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Gap-junctional Hemichannels Are Activated by ATP Depletion in Human Renal Proximal Tubule Cells

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Abstract. We present evidence suggesting that gap-junctional hemichannels (GJH) may be involved in acute ischemic injury of human renal proximal tubule cells (hPT cells). Two GJH, from neighboring cells, join to form an intercellular gap junction channel (GJC). Undocked GJH are permeable to hydrophilic molecules up to 1 kDa, and their opening can significantly alter cell homeostasis. Both GJC and GJH formed by connexin 43 (Cx43) are activated by dephosphorylation. Hence, we tested whether GJH activation during ATP depletion contributes to cell damage in renal ischemia. We found that hPT cells in primary culture express Cx43 (RT-PCR and Western-blot analysis) at the plasma membrane region (immunofluorescence). Divalent-cation removal or pharmacological ATP depletion increased cell loading with the hydrophilic dye 5/6 carboxy-fluorescein (CF, 376 Da) but not with fluorescein-labeled dextran (>1500 Da). Endocytosis and activation of P2X channels were experimentally ruled out. Several GJC blockers inhibited the loading elicited by PKC inhibition. Double labeling (CF and propidium iodide) showed that both Ca^{2+} removal and ATP depletion increase the percentage of necrotic cells. Gadolinium reduced both the loading and the degree of necrosis during divalent-cation removal or ATP depletion. In conclusion, GJH activation may play an important role in the damage of human renal proximal tubule cells during ATP depletion. These studies are the first to provide evidence supporting a role of GJH in causing injury in epithelial cells in general and in renal-tubule cells in particular.

Key words: Connexin 43 — Human kidney — Ischemia — Hypoxia — Cell injury

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Introduction

Renal ischemia is the most common cause of intrinsic acute renal failure (ARF). This form of ARF involves direct damage to the renal parenchyma and is associated with a clinical entity known as acute tubular necrosis (ATN). The late proximal tubule appears to be the principal target of renal ischemia. At the cellular level the lack of adequate oxygen supply results in a critical reduction in cell ATP that initiates a chain of events leading to cell dysfunction, sub-lethal injury and ultimately cell death (Brady et al., 2000). Here, we present evidence suggesting that the activity of gap-junctional hemichannels (GJH) may be involved in the process of cell damage.

Gap junctions (GJ) are cell-to-cell conductive pathways formed by apposed connexons (or hemichannels) from two neighboring cells (Kumar & Gilula, 1996). Each connexon is a hexamer of transmembrane proteins called connexins (Cx), which are arranged as a barrel with the pore in the center. Most connexons are assembled at the trans-Golgi network (Laird, 1996; Evans et al., 1999), inserted in the plasma membrane at random places (Lauf et al., 2002) and diffuse laterally until they either dock with a connexon from a neighboring cell, forming an intercellular gap junction channel (GJC), or are retrieved by endocytosis (Laird, 1996). Before docking, connexons are thought to remain closed (impermeable) because of the blocking effect of normal extracellular Ca^{2+} concentrations (reviewed by Harris, 2001). Assembled gap junctions associate with the cytoskeleton and with other gap junctions in the plasma membrane to form stable pseudo-crystalline arrays or plaques (Kumar & Gilula, 1996). It has been observed that in heterologous expression systems and following reductions in extracellular calcium, it is possible to record GJH currents (Paul et al., 1991; DeVries & Schwartz, 1992; Ebihara & Steiner, 1993; Gupta et al., 1994; Ebihara, Berthoud &

Beyer, 1995; Ebihara, 1996; Pfahnl et al., 1997; Ebihara et al., 1999; Zampighi et al., 1999). These GJH currents represent the functional expression of undocked connexons and exhibit properties concordant with those of GJC, including large conductance and low ion selectivity. Both GJC and GJH are permeable not only to inorganic ions, but also to hydrophilic molecules up to a MW near 1 kDa. Hydrophilic dyes (Paul et al., 1991; DeVries & Schwartz, 1992; Li et al., 1996) as well as second messengers and other small molecules (Bevans et al., 1998) permeate, whereas larger molecules (e.g., proteins or high molecular-weight dextran) do not. In contrast to GJC, which communicate with neighboring cells and do not contribute to the plasma membrane permeability, GJH communicate the cell interior with the extracellular space. Because of their high permeability and low selectivity, opening of GJH would greatly alter intracellular homeostasis, causing cell depolarization, Na^+ and Ca^{2+} loading, and loss of K^+ and intracellular metabolites such as ATP and second messengers. The properties of GJH and the observation of endogenous GJH activity after Ca^{2+} -removal in cell lines and in native tissues (DeVries & Schwartz, 1992; Ebihara, 1996; Li et al., 1996; John et al., 1999), including epithelia (Vanoye et al., 1999), suggest the possibility of a pathophysiological role for GJH.

John et al. (1999) reported that in cardiomyocytes, as well as in cells transfected with Cx43 (the type of Cx most abundantly expressed in contractile myocardium), pharmacologically induced ATP depletion results in activation of large nonselective currents and uptake of gap junction-permeable hydrophilic dyes. These changes in permeability, identical to those induced by removal of extracellular Ca^{2+}, were attributed to Cx43-formed GJH. Contreras et al. (2002) reported that ATP depletion increases the dye permeability of rat-brain astrocytes.

Pharmacologically induced ATP depletion has been used as a model system reproducing some characteristics of renal-tubule ischemia (Sheridan et al., 1993; Lieberthal et al., 1998). It has been recently shown that depletion of ATP can lead to cell death in primary cultures of mouse proximal tubule cells; moderate depletion causes apoptosis, whereas severe and acute ATP depletion results in rapid and massive cell necrosis (Lieberthal et al., 1998). We demonstrated GJH activation in *Necturus* urinary bladder epithelial cells (Vanoye et al., 1999) and reasoned that this phenomenon may also take place in other epithelial cells that express connexins. Among them, renal proximal tubule cells express Cx43. We tested the possibility of GJH activation in hPT cells taking advantage of the fact that these channels are permeable to hydrophilic solutes of up to 1,000 Da, which confers a fingerprint for their identification by dye-loading experiments.

Our data strongly suggest that GJH activation plays a role in the damage of human renal proximal tubule cells under ATP depletion. These studies are the first to provide evidence supporting a role of GJH in causing injury in epithelial cells in general and in renal-tubule cells in particular.

Materials and Methods

Tissue Culture

Human proximal-tubule fragments were obtained by gradient centrifugation from thin sections of normal areas of kidneys removed because of cancer. Fragments were isolated as reported elsewhere (Bello-Reuss and Weber, 1986; Bello-Reuss, Holubec & Rajaraman, 2001) and cultured at 37°C in 5% CO_2/95% air in a mixture of 1:1 Ham's F-12 and Dulbecco's modified Eagle's medium supplemented with, in mM: 25 $NaHCO_3$, 4 L-glutamine; in mg/liter: 10 insulin, 5.5 transferrin, 2 ethanolamine; plus 5 μg/liter sodium selenite (ITES, Sigma-Aldrich, St. Louis, MO, USA) and non essential aminoacids (Bello-Reuss & Weber, 1986). On passage 3, the cells were plated on collagen-coated transparent permeable supports (Falcon). Experiments were performed on confluent monolayers of hPT cells, i.e., after 5–7 days. Only cells at passage 3 were used to avoid variability due to changes in phenotype, including cell senescence.

