TRPP2 channels regulate apoptosis through the Ca^{2+} concentration in the endoplasmic reticulum

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Ca^{2+} is an important signalling molecule that regulates multiple cellular processes, including apoptosis. Although Ca^{2+} influx through transient receptor potential (TRP) channels in the plasma membrane is known to trigger cell death, the function of intracellular TRP proteins in the regulation of Ca^{2+} -dependent signalling pathways and apoptosis has remained elusive. Here, we show that TRPP2, the ion channel mutated in autosomal dominant polycystic kidney disease (ADPKD), protects cells from apoptosis by lowering the Ca^{2+} concentration in the endoplasmic reticulum (ER). ER-resident TRPP2 counteracts the activity of the sarcoendoplasmic Ca^{2+} ATPase by increasing the ER Ca^{2+} permeability. This results in diminished cytosolic and mitochondrial Ca^{2+} signals upon stimulation of inositol 1,4,5-trisphosphate receptors and reduces Ca^{2+} release from the ER in response to apoptotic stimuli. Conversely, knockdown of TRPP2 in renal epithelial cells increases ER Ca^{2+} release and augments sensitivity to apoptosis. Our findings indicate an important function of ER-resident TRPP2 in the modulation of intracellular Ca^{2+} signalling, and provide a molecular mechanism for the increased apoptosis rates in ADPKD upon loss of TRPP2 channel function.

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Introduction

Calcium (Ca^{2+}) is a highly versatile signalling molecule controlling numerous functions during life and death of cells (Berridge *et al*, 2000). Recent studies have emphasized the central function of Ca^{2+} in the regulation of programmed cell death (Orrenius *et al*, 2003; Rizzuto *et al*, 2003). Increases of cytosolic Ca^{2+} during cell death can arise from several sources, including damage of external membranes, hyperac-

tivation of cation channels, or release from Ca^{2+} stores in the endoplasmic reticulum (ER) (Szalai *et al*, 1999; Orrenius *et al*, 2003). Mounting evidence indicates a highly coordinated communication between ER Ca^{2+} stores and mitochondria (Rizzuto *et al*, 1998). Apoptotic stimuli, such as thapsigargin or ceramide, elicit Ca^{2+} release from the ER and thereby increase the mitochondrial Ca^{2+} concentration (Pinton *et al*, 2001). During cell death, mitochondrial Ca^{2+} signals activate the permeability transition pore, resulting in mitochondrial depolarization and the release of cytochrome c (Orrenius *et al*, 2003). Notably, the Ca^{2+} content in the ER determines the amplitude of mitochondrial Ca^{2+} signals and thus regulates apoptosis sensitivity (Pinton and Rizzuto, 2006).

Proteins that regulate apoptosis include the Bcl-2 protein family, which consists of anti- and proapoptotic members. Many of these proteins function at the level of mitochondria, but recently a prominent function of these proteins in the ER has emerged (Pinton and Rizzuto, 2006). Specifically, recent evidence shows that a delicate balance between pro- and antiapoptotic Bcl-2 family members fine-tunes the Ca^{2+} concentration in the ER. Whereas Bcl-2 has been shown to decrease the ER Ca^{2+} content and thus protects cells against apoptosis (Foyouzi-Youssefi *et al*, 2000; Pinton *et al*, 2000), proapoptotic proteins, such as Bax and Bak, have an opposing function (Scorrano *et al*, 2003).

TRPP2 is a member of the transient receptor potential (TRP) superfamily of cation channels (Montell, 2005) that is mutated in autosomal dominant polycystic kidney disease (ADPKD) (Mochizuki *et al*, 1996). ADPKD is among the most common hereditary diseases and is caused by mutations in either *PKD1* or *PKD2*, the genes encoding polycystin-1 and TRPP2 (previously termed polycystin-2), respectively (Mochizuki *et al*, 1996; Boletta and Germino, 2003). TRPP2 functions as a Ca^{2+} -permeable cation channel and has been detected in different subcellular compartments: the ER, the plasma membrane, and the primary cilium (Köttgen and Walz, 2005). We have shown that the trafficking of TRPP2 between the ER and the plasma membrane is controlled by phosphorylation-dependent interaction with the adapter proteins PACS-1 and PACS-2 (Köttgen *et al*, 2005). The regulated trafficking of TRPP2 is an important mechanism to control channel function. Yet, its precise biological function in distinct subcellular compartments is poorly understood. TRPP2 has been implicated in various physiological functions, including mechanosensation, proliferation, and sperm guidance. However, the mechanisms underlying translation of different compartment-specific TRPP2 functions into specific cellular outcomes have remained elusive (Köttgen, 2007).

