transduction of calcium waves in urinary bladder cancer cells. We suggest that improved intercellular calcium signaling is due to the PKCα and βI isoenzyme-inhibition leading to an increase in the amount of functional Cx26 in cell membranes and speculate that this happens through post-translational modification.

METHODS

TCC Cell Culture

Human transitional cell carcinoma cell line 5637 was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) containing 1.8 mM Ca^{2+} and supplemented with 10% fetal calf serum (FSC) (Hyclone, Logan, UT), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were grown in an incubator with 5% CO_2 atmosphere and $+37^{\circ}$C.

Normal Human Urothelial Cell Line Isolation and Culture

Human urothelial tissue biopsies were obtained from three healthy women undergoing cystoscopy for vaginal urethrocystopexy. The sample collection was made with the appropriate approval of the Joint Ethical Committee of Oulu University Hospital. Cell culture reagents were obtained from Invitrogen Corporation (Invitrogen, Carlsbad, CA) if not mentioned otherwise. 4–5 biopsy specimens from each patient were transported in Hank's Balanced Salt Solution containing 10 mM HEPES and 10,000 kIU/500 ml of aprotinin and processed within 15 min from removal. The specimens were digested in collagenase type IV (50,000 kIU/100 ml) for 2 h. The solution was homogenized with a pipette and centrifuged for 5 minutes at 1200 rpm. Cells were then resuspended in Keratinocyte Serum-Free Medium (K-SFM) containing 0.05 mM Ca^{2+} supplemented with 5 ng/ml of epidermal growth factor, 50 μg/ml of bovine pituitary extract, 30 ng/ml of cholera toxin and 1% antibiotic solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) and cultured in cell culture flasks (Becton Dickinson, Cowley, UK) to near-confluence. The medium was changed to defined K-SFM, and the calcium concentration was increased to 1.8 mM to differentiate the urothelial cells 24 h prior to experimentation.

Chemicals

Gap junction blocker 1-heptanol (Sigma-Aldrich, St. Louis, MO) was used at 2 mM and 50 U/ml ATP dephosphorylase apyrase (Sigma-Aldrich, St. Louis, MO) in was used to block the extracellular communication pathway. PKCα and βI isoenzyme -inhibitor, Gö6976, was purchased from Calbiochem (La Jolla, CA) and dissolved in DMSO. In experiments the cells were treated with 1 μM Gö6976 or an equal volume of DMSO for 24 h.

Antibodies

Mouse monoclonal antibodies against connexins 26 (Clone Cx-12H10), 32 (Clone CX-2C2) and 43 (Clone CX-1B1), and rabbit polyclonal antibodies against connexins 26, 32 and 43 were obtained from Invitrogen (Carlsbad, CA).

Western Transfer Analysis

Cells were grown to subconfluency on 6 cm diameter cell dishes, washed with PBS and lysed by

scraping with a rubber policeman in 400 μl of 10 mM Tris-HCl, pH 7.4 containing 1% SDS and 1 mM sodium orthovanadate. The samples were heated 5 min at 95°C and centrifuged for 16000 g for 5 min, to remove the debris. Protein concentration was measured using DC Protein Assay (BioRad, Hercules, CA) and equal amounts of protein were subjected to SDS-PAGE on a 12% gel. The proteins were then electrophoretically transferred to PVDF membrane and processed for immunoblotting. Membranes were first blocked with 5% BSA/PBS + 0.05% Tween-20 and immunolabeled with monoclonal or polyclonal antibody against connexin 26, 32 or 43. As secondary antibodies, goat anti-mouse (Amersham Biosciences, Little Chalfont, United Kingdom), or goat anti-rabbit (Cell Signaling Technology, Danvers, MA) horseradish peroxidase-conjugated antibodies were used. The bound antibodies were detected with enhanced chemiluminescence (ECL) (Amersham Life Sciences, Little Chalfont, United Kingdom). Equal loading of each lane was evaluated by immunoblotting the same membranes with β-actin antibodies after stripping the membrane.