Cell Dissociation

Confluent monolayers were incubated at room temperature for 30 min in physiological salt solution (PSS, in mM: NaCl 140, KCl 5, $CaCl_2$ 1.3, $MgCl_2$ 1, HEPES 10 and 1.4 g/l of glucose, equilibrated with air), which contained 1 mg/ml of both collagenase type IV and protease type XIV (Sigma). We first used the method described here to obtain isolated polarized epithelial cells in amphibian epithelia (Torres et al., 1996; Vanoye et al., 1999). After incubation, the cells were quickly and gently pipetted in ice-cold Ca^{2+}- and Mg^{2+}-free PSS containing 0.5 mM EDTA, washed in cold regular PSS and stored in suspension at 7°C until use (less than 2 hrs).

RT-PCR

cDNA was obtained from total hPT-cell RNA using the Avian Myeloblastosis Virus (AVM) reverse transcriptase. For Cx43, Cx32 and Cx26, the PCR reaction was performed using the conditions and primers described by Pozzi et al. (1995). To avoid amplification of contaminating genomic DNA, primer pairs were chosen to include a 3.5-kb intron in the genomic sequence.

Connexin-Isoform Identification

Western Blot Analysis

Cx43 expression was investigated in whole-cell homogenates using a polyclonal rabbit anti-rat Cx43 primary antibody (Zymed, South San Francisco, CA). Positive controls were homogenates from rat heart and rat brain.

Immunocytochemistry

Confluent monolayers were fixed and permeabilized with 4% paraformaldehyde and 0.2% triton X-100 and labeled with a rabbit

anti-rat Cx43 polyclonal primary antibody (Zymed). A goat anti-rabbit secondary antibody conjugated to Alexa-488 or Alexa-594 was used for indirect immunofluorescence labeling. In Western blots and immunocytochemistry, antibody specificity was tested by immunostaining in the presence of the corresponding antigenic peptide, which should prevent only Cx43-specific labeling.

Dye-Uptake Measurements

Experiments were performed on isolated cells in suspension or in confluent monolayers grown on permeable supports. Cells were incubated in the presence of 5/6-carboxyfluorescein (CF, MW 376) or fluorescein-dextran (MW 3,000 Da, range 1,500–4,500) in either normal PSS (control) or exposed to one of the following treatments: (a) nominally Ca^{2+}- and Mg^{2+}-free PSS for 20 min (suspensions) or 30 min (monolayers), (b) UV light-activated PKC blocker calphostin C (1.5 μM, 2 h), or (c) ATP-depletion solution (described separately below). The cells were exposed to dye during the period of exposure to the experimental solutions; dye concentrations were between 0.5 and 2 mM, as indicated in the figures. After dye loading, efflux following the removal of extracellular dye was prevented by using cold PSS containing 10 μM Gd^{3+} in the monolayer experiments. Dye-loading results were compared among the experimental cell population using the total cell fluorescence intensity or the number of fluorescent cells above an arbitrary threshold. We used several gap-junction channel blockers (*see* Rozental, Srinivas & Spray, 2001, for a review) added from stocks in DMSO or Ringer's, to the following final concentrations: 18-β-glycyrrhetinic acid (Aldrich, Milwaukee, WI), 20 μM; octanol (Sigma, St. Louis, MO), 1 mM; halothane (Sigma), 2 mM and Gap27 (Tocris Cookson, Ellisville MO) 1 mg/ml.

Fluorescence Microscopy

Immunofluorescence studies were performed using an E800 upright epifluorescence microscope (Nikon Instruments, Lewisville, TX) equipped with a CoolSNAP FX digital monochrome camera (Roper Scientific, Tucson, AZ). The uptake of CF, PI and fluorescent dextran was measured with a laser scanning confocal microscope (OZ with Intervision, NORAN Instruments, Middleton, WI, or Zeiss LSM510meta, Zeiss, Jena, Germany) using excitation laser lines at 488 and 568 nm in the Noran instrument and 488 and 543 in the Zeiss.

Temperature Dependence of Fluid-Phase Endocytosis

Confluent monolayers of hPT cells grown on transparent permeable supports were incubated during 20 min in normal PSS in the presence of 1 mM Texas-red-dextran (MW 10,000) at 4°C or 37°C. Then they were washed with fresh PSS and inspected for fluorophore uptake.

ATP Depletion

Confluent monolayers were preincubated for 2 hours at 37°C in glucose-free PSS containing 2 mM glutamine, except for the control group, which was incubated in normal PSS. Subsequently, graded levels of ATP depletion were obtained by exposing the cells to different combinations of metabolic inhibitors: Glycolysis inhibitors, 2-deoxyglucose (2-DOG) or iodoacetic acid (IAA); or mitochondrial inhibitors: a cytochrome bc1 complex blocker (Antimycin A), and an uncoupler of oxidative phosphorylation (carbonyl-cyanide p-(trifluoromethoxy) phenyl-hydrazone, FCCP). ATP was measured by the luciferin-luciferase method (bioluminescence ATP assay kit, Sigma). We report experiments with two degrees of ATP depletion: moderate (ca. 50%, low glucose concentration, 2-DOG and Antimycin A) and severe (<5%, FCCP and IAA).

Dye Uptake after Metabolic Inhibition

Following ATP depletion at 37°C, intact monolayers were incubated at 6°C for 30 min in glucose-free PSS containing 1 or 2 mM CF. Extracellular dye was then removed by several washes with a large volume of cold PSS in the presence of 10 μM Gd^{3+} (to prevent dye efflux; *see* below). Control experiments (*not shown*) demonstrated that in the conditions described ATP levels do not change for up to 1 h after removing the inhibitors, thus allowing for the dye loading to be performed at the desired levels. After the loading, Gd^{3+} was included in all washes and in the final extracellular solution to prevent dye efflux until the time of observation in the confocal microscope, which did not exceed 30 min. Control measurements done at different times indicated that in these conditions there was no significant decrease in the intracellular fluorescence after the incubation.

Assay for Cell Necrosis

To identify necrotic cells, monolayers were exposed to 150 μM propidium iodide (PI) at room temperature for 5 min in the presence of Gd^{3+} (to prevent PI uptake via GJH). After washing out the PI, the monolayer was examined in a video-rate laser-scanning confocal microscope (OZ with Intervision, Noran Instruments, Middleton, WI). The loading was measured as the average fluorescence per field observed in confluent monolayers, and areas of cell detachment were excluded from the observations. Untreated monolayers were used as controls. To confirm the effect of Gd^{3+} on dye loading, the blocker was included in all incubation steps starting with the exposure to metabolic inhibitors. Autofluorescence was measured in unloaded cells and this value used to correct all measurements. Results are expressed as percentages of the respective control group for each experiment.

Statistics

Data are expressed as mean ± SEM; significance was assessed by Student's *t*-test of paired or non-paired data, as indicated.

Results

The hPT cells retain features of the original renal proximal tubule epithelium. They exhibit intercellular tight junctions, apical microvilli, lateral interdigitations and dilated lateral intercellular spaces, a morphology denoting fluid absorption. When grown on impermeable supports, the monolayers form domes that increase in number with cAMP stimulation. They are sensitive to parathyroid hormone but not to vasopressin. They functionally express P-glycoprotein (MDR1) and synthesize 1,25-OH-VitD3. (E. Bello-Reuss, unpublished observations).