Genetic diseases involving dysfunction of ion channels (channelopathies) have been instrumental in unravelling physiological function for many of them (Jentsch *et al*, 2004; Nilius *et al*, 2007). Apoptosis is causally linked to cystogenesis: deletion of antiapoptotic genes encoding Bcl-2 and AP-2 β and overexpression of proapoptotic c-Myc in mice

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result in renal cystic disease (Veis *et al*, 1993; Moser *et al*, 1997; Trudel *et al*, 1997). In ADPKD patients and several animal models of polycystic kidney disease (PKD), increased apoptosis rates have been reported (Woo, 1995; Ecdar *et al*, 2002; Kip *et al*, 2005; Bukanov *et al*, 2006; Starremans *et al*, 2008). Thus, we hypothesized that TRPP2 might function as an antiapoptotic ion channel. This appears rather counter-intuitive, as non-selective cation channels have generally been implicated in promoting cell death rather than survival (Miller, 2006). However, the predominant localization of TRPP2 in the ER (Cai *et al*, 1999) prompted us to investigate its function in the ER Ca^{2+} gateway to apoptosis. In this study, we address the function of TRPP2 in intracellular Ca^{2+} signalling and show that TRPP2 functions as an antiapoptotic ion channel that regulates the Ca^{2+} concentration in the ER. Our results not only elucidate a new function of ER-resident TRPP2, but they may also explain why disruption of Bcl-2 causes PKD in mice.

Results

TRPP2 diminishes Cl^- currents activated by ER Ca^{2+} release in *Xenopus* oocytes

To study the channel function of TRPP2, we expressed the protein in *Xenopus* oocytes and monitored whole cell cur-

rents under voltage clamp conditions. TRPP2 did not affect whole-cell steady-state conductive properties (Figure 1A and B). This is consistent with recent studies reporting no detectable TRPP2 channel activity at the plasma membrane upon heterologous expression in different cell types (Chen *et al*, 2001; Köttgen *et al*, 2005).

To test whether TRPP2 might function in an intracellular compartment and thereby modulate Ca^{2+} signalling, we measured endogenous Ca^{2+} -activated Cl^- currents in oocytes to monitor dynamic changes in the cytosolic Ca^{2+} concentration (Köttgen *et al*, 2003). Stimulation of endogenous G protein-coupled receptors (GPCRs) with trypsin activated outwardly rectifying Ca^{2+} -activated Cl^- currents in water-injected control oocytes (Figure 1C and E). In oocytes expressing TRPP2, however, these Ca^{2+} -activated Cl^- currents were strongly reduced (Figure 1D and E). The trypsin-induced whole-cell conductance was significantly decreased in oocytes expressing TRPP2 (Figure 1F; control: $27.88 \pm 5.79 \mu\text{S}$, $n = 9$; TRPP2: $6.47 \pm 1.42 \mu\text{S}$, $n = 11$). Trypsin receptors in *Xenopus* oocytes transduce signals through $\text{G}_{\alpha\text{q}}$ -protein-mediated activation of phospholipase C, which results in IP_3 -induced release of Ca^{2+} from intracellular stores. Therefore, reduction in the amplitude of the Ca^{2+} -activated Cl^- currents in TRPP2-expressing cells may result either from a reduction in the abundance or activity of

Figure 1 TRPP2 channel function in *Xenopus* oocytes. (A) Current-voltage (I-V) relations for oocytes expressing TRPP2 (dashed line) or water-injected control cells (solid line). (B) Group data from (A). Whole cell conductances (G) were calculated according to Ohm's law. (C) Current-voltage relations for control oocytes in control solution (dashed line) or after stimulation with trypsin (10 $\mu\text{g}/\text{ml}$; solid line). (D) Current-voltage relations in oocytes expressing TRPP2 in control solution (dashed line) or after stimulation with trypsin (solid line). (E) Time-course of the trypsin-induced whole cell currents in control cells and cells expressing TRPP2. Currents were recorded under voltage clamp conditions. Voltage clamp (V_c) protocol as depicted. (F) Group data from (E). Trypsin-induced whole cell conductances (black bars) were calculated at peak currents (grey bars: conductances in control solution). * and § indicate statistically significant differences as indicated.

the proteins involved in this signalling cascade or from a decrease of releasable free Ca^{2+} in the ER Ca^{2+} stores. To distinguish between these possibilities, we studied the influence of TRPP2 on Ca^{2+} signalling in several mammalian cell lines.