Immunofluorescence

Cells were cultured on glass coverslips and rinsed with PBS prior to fixation with −20°C methanol for 5 min. The cells were washed three times with PBS and incubated with monoclonal or polyclonal antibodies against connexins 26, 32 or 43. The cells were washed with PBS and incubated with Alexa 568 conjugated goat anti-mouse or anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA). Hoechst was used to stain nuclei of the cells. Cells were observed with UV microscope including appropriate filters for Alexa 568 fluorescence and hoechst, and photographed using Qimaging MicroPublisher 5.0 RTV digital camera. Immunofluorescence and nuclei were photographed separately and composed digitally with Qcapture Pro v5.0.1.26. All photos were taken with identical settings.

Calcium Imaging

Cells were grown to confluent on 3 cm diameter dishes and treated with Fluo-3 AM (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the cells were washed three times with DMEM and treated with 2 μM Fluo-3 AM supplemented with 0.02% Pluronic F-127 (Invitrogen, Carlsbad, CA) for 30 min. The cells were washed with DMEM and subsequently incubated with DMEM (in controls and in apyrase experiments), DMEM supplemented with 2 mM heptanol or DMEM supplemented with 1 μM Gö6976 or an equal amount of DMSO for 30 min. The medium was then replaced with DMEM (controls), DMEM supplemented with 2 mM heptanol, DMEM supplemented with 50 U/ml apyrase or DMEM supplemented with Gö6976 or an equal amount of DMSO. Calcium imaging was performed with Zeiss argon/helium-neon confocal scanning laser microscope connected to a computer. Objective used was Plan-Neofluar 20x/0.5 and laser power was limited from 1 to 3% of the total 25 mW maximum power. Optical slice was 1.1–1.5 μm and pinhole was 1.54–2.16. Images were taken at 2-second intervals and the total number of images was 100–200. After the first acquired images a single cell was stimulated with micro injector capillary attached to Eppendorf Micromanipulator 5170 and the response was recorded to hard drive with 512 × 512 resolution images. Images were processed with Zeiss LSM510 v.2.5 SP2 software and the number of the responding cells or the distance, speed and intensity of calcium waves were calculated. The maximum length of the calcium wave was measured in micrometers and in cell rows from the initiation point. Micrometer calibration for confocal microscope was performed prior to calculations. Speed of calcium wave was calculated using the length (micrometer) and time (sec).

Fluorescence Recovery After Photobleaching (FRAP)

Normal urothelial cells were grown on 3 cm diameter dishes and differentiated with 1.8 mM calcium.

Then cells were loaded with 10 μM calcein (Invitrogen, Carlsbad, CA) for 30 min, washed with DMEM, incubated 30 min in DMEM and rewashed. A random cell cluster was selected and one of the cells bleached with laser. Recovery of the fluorescence was observed at 1-minute intervals the total number of images being 11. The experiment was repeated six times.

Apyrase Efficiency

Normal urothelial cells were seeded on two dishes, loaded with Fluo-3 and 100 μM ATP was added to the cells while changes in calcium concentration were monitored with confocal microscope. One of the dishes was treated with 50 U/ml apyrase prior to the experimentation.

Statistical Analyses

Statistical analyses were performed with SPSS v.14 and calculated from experiments with normal urothelial and 5637 TCC cells. In 1-heptanol and apyrase experiments, the number of responding cells was counted and post hoc Dunnett's test was used to compare the statistical difference between control group and 1-heptanol or apyrase treated group. To compare normal urothelial and TCC cells in Figure 1, independent samples *t*-test was used. To test statistical difference between Gö6976- and Gö6976+ treated groups in distance, speed and intensity independent samples *t*-test was used.

RESULTS

Intercellular Calcium Signaling Is Gap Junctional in Urothelial Cells

In normal urothelial cell culture, a mechanical stimulation of a single cell initiated propagation of a calcium wave from one cell to another. More specifically, almost all cells within the monitored area responded to the initial stimulus by elevating their cytoplasmic calcium levels. When gap junctional intercellular communication was blocked using heptanol, only a few cells surrounding the epicenter elevated intracellular calcium concentration in normal urothelial cells ($p < 0.05$). In contrast, treatment with apyrase, an ATPase, did not have any apparent effect on calcium wave propagation in this experimental system (Table 1).

TABLE 1 The number of cells increasing their cytosolic calcium concentration after mechanical stimuli

	Normal cell culture The number of responding cells	TCC cell cultures The number of responding cells
(−)	57 ± 16 (12)	9 ± 1 (6)*
1-heptanol	2 ± 1 (8)*	0 ± 0 (3)***
Apyrase	80 ± 19 (7)	3 ± 1 (3)***

mean ± S.D. (n experiments)
Statistical differences: *refers to $p < 0.05$ between 1) no-treatment and treatment groups or 2) no-treatment groups between normal and TCC cell cultures.
***refers to $p < 0.001$ between no-treatment and treatment groups.