Human Proximal-Tubule Cells Express Cx43

We found expression of Cx43 at the mRNA level by RT-PCR, as demonstrated by the presence of a

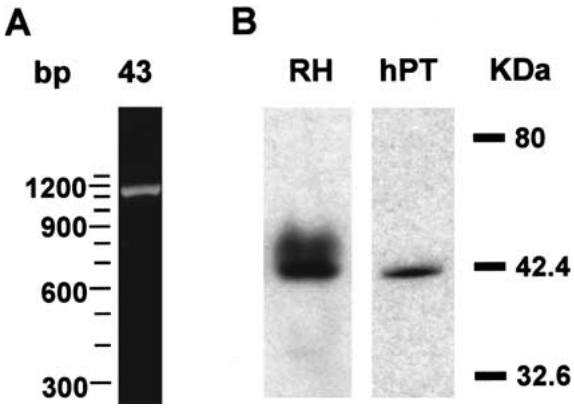

Fig. 1. (*A*) RT-PCR demonstrates the expression of Cx43 at the mRNA level (1.1–1.2 kbp band). (*B*) Western-blot analysis demonstrates protein expression with anti-rat Cx43 polyclonal antibody. A homogenate from rat heart (RH) was used as positive control. Not shown: no band was observed in the presence of excess antigenic peptide.

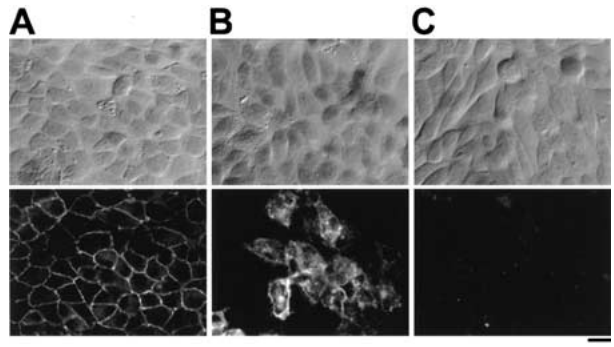

Fig. 2. Indirect immunofluorescence experiment using a polyclonal anti-human Cx43 primary antibody in a confluent hPT cell monolayer. Top and bottom frames depict differential interference contrast (DIC) and fluorescence images, respectively, of the same field. (*A*) Staining with a polyclonal anti-ZO-1 antibody shows homogeneous expression in the intercellular regions throughout the monolayer. (*B*) Heterogeneous level of Cx43 expression in the monolayer. The labeling in positive cells can be observed predominantly along the regions of intercellular contact, although there is also staining in perinuclear cytosolic regions. (*C*) Specificity was demonstrated by blocking with excess antigenic peptide. Calibration bar, 30 μm.

1.1–1.2 kbp band, consistent with the predicted size of 1158 bp (Fig. 1*A*). A positive control experiment was done with rat heart (*not shown*). At the protein level, we demonstrated Cx43 expression by a single band of ca. 42 kDa in Western-blot analysis, using an anti-rat Cx43 rabbit polyclonal antibody (Fig. 1*B*). The width of the band observed in the positive control from rat heart is consistent with varying levels of phosphorylation (Musil et al., 1990; Musil & Goodenough, 1991). In other experiments, broader signals were also observed in hPT cells. We obtained similar results using an anti-human Cx43 polyclonal antibody (*not shown*). The immunofluorescence study depicted in Fig. 2 suggests that Cx43 is expressed at the plasma membrane as well as at one or more intracellular compartments. The level of Cx43 expression was found to vary among hPT cells in the monolayer. Among the cells that express Cx43, a bright punctate pattern is observed in the areas of intercellular junctions (Fig. 2*B*). In contrast, the tight-junction-associated protein ZO1, studied simultaneously and with the same procedure, was shown to be homogeneously distributed (Fig. 2*A*). This observation rules out a staining artifact as the source of Cx43-fluorescence heterogeneity. The apparent variations in ZO-1 fluorescence intensity are caused by small differences in the height of the monolayer. Pre-adsorption of the Cx43 antibody with the antigenic peptide suppressed the labeling (Fig. 2*C*). These experiments confirm that Cx43 is expressed in the areas of intercellular membrane junctions of hPT cells. By RT-PCR, we also found mRNA coding for Cx26 and Cx32. However, expression of these isoforms at the protein level was not detectable by either Western-blot analysis or immunofluorescence.

Dissociated hPT Cells Can Be Loaded with Gap-junction Permeable Hydrophilic Dyes

To test for functional expression of GJH we used the hydrophilic-dye-uptake technique. CF is known to be permeable through gap junctions and has been used to study GJH in several cell types (DeVries & Schwartz, 1992; Li et al., 1996; John et al., 1999; Vanoye et al., 1999; Contreras et al., 2002). In interpreting the results of these experiments, it is very important to note that in dissociated cells assembled gap junctions are internalized as such, and thus they are no longer functional (Laird, 1996). Thus, connexin channels remaining in the cell membrane are hemichannels (*see* Discussion).

Dissociated hPT cells were loaded with CF at low temperature (2–7°C) in a nominally Ca^{2+}-and Mg^{2+}-free medium without Ca^{2+} chelators (Fig. 3*A*). Whereas under control conditions less than 5% of the cells were loaded, in low-Ca^{2+} medium this number increased significantly to near 25%. Gd^{3+} (10 μM) reduced the uptake of CF to a level not different from that in control [Ca^{2+}] and [Mg^{2+}]. In this and other experiments, we used Gd^{3+}, an effective and reversible blocker of GJH, albeit nonspecific since it blocks other channels (*see* below). With a larger hydrophilic dye, fluorescein-labeled dextran (F-DX3000, Dextran 3000 from Molecular Probes, size range from 1500 to 4500 Da), no loading was observed under similar experimental conditions (Fig. 3*B*). These results are consistent with the notion that removal of extracellular divalent cations activates GJH. As mentioned above, removal of divalent cations has been shown to activate GJH in many cell types, with the exception of