TRPP2 is localized to the ER and reduces Ca^{2+} release from intracellular stores

Indirect immunofluorescence of TRPP2 expressed in human embryonic kidney (HEK) 293 cells showed an intracellular reticular distribution of the protein that co-localized with BAP31.GFP, a marker for the ER (Figure 2A). To determine the function of TRPP2 in the ER, we measured the cytosolic Ca^{2+} concentration $[\text{Ca}^{2+}]_c$ in HEK 293 cells using ratio-metric Ca^{2+} imaging. The basal $[\text{Ca}^{2+}]_c$ was not significantly different between vector-transfected (control) and TRPP2-expressing cells (14.9 ± 2.6 nM and 14.3 ± 2.4 nM, respectively, $n=9$). Stimulation of purinergic GPCRs with ATP, however, showed a marked decrease in the amplitudes of Ca^{2+} signals in cells expressing TRPP2 or Bcl-2 (Figure 2B and C; control: 183.31 ± 17.75 nM, $n=9$, TRPP2: 92.39 ± 8.79 nM, $n=9$ and Bcl-2: 83.94 ± 7.01 nM, $n=8$). As these experiments were performed in the nominal absence of extracellular Ca^{2+} , the decreased cytosolic Ca^{2+} signals reflect a reduction of Ca^{2+} release from intracellular stores. We confirmed that expression of TRPP2 in HEK 293 cells does not change the levels of the sarcoendoplasmic Ca^{2+} ATPase

Figure 2 TRPP2 localizes to the ER and modulates Ca^{2+} signalling. (A) HEK 293 cells transfected with TRPP2 and the ER marker BAP31.GFP (middle panel) were stained with anti-TRPP2 antibody (left panel, scale bar: 10 µm; blue: nuclear staining with Hoechst 33342). (B) Fura-2 Ca^{2+} measurements: effect of transient TRPP2 or Bcl-2 expression on Ca^{2+} release from intracellular stores in HEK 293 cells. Purinergic receptors were stimulated with 10 µM ATP in the absence of extracellular Ca^{2+} . (C) Group data from (B) at peak Ca^{2+} increase (ATP-induced peak-baseline). (D) The levels of SERCA2 and Bcl-2 in the lysates of HEK 293 cells transfected with TRPP2-encoding plasmid or empty vector were analysed by western blotting.

(SERCA) and Bcl-2, two proteins that were earlier shown to affect ER Ca^{2+} content (Pinton *et al*, 2001; Pinton and Rizzuto, 2006; Figure 2D).

To exclude cell-type-specific effects and potential artefacts due to toxicity of transient overexpression, we generated HeLa cells stably expressing TRPP2 using retroviral gene transfer (western blot shown in Figure 3A). HeLa cells stably expressing TRPP2 showed a significant reduction of Ca^{2+} release from intracellular stores upon the stimulation of G_q -coupled receptors, confirming the results from *Xenopus* oocytes and HEK 293 cells (Supplementary Figure 1). To test whether this decrease in the cytosolic Ca^{2+} signals was due to modulation of proteins in the GPCR signalling pathway or due to a reduction of the Ca^{2+} concentration in the ER ($[\text{Ca}^{2+}]_{\text{ER}}$), we bypassed the GPCR signalling pathway by releasing Ca^{2+} from intracellular stores in a receptor-independent manner (in the absence of extracellular Ca^{2+}). Inhibition of SERCA by thapsigargin led to the release of Ca^{2+} from the ER due to passive leak. As $[\text{Ca}^{2+}]_{\text{ER}}$ determines the driving force for Ca^{2+} release, the amplitude of the cytosolic Ca^{2+} increase indirectly reflects the ER Ca^{2+} content. The peak amplitude after application of thapsigargin in HeLa cells stably expressing TRPP2 was significantly reduced compared with control cells (Figure 3B and D; control: 184.23 ± 22.81 nM and TRPP2: 112.64 ± 17.08 nM, $n=11$). Likewise, Ca^{2+} release from intracellular stores induced by the ionophore ionomycin was decreased in HEK 293 cells expressing TRPP2 (Figure 3C and E; control: 2.27 ± 0.35 µM, and TRPP2: 1.01 ± 0.10 µM, $n=9$). Thus, our data point to the function of TRPP2 in decreasing the ER Ca^{2+} load. Acute reduction of the ER Ca^{2+} concentration results in activation of capacitative Ca^{2+} influx (Hofer *et al*, 1998). However, previous studies showed that long-term reduction of ER Ca^{2+} levels by Bcl-2 overexpression downregulates capacitative

Figure 3 TRPP2 decreases GPCR-independent release of Ca^{2+} from the ER. (A) Expression of TRPP2 in HeLa stable cell lines was examined by western blotting using anti-TRPP2 antibody (control: HeLa cells transduced with retrovirus generated from empty vector pLXSN; TRPP2: HeLa cells transduced with retrovirus encoding TRPP2). Levels of 14-3-3 proteins were analysed as a loading control. (B) Fura-2 Ca^{2+} measurements: effect of stable expression of TRPP2 in HeLa cells on the release of Ca^{2+} from the ER using thapsigargin (TG, 10 µM). (C) Effect of transient expression of TRPP2 in HEK 293 cells on the release of Ca^{2+} from the ER using ionomycin (Iono, 5 µM). Experiments in (B) and (C) were performed in the absence of extracellular Ca^{2+} . (D) Group data from (B) at peak Ca^{2+} increase (TG-induced peak-baseline) ($n=11$). (E) Group data from (C) at peak Ca^{2+} increase ($n=9$).