Calcium Based Signal Transduction Is Altered in Bladder Cancer Cells Compared to Normal Urothelial Cells

In 5637 TCC cells, the propagation of calcium wave was decreased, compared to normal urothelial cells ($p < 0.05$). Specifically, only nine neighboring cells responded to the stimulus elicited by a single mechanically targeted cell. When 5637 TCC cells were treated with heptanol, the transmission of the calcium wave was entirely blocked ($p < 0.001$) while apyrase treatment only decreased the calcium signal transduction and three cells surrounding the mechanically stimulated cell responded ($p < 0.001$) (Table 1). These findings suggest that in 5637 TCC cells intercellular calcium signaling is dependent on GJIC but not on the extracellular route although both are operational when no

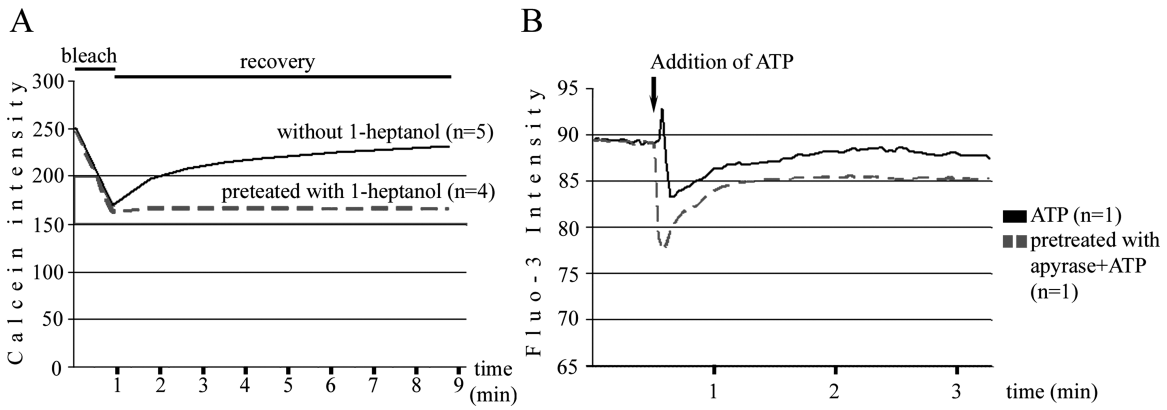

Figure 1. Method controls for gap junctional and ATP mediated calcium wave blockers. A) Normal urothelial cells were grown on 3 cm diameter dishes and differentiated with 1.8 mM calcium. The cells were loaded with 10 μM calcein and a random cell cluster was selected. One of the cells was bleached with laser and the recovery of the fluorescence was observed at 1-minute intervals the total number of images being 11. The experiment was repeated five times for no treatment and four times for 1-heptanol treatment. B) Normal urothelial cells were seeded on two dishes, loaded with Fluo-3 and 100 μM ATP was added to the cells while changes in calcium concentration were monitored with confocal microscope. One of the dishes was treated with 50 U/ml apyrase prior to the experimentation.

inhibitors are used. However, the regulation of intercellular calcium signaling is very complex and there may be some other regulatory mechanisms also present.

Method Control for Gap Junction and ATP Mediated Calcium Wave Block

As a method control, fluorescence recovery after photobleaching (FRAP) was used to monitor calcein dye transfer in urothelial cells with and without heptanol. The results showed that in the presence of heptanol, no recovery of the fluorescence after bleaching was observed, while cells with no treatment restored almost original fluorescence level during the 9 min surveillance period. Apyrase efficiency was tested by adding 100 μM ATP to normal urothelial cells with and without apyrase pretreatment. Cells with no apyrase pretreatment increased their cytosolic calcium concentration simultaneously and immediately after addition of ATP, while apyrase treated cells did not respond to ATP application. Application of extra medium with ATP influenced long lasting decrease in fluorescence seen in both experiments (Fig. 1).