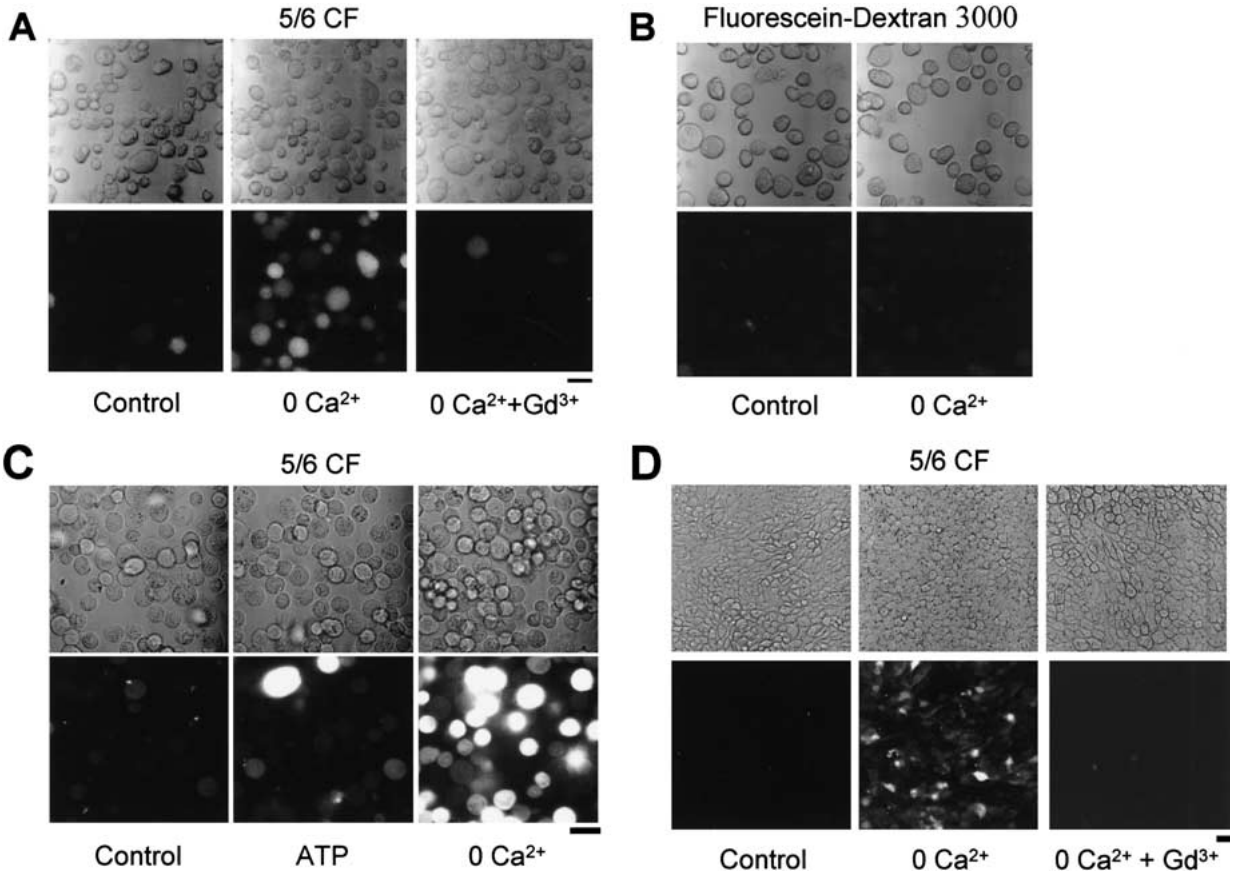

Fig. 3. Effect of extracellular Ca^{2+} removal and ATP on dye-uptake experiments at 4°C. Upper panels are transmission images, lower panels are confocal-fluorescence images; calibration bars, 30 μm. (*A*) Isolated hPT cells were exposed to 0.5 mM CF in the following conditions: normal Ringer's solution (*Control*), nominally divalent-cation-free Ringer's (0 Ca^{2+}) and Ca^{2+}-free plus 10 μM gadolinium (0 Ca^{2+} + Gd^{3+}). In control conditions, 3.6 ± 1.6% of the cells were positive for loading in comparison to 23.7 ± 3.6% for 0 Ca^{2+} and 5.6 ± 1.8 for 0 Ca^{2+} + Gd^{3+}. (*B*) Representative images showing no loading after incubation with 1 mM fluorescein-Dextran 3000 in control and 0-Ca^{2+} solutions. (*C*) Effect of 500 μM ATP on dye loading. Percentages of loaded cells were 2.3 ± 0.3 for control, 7.5 ± 1.9 for ATP and 29.6 ± 7.9 for 0 Ca^{2+}. (*D*) Dye loading in confluent monolayers of hPT cells grown on collagen-coated permeable supports. Experimental conditions similar to *A*. Instead of counting positive cells, the dye loading in monolayers was quantified by measuring the average fluorescence intensity in randomly selected confluent areas. The results in arbitrary units were 100 ± 16.7, 408 ± 41.7 and 80.6 ± 22.2 for Control, 0 Ca^{2+} and 0 Ca^{2+} + Gd^{3+} respectively.

rat-brain astrocytes (Contreras et al., 2002). However, the exposure to dye lasted only 2 min in these studies. In contrast, in an astrocyte cell line, a more prolonged Ca^{2+} removal resulted in significant dye loading (Hofer & Dermietzel, 1998).

Extracellular ATP Does not Elicit Dye Loading in hPT Cells

In addition to GJH, certain purinergic receptor channels of the P2X family have been reported to form pores permeable to hydrophilic dyes when stimulated with ATP at high-micromolar concentrations (Khakh et al., 1999; Virginio et al., 1999). Therefore, it appeared necessary to rule out a role of purinergic-receptor channels in our studies. We tested this possibility by exposing isolated hPT cells and monolayers to 500 μM ATP. As shown in Fig. 3*C*, there was only a small increase in CF loading, clearly less than those observed with removal of divalent cations (Fig. 3*A*) or with ATP depletion (*see* Fig. 7, below).

Monolayers of hPT Cells also Take up Hydrophilic Dyes

To ascertain whether or not GJH can be activated under conditions in which the cells can form gap junctions, we performed CF-uptake studies in confluent monolayers. These experiments were performed at low temperature in order to inhibit endocytosis (*see* below); cell detachment was ruled out by microscopic observation. A positive result would indicate that the expression of uncoupled connexons is not just an artifact resulting from the cell-isolation procedure (i.e., enzymatic treatment or