Ca^{2+} influx (Pinton *et al*, 2000). We investigated the effect of TRPP2 expression on capacitative Ca^{2+} entry in HEK 293 cells. Following depletion of stores with thapsigargin in the absence of extracellular Ca^{2+} , capacitative influx was observed after readdition of Ca^{2+} to the bath solution. In cells expressing TRPP2, capacitative Ca^{2+} entry was significantly downregulated (Supplementary Figure 2), adding to the list of functional similarities between Bcl-2 and TRPP2.

TRPP2 decreases the Ca^{2+} concentration in the ER

The data from our cytosolic Ca^{2+} measurements are suggestive of a reduced ER Ca^{2+} concentration, but do not provide the actual $[\text{Ca}^{2+}]_{\text{ER}}$. To substantiate the evidence for a reduction of the ER calcium concentration in cells expressing TRPP2, we measured $[\text{Ca}^{2+}]_{\text{ER}}$ directly using a genetically encoded ER-targeted Ca^{2+} sensor (Yellow Cameleon) (Miyawaki *et al*, 1997). The calreticulin signal peptide and KDEL sequence targeted the low-affinity Ca^{2+} sensor YC4_{ER} to the ER where it colocalized with TRPP2 (Figure 4A). Cameleon dyes consist of cyan fluorescent protein (CFP), calmodulin, the calmodulin-binding domain of myosin light

chain kinase (M13), and a yellow fluorescent protein (YFP). Ca^{2+} binding to calmodulin induces its intramolecular interaction with M13, which increases the efficiency of fluorescence resonance energy transfer between CFP and YFP. Thus, calcium binding can be visualized by exciting CFP at 440 nm and recording the change in the emission ratio 535/480 nm (Figure 4B). We directly measured the steady-state concentration of Ca^{2+} in the ER by calibrating the ratiometric fluorescence signal in ' Ca^{2+} -permeabilized' cells (10 μM ionomycin, 0.5 $\mu\text{g}/\text{ml}$ digitonin) that were exposed to defined extracellular Ca^{2+} concentrations (Figure 4B). $[\text{Ca}^{2+}]_{\text{ER}}$ was significantly reduced in cells expressing TRPP2 (Figure 4B and C; control: $737.74 \pm 74.63 \mu\text{M}$, $n = 16$ and TRPP2: $511.91 \pm 70.93 \mu\text{M}$, $n = 18$).

Reduction of mitochondrial Ca^{2+} signals

The ER and mitochondria are interconnected both physically and physiologically (Rizzuto *et al*, 1998). Ca^{2+} that is released from the ER is rapidly taken up by closely juxtaposed mitochondria. To study the influence of TRPP2 on mitochondrial Ca^{2+} dynamics in living cells, a Cameleon dye targeted to mitochondria ($\text{YC4.1}_{\text{mito}}$) was expressed in HeLa cells

Figure 4 TRPP2 decreases $[\text{Ca}^{2+}]_{\text{ER}}$ and mitochondrial Ca^{2+} signals. (A) The ER-targeted Cameleon (YC4_{ER}) co-localized with TRPP2 (immunodecorated with anti-TRPP2 antibody) in HeLa cells (scale bar: 10 μm). (B) Calibration of the normalized fluorescence emission ratio (535/480 nm) of YC4_{ER} in cells co-transfected with empty vector (control) or TRPP2 (10 μM ionomycin, 0.5 $\mu\text{g}/\text{ml}$ digitonin with either 20 mM Ca^{2+} or 5 mM EGTA). Note the decreased steady-state value in cells expressing TRPP2 (dashed orange line). (C) Steady-state Ca^{2+} concentration in the ER ($[\text{Ca}^{2+}]_{\text{ER}}$) in control and TRPP2-expressing cells calculated from a series of experiments as depicted in (B) ($n = 16$ and 18, respectively). (D) A cameleon targeted to mitochondria ($\text{YC4.1}_{\text{mito}}$) was expressed in HeLa cells (scale bar: 10 μm). (E) The fluorescence emission ratio (535/480 nm) of $\text{YC4.1}_{\text{mito}}$ was recorded in cells transfected with TRPP2 or empty vector (control) (10 μM histamine). (F) Group data from (E) at peak Ca^{2+} increase.