PKC α and βI Isoenzyme Inhibition Increases Calcium Wave Propagation in Bladder Cancer Cells

Statistical analyses of calcium waves in Table 2 demonstrate that in normal urothelial cells the average distance of calcium wave propagation was 239 ± 29 μm and that this property remained unaltered on exposure to Gö6976 (253 ± 26 μm). The speed of the calcium wave in non-treated normal cells was 15 ± 6 μm s^{-1}, and 8 ± 3 μm s^{-1} in Gö6976 treated normal cells. The rise of intracellular calcium concentration after mechanical stimulation was 105 ± 13 units on a scale from 0 to 255. After Gö6976 treatment this value was 87 ± 10 units. There was no statistical difference ($p > 0.05$) between Gö6976 and no-treatment groups. In 5637 TCC cells, the distance of calcium wave propagation was 81 ± 8 um. Gö6976 treatment increased the distance of calcium wave to 164 ± 18 μm ($p < 0.001$). Speed of the calcium wave was 8 ± 1 μm s^{-1} before and after (no statistical difference) Gö6976 treatment 15 ± 4 μm s^{-1}. In 5637 TCC cells, the rise of intracellular calcium concentration after mechanical stimulation was 49 ± 8 units in a scale from 0 to 255. After Gö6976 treatment this value was 45 ± 8 units.