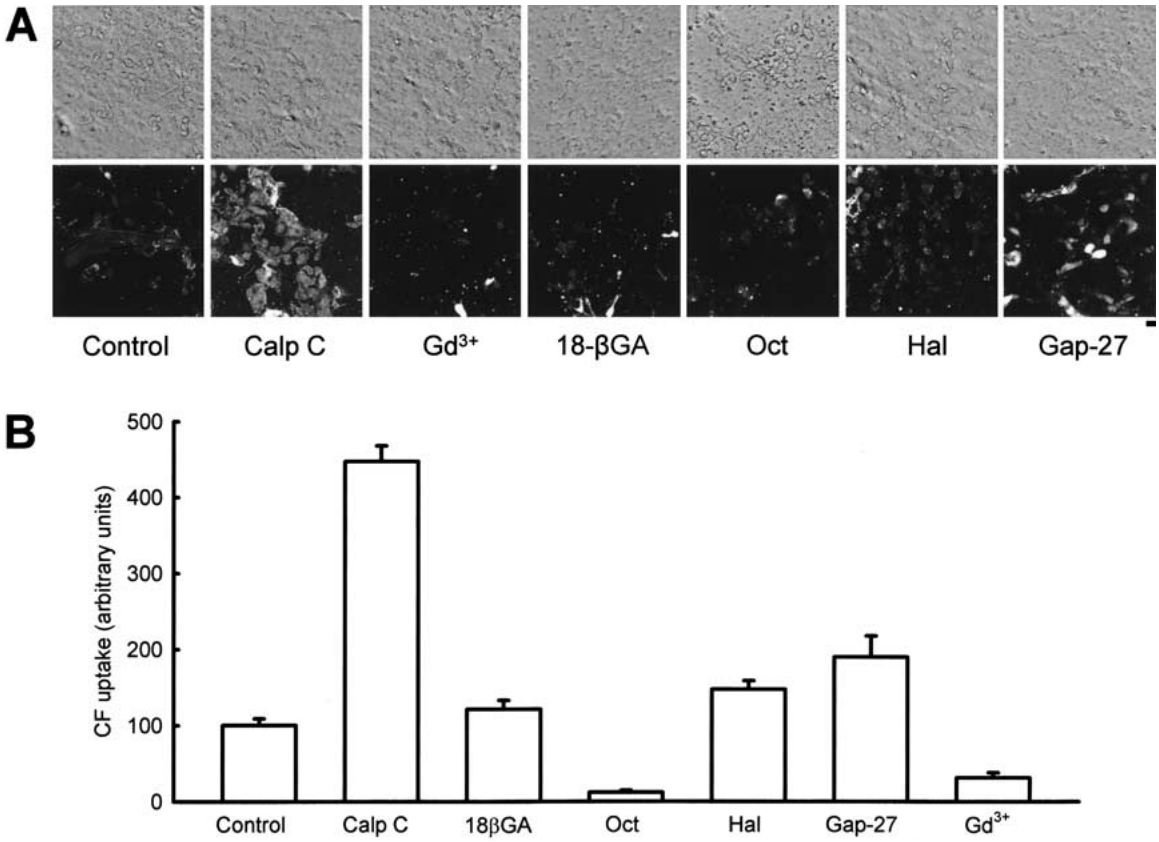

Fig. 4. Calphostin C-induced uptake of CF in hPT cells growing on transparent permeable supports and the effects of gap-junction blockers. (*A*) Transmission images (*upper panels*) and corresponding representative confocal fluorescence images (*lower panels*). The cells were exposed to control Ringer's solution either in the absence (*Control*) or in the presence of 1.5 μM calphostin C alone (*Calp C*), or to one of the following blockers: 10 μM $GdCl_3$ (Gd^{3+}), 20 mM 18-β-glycyrrhetinic acid (18-β*GA*), 1 mM octanol (*Oct*), 2 mM halothane (*Hal*) or 1 mg/ml Gap27 peptide (*Gap-27*). (*B*) Summary of the effects of blockers. Means ± SEM are depicted. Calp C was significantly different from the control group and from the blockers ($P < 0.005$, unpaired *t*-test).

mechanical damage). We used two methods to activate GJH (at 6–7°C to prevent endocytosis): removal of divalent cations (for 30 min) and treatment with the PKC blocker calphostin C (1.5 μM for 2 h). A significant fraction of the cells was loaded under the two conditions (Fig. 4). Gd^{3+} inhibited the loading elicited by removal of divalent cations and the loading elicited by PKC block was also inhibited by four other agents that inhibit gap-junction communication (octanol, halothane, 18-β-glycyrrhetinic acid and the peptide Gap27). The rationale for the use of several blockers of diverse structures in these experiments is that none of them are demonstrably specific, but the fact that all of them have the expected blocking effect limits the likelihood that the loading is not mediated by GJH. Thus, these results strongly support the notion that the permeation pathway responsible for CF uptake is constituted by uncoupled connexons. The functional presence of uncoupled connexons in the plasma membrane of hPT cells is consistent with and extends the significance of the recent observation that vesicles containing GJH fuse randomly with the plasma membrane (Lauf et al., 2002).

Endocytosis Is Inhibited at 4°C

To quantify endocytosis in hPT-cell monolayers at low temperature, we measured the uptake of Texas-Red-labeled Dextran-10000 (1 mM, Molecular Probes, TxRd-DX10000), a hydrophilic dye that does not permeate GJC or GJH. As seen in Fig. 5, dye uptake was observed at 37°C but not at 4°C, which is the temperature used to measure CF uptake via GJH. In addition, the uptake observed at 37°C occurred in the presence of 1.3 mM extracellular Ca^{2+}. Furthermore, the cells loaded by labeled dextran display a punctate pattern as expected for a vesicle-mediated uptake, in contrast with the diffuse loading observed in the experiments with smaller hydrophilic dyes at 4°C. The results shown in this figure rule out a contribution of endocytosis to the uptake of CF, which we attribute to GJH.

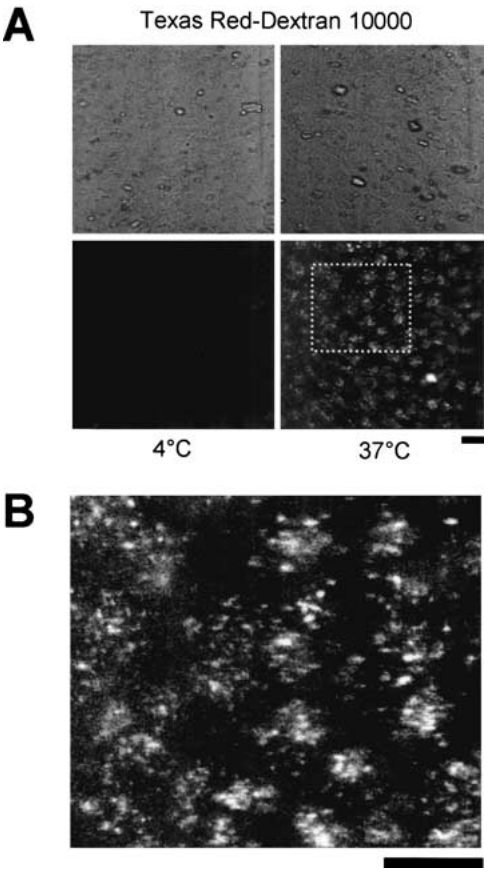

Fig. 5. (*A*) Temperature dependence of endocytosis in hPT cell monolayers assessed by incubating confluent cells with 1 mM Texas Red-Dextran (MW 10,000) during 30 min at the temperatures indicated. *Top*: transmission images; *bottom*: fluorescence images. Endocytosis was clearly revealed as dye uptake at 37°C (*right*), but not at 4°C (left). (*B*) Amplified inset shows punctate fluorescence, typical of endocytosis. Calibration bars, 30 μm.

Taken together, these experiments conclusively demonstrate that the permeability pathway responsible for CF loading has a molecular-size cut-off below 1,500 Da, and is sensitive to several blockers of gap-junction communication, thus ruling out a transient non-specific permeabilization of the plasma membrane by Ca^+ removal and/or mechanical stress during experimental manipulation.