(Arnaudeau *et al*, 2001) (Figure 4D). Cells expressing TRPP2 showed a diminished increase in the mitochondrial $[Ca^{2+}]$ after stimulation with histamine (10 μ M, Figure 4E and F), reflecting the reduced amount of releasable Ca^{2+} in the ER.

TRPP2 knockdown augments the amount of releasable ER Ca^{2+}

Subsequently, we investigated whether endogenous TRPP2 channels regulate the ER Ca^{2+} load, as we observed with overexpressed TRPP2. Using lentiviral gene transfer, we generated polyclonal lines of epithelial Madin–Darby canine kidney (MDCK) cells expressing TRPP2-directed (TRPP2 knockdown (kd)) shRNA or non-targeting shRNA (control) cassette, each combined with a GFP marker, in a tetracycline-inducible manner. Cultivation of the TRPP2 kd cells in the medium containing 5 μ g/ml tetracycline for 4 days resulted in an almost complete loss of TRPP2 expression (Figure 5A and D). In contrast, TRPP2 levels in control cells remained unaffected upon addition of tetracycline. In addition, inducible expression of the shRNA cassette in either cell line was evidenced by the appearance of the GFP fluorescent signal (Figure 5A). The cells grown in the presence of tetracycline

were analysed for their cytosolic Ca^{2+} responses using Fura-2 imaging. TRPP2 kd cells had significantly higher amplitudes of Ca^{2+} transients upon stimulation with ionomycin (Figure 5B) or ATP (Figure 5C) than control cells, indicating that depletion of TRPP2 augments the amount of releasable Ca^{2+} in the ER. We confirmed that the steady-state levels of SERCA2 and Bcl-2 were not altered in the TRPP2-depleted cells (Figure 5D). These results corroborated our previous findings obtained with the TRPP2-overexpression system.

We next investigated whether depletion of TRPP2 affects Ca^{2+} pumping into the ER by SERCA or passive Ca^{2+} leak from this organelle. The cells were loaded with the low-affinity calcium indicator Mag-Fura-2 AM, under conditions that favour its accumulation in the intracellular stores, and their plasma membranes were permeabilized with digitonin (Figure 5E, panel a). Following depletion of the intracellular stores by perfusion with an EGTA-containing buffer, the stores were refilled with Ca^{2+} by the action of SERCA activated with ATP and Mg^{2+} , as evidenced by an increase in the 340/380 fluorescence ratio of the ER-localized Mag-Fura-2 (panels b and c of Figure 5E and F). The Ca^{2+} loading of the stores reached a steady-state level during ATP applica-

Figure 5 Ablation of TRPP2 in MDCK cells reduces the ER Ca^{2+} leak and results in increased amount of releasable Ca^{2+} . (A) Analysis of MDCK cells conditionally expressing TRPP2-targeting (TRPP2 kd) or control shRNA cassettes, together with a GFP marker. The lysates prepared from the cells grown in the absence (– tet) and presence (+ tet) of tetracycline were analysed by western blotting. Inducible expression of GFP was also visualized by fluorescence microscopy (green). The nuclei were stained with Hoechst 33342 (blue). Scale bars: 10 μ m. (B) Cytosolic Ca^{2+} imaging of TRPP2-depleted (TRPP2 kd) and control cells upon treatment with 2 μ M ionomycin (Iono) in the absence of extracellular Ca^{2+} . Representative traces and group data are shown (peak values–baseline; $n = 7$). (C) Experiment was performed as in (B), except that 10 μ M ATP was used ($n = 6$). (D) The levels of SERCA2 and Bcl-2 in the lysates prepared from control and TRPP2 kd cells were analysed by western blotting. (E) MDCK cells were loaded with the low-affinity Ca^{2+} indicator Mag-Fura-2 AM and permeabilized with digitonin to analyse the kinetics of Ca^{2+} pumping into the ER and its leak. The pictures show the fluorescence of Mag-Fura-2 (excited by 340 nm wavelength) localized in the intracellular stores after permeabilization (a), as well as their Ca^{2+} loading state (340/380 nm ratio is presented in pseudocolor) before (b) and after (c) refilling. (F) The graphs show the changes in the Ca^{2+} loading state of the intracellular stores (340/380 nm ratio R divided by the minimal ratio R_0) in control and TRPP2-depleted (TRPP2 kd) cells. Refilling was initiated by the addition of 1.5 mM ATP and the Ca^{2+} leak was observed after inhibition of SERCA with 30 μ M CPA. The minimal ratio R_0 was found after depleting the stores with ionomycin (Iono; 2 μ M). (G) Statistical analysis of the ER refilling and leak in the $n = 64$ control cells and $n = 64$ TRPP2-depleted cells from six and seven independent measurements, respectively. The calcium leak rate in each cell was plotted against R/R_0 at the start of the CPA treatment, and the data were fitted into linear regression lines with the Excel software ($y = bx + a$, where b is 0.0054, a is –0.005, and correlation $r = 0.835$ for control cells; b is 0.0038, a is –0.0032, and $r = 0.661$ for TRPP2-depleted cells). The bar chart on the left shows the difference between the two regression slopes, and their standard errors ($P = 0.025$). The remaining bar charts show the mean rates of the leak and refilling, and their standard errors.