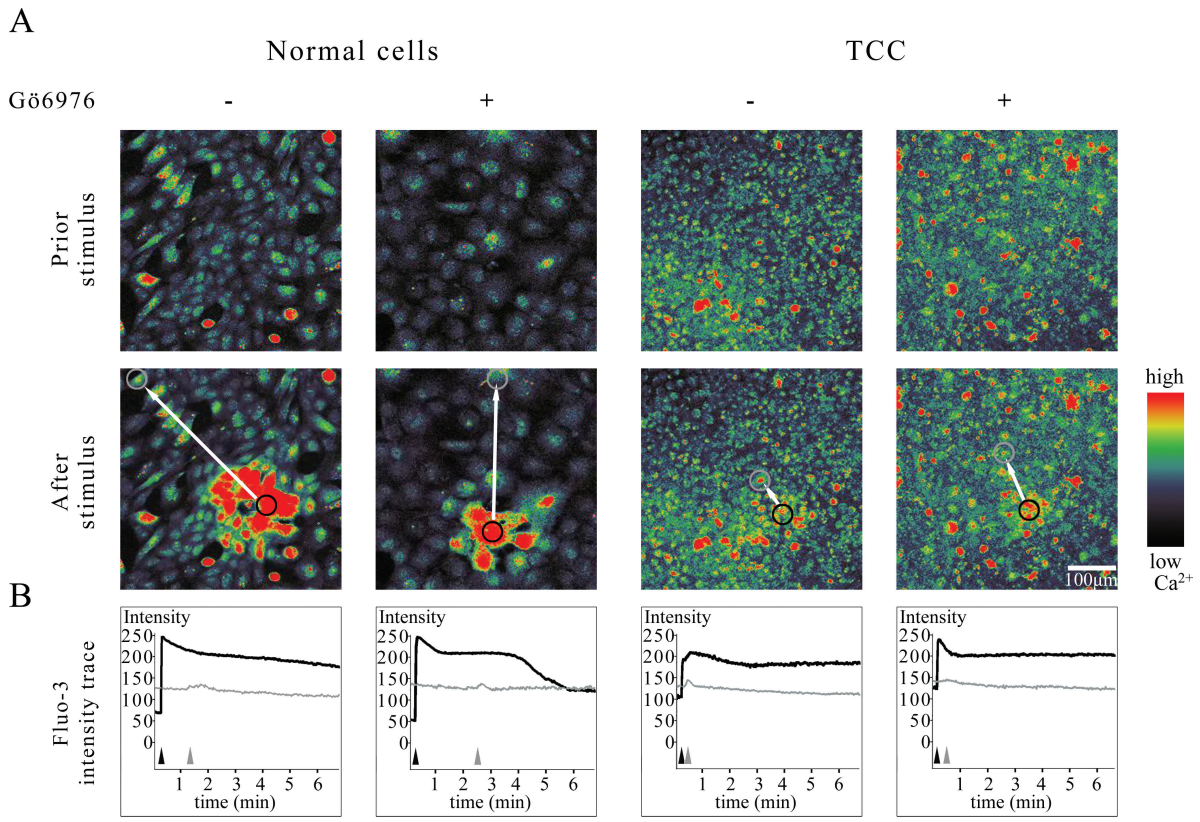

Figure 2. Calcium mediated signal transduction in normal urothelial and 5637 TCC cells with and without Gö6976. A) Upper image is taken prior to micromanipulator stimulation of one cell and lower image immediately after the calcium wave had reached its maximal distance (white arrows). Pseudocolors from black through rainbow colors to red are used to illustrate changes in the intracellular calcium concentration. B) Curves beneath images illustrate changes in cytosolic calcium concentrations within selected cells: black line for stimulated cell and gray line for the most peripheral reacting cell. Black and gray arrowheads indicate the time point where cells start to raise their cytosolic calcium concentration. All images are presented with the same magnification.

Appearance of Normal Urothelial and TCC Cell Cultures

Representative experiments on calcium wave propagation in normal and 5637 TCC cell cultures with and without Gö6976 are presented in Figure 2. In normal urothelial cell culture, cells grew regularly and the cell borders were easily recognizable. Gö6976 treatment enlarged the sizes of normal urothelial cells as estimated by the area covered by a single cell (Fig. 2). 5637 TCC cells differed from normal cells by their smaller size and irregular growth pattern. Gö6976 treatment partly restored normal cell clustered morphology as described previously by Koivunen et al. 2004.

The Length of Calcium Wave Propagation Estimated in Cell Rows

Because of the morphological and size alterations between cells we further analyzed the length of calcium wave in cell rows. The average length of calcium wave in normal urothelial cell culture without Gö6976 was 6.67 ± 2.93 cell rows and 5.77 ± 2.13 cell rows with Gö6976. In TCC cell culture the lengths were 3.23 ± 1.54 and 5.10 ±

TABLE 2 The influence of PKC α and βI isoenzyme inhibition on distance, velocity and $\triangle$ intensity of calcium waves

	Normal cell culture			TCC cell culture		
	Distance (μm)	Speed (μm/s)	Intensity rise	Distance (μm)	Speed (μm/s)	Intensity rise
Gö6976–	239 ± 29 (12)	15 ± 6 (12)	105 ± 13 (12)	81 ± 8 (13)	8 ± 1(13)	49 ± 8 (13)
Gö6976+	253 ± 26 (13)	8 ± 3 (13)	87 ±10 (13)	164 ± 18 (11)***	15 ± 4 (11)	45 ± 8 (11)

mean ± S.D (n experiments).
Statistical differences: ***refers to $p < 0.001$ between Gö6976 treatment and without Gö6976 treatment groups.

1.60, respectively (Table 3). The results are in good agreement with the results demonstrated in Table 2.

Connexin 26 Displays Similar Steady-state Levels in Normal and 5637 TCC Cells which Are Not Influenced by PKC α and βI Inhibition

Previous reports have shown that expression levels of Cx proteins can be altered in urinary bladder cancer cells (7). In the present study we studied if the steady-state levels of connexins 26, 32 and 43 were altered in the 5637 TCC cell line compared to normal urothelial cells and if the levels of proteins could be stimulated by Gö6976 treatment. The results showed that there was no significant alteration in the levels of Cx26 protein between normal urothelial and 5637 TCC cells. Gö6976 treatment did not markedly increase Cx protein levels in either group. On the contrary Gö6976 treatment decreased the steady state levels of Cx43 protein in normal urothelial cells. In 5637 TCC cells the levels of Cx43 and Cx32 were elevated compared to normal urothelial cells. A double band for Cx43 was seen with normal urothelial cells. Beta-actin labeling shows only minor alterations in sample loading. (Fig. 3A)

To study whether Gö6976 treatment increases Cx26 levels in 5637 TCC cells we repeated western blot three times and no significant increase was detected in the protein levels as demonstrated in Figure 3B. On the contrary, the intensity measurements of western blot bands revealed a minor decrease in response to Gö6976 treatment.

TABLE 3 The length of calcium wave measured in cell rows

	Normal cell culture Average cell rows	TCC cell culture Average cell rows
Gö6976-	6.67 ± 2.93 (12)	3.23 ± 1.54 (13)
Gö6976+	5.77 ± 2.13 (13)	5.10 ± 1.60 (10)

mean ± S.D (n experiments).