Moderate ATP Depletion Results in Dye Uptake by hPT-Cell Monolayers in Normal Extracellular Ca^{2+}

Although useful as a test for GJH activation, removal of extracellular divalent cations is of no physiological or pathophysiological significance. However, Cx43 phosphorylation has been shown to regulate gap-junctional intercellular communication (Lau et al., 1996) and our studies with calphostin C suggest that Cx43 GJH are activated by dephosphorylation of

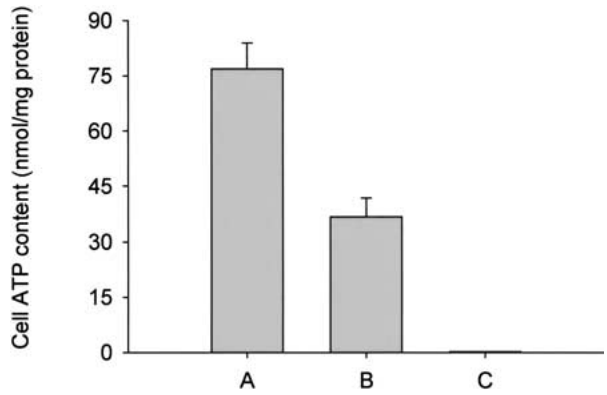

Fig. 6. Cell ATP content after incubation of confluent hPT-cell monolayers under control conditions (*A*), after a 1-h incubation in medium containing 2 mM glucose, 2 mM DOG and 10 μM Antimycin A (*B*), and after a 15-min incubation in medium containing 10 μM FCCP and 1 mM IAA (C). The change in ATP content was significant in B and C and the level in C did not differ significantly from 0.

one or more PKC consensus sites. In addition, it was recently reported that pharmacologically induced ATP depletion activates gap-junctional hemichannels in cardiomyocytes, Cx43 transfected HEK293 cells (John et al., 1999) and brain astrocytes (Contreras et al., 2002). Hence, we tested whether in normal extracellular $[Ca^{2+}]$ and $[Mg^{2+}]$ the connexons putatively expressed on the plasma membrane of renal-tubule cells can be activated by ATP depletion. Because the mode of cell damage depends on the time course and magnitude of ATP depletion, we adapted protocols developed by others (Lieberthal et al., 1998) to be able to obtain several different ATP levels, ranging from ca. 50 (moderate) to virtually 100% (severe) depletion (Fig. 6). Monolayers of hPT cells treated with 2 mM 2-DOG and 10 μM Antimycin A in 2 mM glucose medium, had moderate ATP depletion, a level shown to cause apoptosis in mouse proximal tubule cells in culture (Lieberthal et al., 1998). Hence, we depleted the cells of ATP at 37°C with this protocol and then exposed them to CF at 6–7°C. The ATP-depleted cells exhibited significantly higher levels of fluorescence than the control cells, and this difference was abolished by exposure to Gd^{3+} (Fig. 7). Not shown, the fluorescence persisted after cell dissociation, confirming the intracellular location of the dye. Notably, the fluorescence was distributed in a heterogeneous pattern (Fig. 7*A*). These results support the hypothesis of CF uptake via GJH activated by moderate ATP depletion.

To test whether CF loading was the result of irreversible membrane permeabilization, we stained the cells with propidium iodide (PI) in the presence of $GdCl_3$ and $CaCl_2$, after the incubation with CF. The CF loading was heterogeneous and the CF-loaded cells excluded PI. This denotes that the CF-loaded

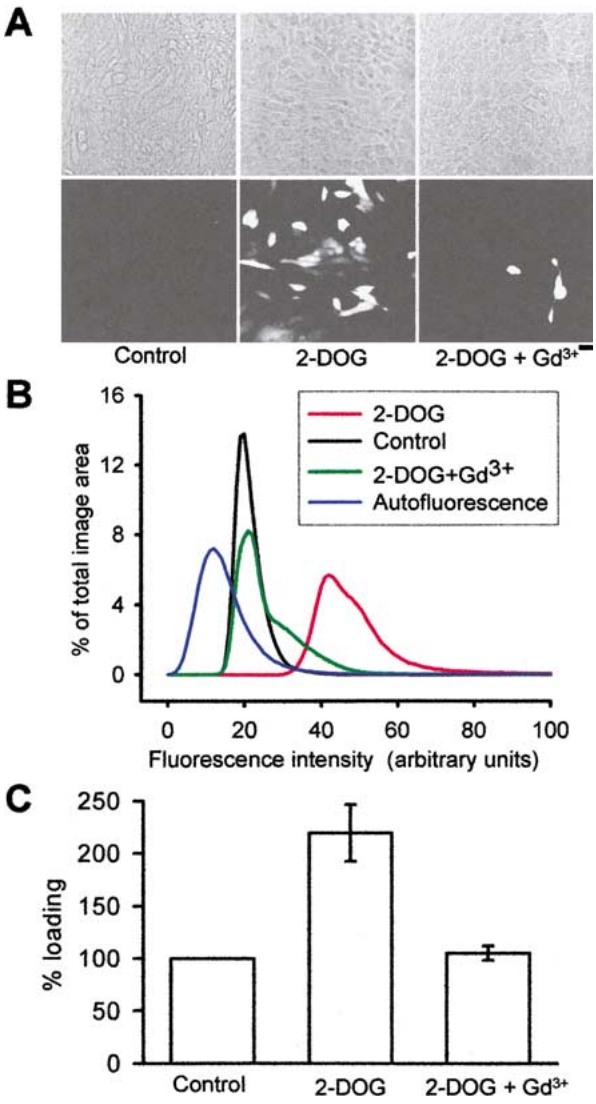

Fig. 7. ATP-depletion induces uptake of CF in confluent hPT cell monolayers. (*A*) Representative pictures of monolayers exposed to CF in control conditions (*left*), or after ATP depletion in absence (*center*) or presence of 10 μM $GdCl_3$ (*right*). Transmission images (*upper panels*) and corresponding representative confocal fluorescence images (*lower panels*) are shown. (*B*) Representative experiment depicting the distribution of pixel fluorescence intensity measured using Metamorph 4.0 (Universal Imaging Corp. Downingtown, PA) in monolayers after incubation with CF under control conditions or after ATP depletion in absence or presence of $GdCl_3$, compared with autofluorescence, measured in unloaded cells. Each curve summarizes data from 5 images. Note that ATP depletion (2-DOG) displaces the fluorescence distribution to higher values, but the peak fluorescence approaches the control level with the addition of Gd^{3+}. The fluorescence distribution of ATP-depleted cells is bimodal, suggesting two cell populations. (*C*) Average fluorescence intensity measured in areas of total cell confluency and corrected by background (autofluorescence). Control is compared with ATP-depleted cells, in the presence ($n = 5$) or absence ($n = 3$) of 10 μM Gd^{3+}. Data expressed as percentage of the control group. ATP-depleted *vs.* control $P < 0.005$, and *vs.* Gd^{3+}, $P < 0.05$, unpaired *t*-test.

cells are viable and that the loading is not caused by irreversible rupture of the plasma membrane. Necrotic cells, that cannot exclude PI, cannot retain CF (*see* below).

Removal of Divalent Cations and Moderate ATP Depletion Cause Cell Death

We also carried out experiments in which cells were stained with PI, after CF loading, under conditions that increase hemichannel permeability, i.e., Ca^{2+} removal and ATP depletion. Both conditions increased significantly the percentage of necrotic (PI stained) cells, by 3-fold and 4-fold by removal of divalent cations and moderate ATP depletion, respectively (Fig. 8). In both experiments, the cell damage was confirmed by measurements of the release of lactic dehydrogenase (*data not shown*). Exposure to Gd^{3+} prevented the increase in the percentage of necrotic cells under both experimental conditions. These findings strongly support the view that open GJHs are a determinant of cell death in ATP-depleted renal proximal-tubule cells and that entry of Ca^{2+} via GJH is not the sole mechanism of cell damage during ATP depletion (*see* Discussion). In addition, the results of these double-labeling experiments indicate that in our experimental conditions CF and PI enter the cells via different pathways. CF permeates GJH and is retained in live cells after the addition of Gd^{3+} to prevent the efflux through the same pathway. Gd^{3+} also prevents entry of PI via GJH. PI entry is Gd^{3+}-independent and thus permeates cells that have lost membrane integrity and therefore do not retain CF when exposed to Gd^{3+}.