tion, but it declined after ATP was replaced with the SERCA inhibitor cyclopiazonic acid (CPA; Figure 5F). The calculated rates of Ca^{2+} passive leak from the stores of control and TRPP2-depleted cells positively correlated with the Ca^{2+} levels at the start of CPA treatment (Figure 5G), whereas no such relation was found for the refilling rates and initial Ca^{2+} levels (data not shown). The dependence of the leak rate on the ER Ca^{2+} load in both cell populations could be fitted into linear regression lines with significantly different slopes (0.0054 for control cells, 0.0038 for TRPP2 kd cells; Figure 5G). In addition, whereas the mean leak rates were significantly smaller for TRPP2-depleted cells than for control cells, their mean refilling rates were virtually identical. These results suggest that TRPP2 channels contribute to the passive leak of Ca^{2+} from the ER, which in turn may be responsible for the increased amount of releasable Ca^{2+} in TRPP2-depleted cells.

TRPP2 knockdown augments the sensitivity to select apoptosis inducers

As increased Ca^{2+} load in the ER sensitizes the cells to undergo apoptosis in response to certain stimuli, for example ceramide (Pinton *et al*, 2001), we analysed the progression of apoptosis in TRPP2-depleted (TRPP2 kd) and control MDCK cells by measuring caspase 3 activity. This activity increased

upon treatment of cells with C_2 -ceramide in a time- and dose-dependent manner, indicating induction of apoptosis (Figure 6A). Importantly, application of $20\text{ }\mu\text{M}$ C_2 -ceramide for 9 h resulted in a substantially and significantly higher caspase 3 activity in TRPP2 kd cells, as compared with control cells. Cytoplasmic fractions of ceramide-treated TRPP2 kd cells also contained higher levels of nucleosomes due to apoptotic DNA fragmentation (Figure 6B). In contrast, TRPP2 kd cells were not sensitized to the apoptosis induced by the transcriptional inhibitor actinomycin D, as compared with control cells (Figure 6C). These results suggest that depletion of TRPP2 augments the sensitivity of cells specifically to the apoptotic stimuli that engage the ER Ca^{2+} gateway, such as ceramide (Rizzuto *et al*, 2003), as a result of their increased ER Ca^{2+} load. Our data also indicate that the lack of TRPP2 ER function may be responsible for the increased apoptosis rates observed in polycystic kidneys.

Discussion

TRPP2 functions as a Ca^{2+} -permeable cation channel that has been shown to localize to various subcellular compartments, where it appears to serve distinct functions (Köttgen, 2007). The long-standing controversy whether TRPP2 resides and functions in the plasma membrane or in the ER has been reconciled by the recent demonstration that the subcellular localization and transport of TRPP2 are controlled by multiple interactions with adapter proteins (Hidaka *et al*, 2004; Köttgen *et al*, 2005; Köttgen and Walz, 2005). However, it has remained unknown how the different compartment-specific functions of TRPP2 regulate specific cellular outcomes. Here, we show that TRPP2 regulates the ER Ca^{2+} gateway to apoptosis by decreasing the Ca^{2+} concentration in the ER.