PKC α and βI Isoenzyme Inhibition Increases Gap Junction Plaque Formation in Normal Urothelial and Bladder Cancer Cells

In further studies the gap junction plaque formation by connexin proteins in cell cultures was investigated by indirect immunofluorescence (IIF). In normal urothelial cells, Cx26 was most extensively localized at cell borders of the three connexin proteins studied, but Cx43 formed gap junction plaques also. In contrast the subcellular localization of Cx32 was diffuse and was not associated with gap junction plaques. In 5637 TCC cells, all connexin proteins localized diffusely and no gap junction plaque formation was seen. Gö6976 treatment induced gap junction plaque formation by Cx26 and Cx43 in normal urothelial cells and by Cx26 in 5637 TCC cells (Fig. 4, Normal cells and TCC, Gö6976+). Normal urothelial cells were larger compared to 5637 TCC cells. IIF labeling was repeated with polyclonal antibodies and again (Supplementary Fig. 1) with monoclonal antibodies with similar results.

DISCUSSION

Intercellular Calcium Signaling of Urothelial Cells

The results of the present study show that in normal urothelial cells calcium wave propagation

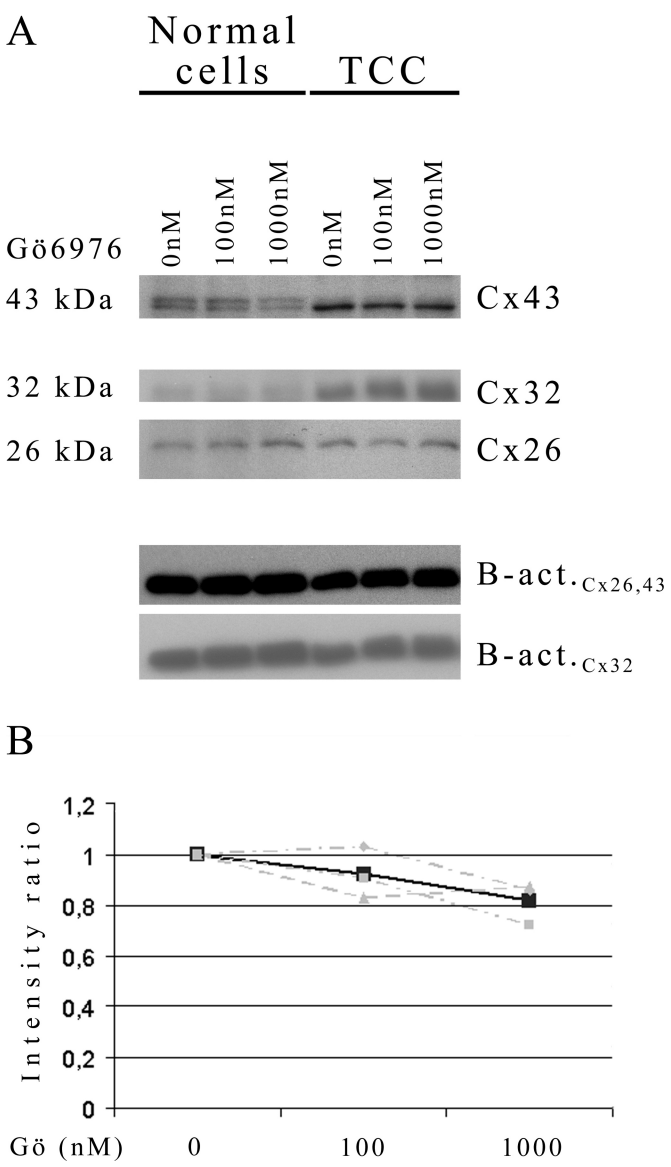

Figure 3. Western transfer analysis of connexin expression. A) Western transfer analysis was performed with samples of normal urothelial cells (Normal cells) and 5637 TCC cells (TCC) treated with different concentrations (0 nM, 100 nM, 1000 nM) of Gö6976 for 24 h. Cx26 and Cx32 detection was done with polyclonal and Cx43 with monoclonal antibody. Beta-actin labeling shows equal sample loading. Western transfer analysis was performed with cell lysate from normal cell culture of one patient. B) To study whether Gö6976 treatment affects Cx26 levels in 5637 TCC cells, western transfer analysis was repeated three times and the intensity ratios of Cx26 and Beta-actin bands were determined. Intensity ratios are presented on y-axis and x-axis demonstrates different concentrations of Gö6976. The image presents three different western blot experiments (grey lines) and an average value presented as black line.