Discussion

The main findings reported in this paper are: (a) hPT cells express Cx43 in the plasma membrane; (b) this connexin forms hemichannels that can be demonstrated by size-restricted hydrophilic-dye uptake following removal of extracellular divalent cations; (c) the above effect is inhibited by several blockers of gap-junctional communication; (d) moderate ATP depletion activates this pathway in the presence of divalent cations, increasing dye loading; both removal of divalent cations and moderate ATP depletion increase the percentage of necrotic cells in the monolayer; (e) exposure to Gd^{3+} in micromolar concentrations reduces the dye loading and the degree of cell death elicited either by Ca^{2+} and Mg^{2+} removal or by ATP depletion.

Our aim was to study a possible pathophysiological role of GJH in human renal proximal tubules using cell monolayers obtained from primary cultures. Morphologically and functionally the cells resemble human proximal tubule cells from S3, the

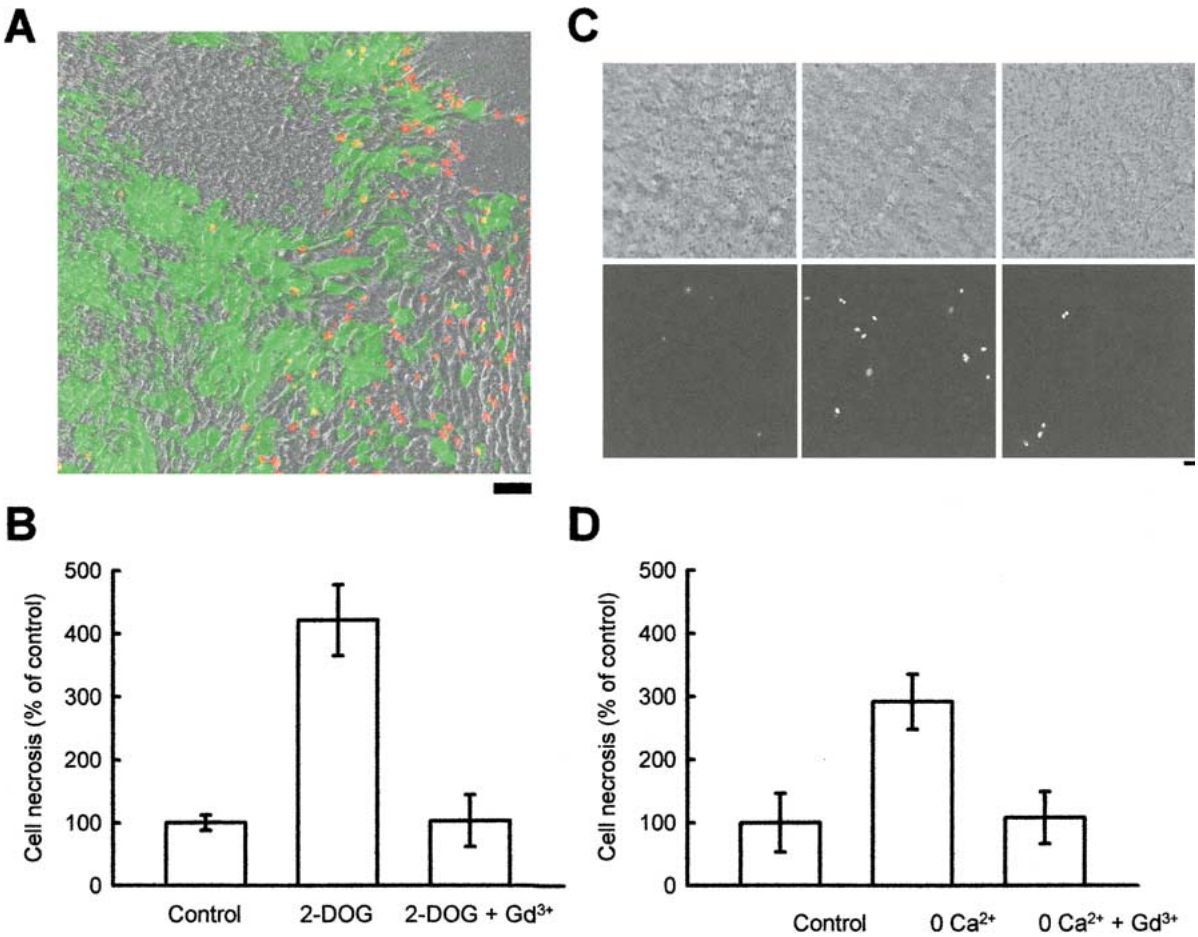

Fig. 8. Moderately ATP-depleted monolayers were loaded with CF in the absence or presence of Gd^{+3} and then with PI in the presence of Gd^{+3}. There was patchy distribution of the CF loading (green fluorescence) as well as of the PI-stained cells (red). Some areas excluded both dyes. In the stained areas, most of the CF-loaded cells excluded PI. The necrotic cells concentrate in certain areas of the ATP-depleted monolayers, but correspond to less than 4% of the total number of cells. (*B*) Effect of metabolic inhibition on cell necrosis. Results are expressed relative to the control group (mean ± SEM). Number of experiments: 8, 8 and 4, respectively. ATP depletion results in a 4-fold increase in the number of necrotic cells, $P < 0.005$ for both ATP-depleted *vs.* control and ATP-depleted *vs.* Gd^{3+}, unpaired *t*-tests. (*C* and *D*) Analogous experiments in which GJH were activated by Ca^{2+} removal. (*C*) Representative images. (*D*) Summary of results, showing a 3-fold increase in cell necrosis upon Ca removal; $P < 0.05$ for both 0 Ca^{2+} *vs.* control and 0 Ca^{2+} *vs.* Gd^{3+}, paired *t*-tests. Number of observations: 5, 8 and 8, respectively.

segment most damaged by renal ischemia or toxins.[1] The human pars recta cells in culture also express Cx43, as indicated by RT-PCR and Western blot analysis and the connexin is properly targeted to the plasma membrane in regions of intercellular contacts, as shown by immunofluorescence of intact monolayers. Thus, we consider these cells to be an acceptable model for the pars recta human proximal tubule cells. It is important to note that the main limitation of the use of cells in culture in studies of ischemic damage resides in their adaptation to anaerobic metabolism. While a valid objection for studies of hypoxia, this issue is not relevant to the observations reported here, where the changes in Cx43 function were achieved by experimental ATP depletion. Of course, further studies in vivo will be needed to assess the relevance of this mechanism to ischemic damage in the kidney. The use of confluent monolayers to estimate GJH activation has the advantage that the preparation is close to the in vivo condition. However, a disadvantage is the possibility that dye loading of a group of cells can occur by a combination of GJH activation and cell-to-cell dye

[1]The hPT cells exhibit short microvilli, extremely dense lysosomes and sparse and small mitochondria; stimulation with forskolin or parathyroid hormone (PTH) increases adenylate-cyclase activity, whereas incubation with arginine vasopressin or calcitonin has no effects. The monolayers also exhibit vectorial fluid transport from apical to basolateral side; PTH and cAMP were also noted to increase microvilli differentiation (Ernest, S., Rajaraman, S. and Bello-Reuss, E. PTH-induced differentiation of human proximal tubule (PT) cells in culture is mediated by cAMP. Abstracts of the XVth International Congress of Nephrology, Buenos Aires, Argentina, p. 1, 1999.)

flux via GJC. However, our studies with depletion of extracellular divalent cations demonstrated loading both in confluent and dissociated cells, suggesting that the observation in confluent monolayers cannot be deemed to be an artifact.