Ca^{2+} has long been recognized as a key signalling molecule in apoptotic pathways (Orrenius *et al*, 2003; Rizzuto *et al*, 2003). Owing to the toxicity of Ca^{2+} ions, a low Ca^{2+} concentration (10–100 nM) must be maintained in the cytoplasm. Most of the cellular Ca^{2+} is stored in the ER. Ca^{2+} is pumped into the ER by SERCA and is released only transiently during short periods of signalling, by the opening of IP_3 - or ryanodine receptors (Berridge *et al*, 2000). A significant fraction of the released Ca^{2+} is sequestered by mitochondria, which are strategically located near the ER Ca^{2+} -release channels (Rizzuto *et al*, 1998) (Figure 7). Mitochondria eventually determine whether Ca^{2+} signals are decoded as life or death signals. The switch from a life to a death cue involves coincidence detection of Ca^{2+} and proapoptotic stimuli, and depends on the amplitude of the mitochondrial Ca^{2+} transient (Szalai *et al*, 1999; Pinton and Rizzuto, 2006). The magnitude of mitochondrial Ca^{2+} signals, in turn, depends largely on the ER Ca^{2+} content (Pinton *et al*, 2000). Notably, several recent studies have shown that the Ca^{2+} content of the ER determines the sensitivity of the cell to apoptotic stress (Pinton *et al*, 2001; Pinton and Rizzuto, 2006). Procedures that decrease the ER Ca^{2+} load, such as genetic ablation of the Ca^{2+} -buffering protein calreticulin or overexpression of plasma membrane Ca^{2+} ATPases, protect cells from apoptosis (Nakamura *et al*, 2000; Pinton *et al*, 2001). Conversely, procedures that increase the ER Ca^{2+} load, such as overexpression of SERCA or calreticulin, sensitize cells to apoptotic stress (Pinton *et al*, 2001; Arnaudeau *et al*, 2002).

Figure 6 Ablation of TRPP2 sensitizes the cells to apoptosis induced by ceramide but not by actinomycin D. (A) The caspase 3-like activity in TRPP2-depleted (TRPP2 kd; grey bars) and control (black bars) MDCK cells was measured after apoptosis induction with 10 or $20\text{ }\mu\text{M}$ C_2 -ceramide for 6 or 9 h. The activity is presented in relative fluorescence units (RFU) per minute and was normalized to 1 mg of total protein ($n=4$). (B) The relative amount of cytoplasmic nucleosomes in apoptotic cells after 9 h of treatment with the indicated doses of C_2 -ceramide was measured by ELISA, and normalized to total protein level in the cell lysates. The bar chart shows the data from one representative measurement. The assay was repeated three times yielding essentially the same results. (C) The apoptosis in TRPP2-depleted and control cells was induced with $0.5\text{ }\mu\text{g/ml}$ actinomycin D (Act D) for 9 h. The caspase 3-like activity was measured and presented as in (A) ($n=3$).

Figure 7 Simplified model of the ER Ca²⁺ gateway to apoptosis. Under physiological conditions, Ca²⁺ continuously cycles between the ER and mitochondria. Ca²⁺ ATPases (SERCA) pump Ca²⁺ into the ER from where it is released by IP₃-gated channels (IP₃R). A Ca²⁺ uniporter (mCU) mediates Ca²⁺ uptake into mitochondria and a mitochondrial Na⁺/Ca²⁺ exchanger (mNCE) releases Ca²⁺. Apoptotic stimuli can release Ca²⁺ from the ER, which results in mitochondrial Ca²⁺ signals. The magnitude of the mitochondrial Ca²⁺ signal and additional proapoptotic stimuli determine whether cytochrome *c* is released to trigger apoptosis. The amplitude of the mitochondrial Ca²⁺ signals depends on the Ca²⁺ content of the ER, which is maintained by the balance between active Ca²⁺ pumping by SERCA and passive Ca²⁺ exit from the ER. TRPP2 and Bcl-2 decrease the Ca²⁺ concentration in the ER ([Ca²⁺]_{ER}) by increasing the passive Ca²⁺ exit pathway. This results in decreased mitochondrial Ca²⁺ signals, which cause reduced sensitivity to apoptosis. According to the 'rheostat model' (Demaurex and Distelhorst, 2003), the ER Ca²⁺ load is regulated by the balance between anti- and proapoptotic Bcl-2 protein family members (Bcl-2 and Bax/Bak, respectively). Here, we introduce the cation channel TRPP2 as a novel antiapoptotic player in this model (modified from the review by Demarex and Distelhorst, 2003).

Furthermore, recent data have shown an unexpected function of Bcl-2 family proteins in the regulation of the steady-state Ca²⁺ concentration in the ER (Pinton and Rizzuto, 2006). A series of elegant studies suggest that the balance between pro- and antiapoptotic Bcl-2 family members determines the ER Ca²⁺ content. Overexpression of the antiapoptotic protein Bcl-2 decreases the ER Ca²⁺ load and thereby protects cells from death (Foyouzi-Youssefi *et al*, 2000; Pinton *et al*, 2000). Conversely, the proapoptotic proteins Bax and Bak were shown to serve an opposing function. Cells from mice deficient in Bax and Bak are resistant to apoptosis due to a reduced ER Ca²⁺ load, which results in decreased mitochondrial Ca²⁺ signals (Scorrano *et al*, 2003) (Figure 7).