is decreased dramatically when gap junctional intercellular communication (GJIC) is blocked. In contrast, extracellular communication block does not alter calcium wave transmission. These results indicate that intercellular calcium based signal transduction in normal urothelial cells is mostly dependent on functional gap junctions, and to a lesser extent on ATP mediated signal transduction. These

findings are in agreement with the earlier hypothesis that gap junctions are the main route for calcium waves after mechanical stimulation. However, the results of the present study demonstrate that both communication pathways are active in urothelial cells since gap junctional block did not entirely prevent calcium wave propagation.

Intercellular Calcium Signaling in TCC Cells

The results of the present study show that calcium mediated signal transduction is impaired in 5637 TCC cells when compared to normal urothelial cells. In addition, IIF labeling with antibodies against connexin proteins 26, 32 and 43 showed that no clusters of gap junctions, known as gap junction plaques (2), are formed between 5637 TCC cells in the culture. This may be a possible explanation for altered intercellular calcium signaling. However, the steady-state level of the primary Cx protein 26 in western blotting remained unaltered compared to normal urothelial cells suggesting a defect in Cx26 translocation or rapid degradation of connexin 26 in the plasma membrane of 5637 TCC cells. However, it should be noted that IIF labeling can not confirm the actual gap junction plaque formation which is why the results are only indicative.

In the present study heptanol blocked entirely GJIC in TCC cells even though gap junction plaques could not be found between cells in the IIF labeling. The reason for this may be found in the incapability of the IIF labeling, as a method, to show all the gap junctions between cells. These gap junctions may provide a pathway to impaired calcium waves. Our results also showed that the extracellular signaling