Gap-junctional hemichannels have been observed after heterologous connexin expression in *Xenopus* oocytes and in cell lines (Paul et al., 1991; DeVries & Schwartz, 1992; Ebihara & Steiner, 1993; Gupta et al., 1994; Ebihara et al., 1995, 1999; Ebihara, 1996; Pfahnl et al., 1997; Zampighi et al., 1999), as well as in cells or multicellular preparations from native tissues with endogenous connexin expression (DeVries & Schwartz, 1992; Ebihara, 1996; Li et al., 1996; John et al., 1999; Vanoye et al., 1999). Although the existence of uncoupled connexons in the plasma membrane has been assumed as part of the life cycle of connexins in gap junctions (Laird, 1996), only recently the presence of connexins in non-junctional regions of the plasma membrane was directly demonstrated by freeze-fracture electron microscopy in *Xenopus* oocytes (Zampighi et al., 1999) and by confocal microscopy of live cells transfected with EGFP-Cx43 fusion proteins (Jordan et al., 1999). It has been proposed that in dissociated cardiomyocytes (John et al., 1999; Kondo et al., 2000) and astrocytes (Contreras et al., 2002) Cx43 GJH are activated by ATP depletion becoming potential contributors to cell injury.

Our studies were based on measurements of dye uptake that revealed that hPT cells express a pathway permeable to hydrophilic molecules smaller than ca. 1,500 Da. This size selectivity is consistent with GJH and perhaps with certain subtypes of P2X receptors (Virginio et al., 1999), but we ruled out a significant contribution of the latter pathway to our observations because of the meager effect of extracellular ATP on dye loading. The pathway in hPT cells is activated by removal of extracellular divalent cations and by ATP depletion, and is inhibited by Gd^{3+}. Since there are no demonstrably specific GJH blockers, in a series of experiments in which the cells were treated with calphostin C we tested the gap-junction blockers 18-β-glycyrrhetinic acid, halothane, octanol and Gap27. All of them inhibited significantly CF uptake. Our results strongly suggest the expression of functional GJH in non-junctional regions of the plasma membrane of hPT cells. The essential arguments for this conclusion are the selective, size-dependent permeation of hydrophilic solutes, which is consistent with the permeability of Cx43 GJC (Koval et al., 1995), and the effects of gap-junction blockers. Supportive observations are the lack of response to extracellular ATP, which rules out purinergic-receptor channels (Virginio et al., 1999), as well as the similar effects of both removal of external Ca^{2+} and depletion of ATP.

To study the effects of renal ischemia at the cellular level, we used a model of pharmacological ATP depletion. We could obtain graded levels of ATP depletion by the combined use of inhibitors of glycolysis and/or oxidative phosphorylation. Moderate ATP depletion activates GJH even in the presence of normal extracellular calcium in intact monolayers and in a time as short as 1 hour. In contrast, the severe ATP depletion elicited by FCCP and IAA occurred in a few minutes and both CF loading and cell death were observed correspondingly earlier.

As argued in Results, the dye-loading results obtained in confluent monolayers have the potential ambiguity of a mixed mechanism, involving CF uptake via GJH and cell-to-cell dye flux via GJC. A significant role of this mechanism was ruled out by parallel studies in dissociated cells.

Although the mechanism of activation of GJH by ATP depletion is not known, one possibility is that the lower intracellular [ATP] results in a reduction in the phosphorylation of Cx43, as indicated, e.g., by studies in brain cells (Li and Nagy, 2000). There is a large body of evidence pointing towards a direct relationship between ATP depletion, Cx43-phosphorylation level and GJH (or GJC) activity (Solez, Morel-Maroger & Sraer, 1979, Swenson et al., 1990, Kwak et al., 1995; Lau et al., 1996; Li et al., 1996). However, in astrocytes expressing Cx43, GJH were also activated by ATP depletion, but by an indirect mechanism involving arachidonic-acid metabolites. Whether or not this mechanism is also operative in other cells is unclear at present. Our results support the notion that Cx43 dephosphorylation mediates the activation of GJH, because the effect was elicited by both ATP depletion and PKC inhibition. Experiments in progress indicate that purified Cx43 forms hemichannels when reconstituted in liposomes and the permeability of these hemichannels can be modulated by PKC-mediated phosphorylation, supporting a direct effect (X. Bao, L. Reuss and G. A. Altenberg, unpublished observations).

In contrast to recent publications (John et al., 1999; Contreras et al., 2002), which report activation of GJH upon treatment with pharmacological agents that induce severe ATP depletion, we found that moderate ATP depletion (ca. 50% of normal ATP content) is enough to induce GJH activation. This suggests that GJH are involved in early stages of cell damage induced by metabolic inhibition. Moderate ATP depletion has been shown to induce apoptosis in mouse proximal tubules (Lieberthal et al., 1998) consistent with our results showing an increase in cell death. Inasmuch as the PI-uptake method detects only necrosis, further studies will be needed to ascertain whether our observations denote primary and/or secondary necrosis. Severe ATP depletion, as obtained by simultaneous exposure to FCCP and IAA for 15 min, also results in increased dye uptake but the level of necrosis is much higher. During ischemic ATN, it has been shown that cell death is not a

generalized process, but is observed as dispersed foci of epithelial destruction along the late proximal tubule (Solez et al., 1979). However, metabolic inhibition may cause more generalized cellular dysfunction and sub-lethal injury not identifiable by morphological studies. In addition to its putative effect on GJH, ATP depletion affects the epithelial barrier function by altering the formation of intercellular junctions and cell adhesion, thus contributing to the pathogenesis of intrinsic ARF by increasing the tubule permeability (back-leak of luminal fluid) and by causing tubule obstruction, mostly by detached cells. In addition, profound ATP depletion may have lethal effects on cells by a number of other mechanisms. We suggest that activation of GJH may amplify the deleterious effect of ATP depletion by permitting dissipative fluxes between intra- and extracellular fluids and also by facilitating the loss of intracellular metabolites including ATP and other nucleotides. The loss of ADP and AMP will impair the ability of cells to recover after ischemic injury, because these metabolites will not be available when oxygenation is restored (Bonventre, 1993). In addition, gap junctions mediate metabolic cooperation among neighboring cells, and therefore it will be interesting to evaluate the effect of metabolic inhibition on gap-junctional communication and the role of these changes in the pathogenesis of renal-tubule necrosis.

The hypothesis of a pathophysiological role for GJH in the renal tubule cell injury observed after moderate metabolic inhibition may help better understand the pathogenesis of renal-tubule necrosis at the molecular level. It will be important to confirm these results in experiments in which the expression of Cx43 in proximal tubule cells is ablated, to unravel the mechanism by which ATP depletion leads to activation of the hemichannels, to determine what other factors are involved in the regulation of GJH, and to assess the possibility of their activation during other pathological processes such as toxic insult and chronic ischemia.

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