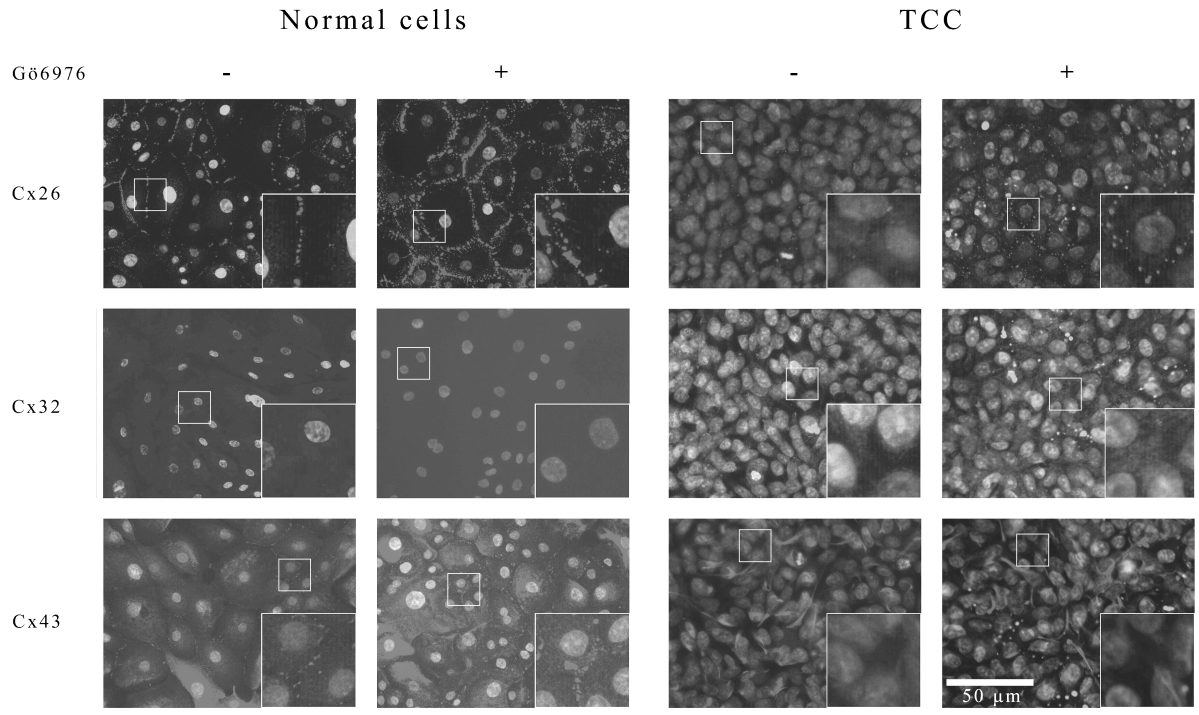

Figure 4. Indirect immunofluorescence labeling using monoclonal antibodies against connexins 26, 32, and 43. Panels in the left demonstrate expression of different connexins in normal urothelial cells culture before (−) and after (+) Gö6976 tretament. Panels in the right demonstrate the expression of different connexins in TCC cell culture without (−) and with (+) Gö6976 treatment. Nuclei have been stained with hoechst (blue) and connexin immunosignal is in red. All images are presented with the same magnification. Small boxes within panels show a demonstrative area of each picture in higher magnification. Scale bar 50 μm.

was not operational in 5637 TCC cell cultures when heptanol was used to block GJIC. However, apyrase treatment decreased intercellular calcium signaling indicating that the extracellular route is operational when GJIC is working properly. These findings suggest that in 5637 TCC cells intercellular calcium signaling is dependent on GJIC, but not on extracellular route, although both are operational when no inhibitors are used.

Cellular Morphology and Intercellular Calcium Signaling

The decreased distance of intercellular calcium signals in 5637 TCC cells may in part be explained by the morphological alterations between normal urothelial and TCC cells. More specifically, in normal urothelial cell culture, a calcium wave extending 100 μm from the starting point must pass only one cell border while in 5637 TCC cell culture the calcium wave must pass two or three cell borders. Also the effect of Gö6976 on the cell morphology may affect the results. However, the data of Table 3 demonstrates that the difference in the length of the calcium wave between normal urothelial cells and TCC cells is present also when the wave length is measured as cell rows. The increase in the length of the calcium wave in TCC cells after Gö6976 treatment is also seen in this experimental system.

The Effect of Gö6976 on Intercellular Calcium Signaling

In the present study we also demonstrate that Gö6976, a known PKC α and βI inhibitor, induced gap junction formation in 5637 TCC cell culture. Previous studies have demonstrated that PKCγ can phosphorylate Cx43 resulting in inhibition of connexin protein transport to the cell membrane and inhibition of gap junction formation (15). In contrast, the current consensus is that Cx26 is not a phosphoprotein (26). However, a recent study suggested that Cx26 has several possible post-translational modification sites and possibly one phosphorylation site in the amino terminal end (27). Considering previous findings (18, 19) and the data presented in the present study, it can be speculated that PKC α and βI inhibition may enhance gap junction plaque formation in 5637 TCC cells through post-translational modification of Cx26 protein.

Furthermore, we noticed that Gö6976 improves the length of intercellular calcium wave propagation in 5637 TCC cells. This is important because the propagation length of the calcium wave indicates how well intercellular calcium signal transduction works in cell cultures. The improved intercellular calcium signaling is understandable since gap junctions play a key role in calcium mediated signal transduction in urothelial cell culture. However, Gö6976 treatment increased gap junction plaque formation by Cx26 also in normal urothelial cells but did not improve intercellular calcium signaling. An explanation for this could be that before treatment with Gö6976 there already were sufficient amounts of gap junction plaques between normal urothelial cells to achieve maximal gap junction communication. The influence of Gö6976 treatment on speed and intensity of the calcium wave was not statistically significant. The reason for this could be that these parameters do not correlate as well with the effectiveness of intercellular calcium signal transduction as length of the calcium wave.

CONCLUSION

In the present study, we characterize the routes of intercellular calcium signaling in urothelial cells and show that calcium wave propagation is decreased in 5637 TCC cells compared to normal urothelial cells. In addition, the present study suggests that the altered intercellular calcium signaling might be due to impaired gap junction formation between 5637 TCC cells and that this resulted from a failure in gap junction plaque formation by Cx26. Furthermore, we hypothesize that classical PKCs may regulate gap junction formation by influencing post-translational modification of Cx26 within cells. These findings are in good agreement with earlier results that have demonstrated regulation of gap junctions by classical

PKCs. Our results highlight the role of PKC as a regulator of intercellular calcium signal transmission in urothelial cells and their malignant equivalents. We believe that classical PKC inhibitors, such as Gö6976, can be used to normalize intercellular calcium signaling in TCC cells and thus in part to counteract the malignant behavior of the TCC cells.

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