In this study, we demonstrate that TRPP2 fulfills the criteria for a protein functioning in the ER Ca²⁺ gateway to apoptosis. In cells expressing TRPP2: (1) [Ca²⁺]_{ER} is decreased, as assessed by direct measurement using an ER-targeted Ca²⁺ sensor; (2) IP₃ receptor stimulation leads to decreased cytosolic and mitochondrial Ca²⁺ signals; (3) Ca²⁺ release from the ER, using stimuli that induce apoptosis, such as ionomycin or thapsigargin, is diminished. In addition, knockdown of TRPP2 (4) reduces passive Ca²⁺ leak from the ER, (5) augments Ca²⁺ release upon store depletion, and (6) increases apoptosis sensitivity.

The *in vivo* relevance of the cellular mechanism for TRPP2 function proposed here is supported by published data on Bcl-2. This protein shares with TRPP2 the same mechanism of apoptosis regulation in the ER (Pinton *et al*, 2000). Even more importantly, *Bcl-2*-deficient mice develop PKD, through an unknown mechanism (Veis *et al*, 1993). The similarity of the physiological roles of TRPP2 and Bcl-2 at the level of ER Ca²⁺ homeostasis provides a mechanistic link for the phenotype that is observed upon loss of function of either protein. From a TRPP2 perspective, this supports an important function for apoptosis in the pathogenesis of PKD; from a Bcl-2 perspective, it may explain the unexpected cystic phenotype of *Bcl-2*-deficient mice. It should be noted that the cystic kidneys of *Bcl-2*-deficient animals and those of ADPKD patients show morphological differences, in particular kidney size, which is increased in ADPKD and decreased in *Bcl-2* knockout mice. This difference may be explained by additional compartment-specific functions of TRPP2, including regulation of proliferation, cell polarity, and differentiation (Köttgen, 2007). Furthermore, it is conceivable that the developmental pattern and timing of expression of either gene will influence specific morphological features of the cystic phenotype.

The mechanism by which Bcl-2 alters ER Ca²⁺ is still unresolved. Even though Bcl-2 was proposed to be an ion channel itself, recent evidence supports the view that Bcl-2 interacts with ER channels, such as the IP₃ receptor, to reduce the ER Ca²⁺ content (Pinton and Rizzuto, 2006). We have preliminary data showing that Bcl-2 and TRPP2 co-immunoprecipitate (M Köttgen, unpublished data). Interestingly, TRPP2 has been recently shown to interact with the IP₃ receptor as well (Li *et al*, 2005). Thus, it is tempting to speculate that TRPP2, Bcl-2, and the IP₃ receptor might form a macromolecular complex that orchestrates the ER Ca²⁺ gateway to apoptosis. To test whether the IP₃ receptor is required for the TRPP2-mediated reduction of releasable Ca²⁺ from the ER, we studied store release in IP₃ receptor knockout cells (Supplementary Figure 3). These experiments showed that IP₃ receptors are not required for TRPP2-mediated reduction of the ER Ca²⁺ load, which suggests that TRPP2 forms a new independent ER leak channel.

Besides its function in the ER Ca²⁺ gateway to apoptosis, the function of the ER-resident TRPP2 may have broader implications in Ca²⁺-dependent cellular processes. It has been recently shown that increasing ER Ca²⁺ leak by long-term treatment with Ca²⁺ mobilizing agents, such as thapsigargin, ATP, or vitamin D₃ compounds, upregulates autophagy, a pathway for the degradation of cytoplasmic components in the lysosome (Hoyer-Hansen *et al*, 2007). This effect could be prevented either by chelating intracellular Ca²⁺ or by expression of the ER-targeted form of Bcl-2 that pre-empted ER stores. Experimentally induced ER Ca²⁺ leak led to an activation of cytosolic Ca²⁺/calmodulin-dependent kinase kinase-β and subsequent inhibition of the mammalian target of rapamycin (mTOR) pathway, the main negative regulator of autophagy. Interestingly, mTOR pathway appears to be aberrantly activated in the cystic epithelial cells of ADPKD patients and in several animal models of the disease (reviewed by Walz, 2006). Therefore, we hypothesize that TRPP2 may contribute to the downregulation of the mTOR pathway by increasing the Ca²⁺ leak from the ER.

It has been suggested recently that TRPP2 may function as a Ca^{2+} release channel in the ER, but the biological consequences have not been addressed in this study (Koulén *et al*, 2002). The authors reported that overexpression of TRPP2 resulted in increased vasopressin-induced Ca^{2+} release from the ER, which is in contrast to our findings. Although we cannot finally resolve this discrepancy, we attempted to confirm that our results do not solely depend on cell type or the mode of Ca^{2+} release from the ER by using different cell lines and stimuli, and by expressing TRPP2 in a transient or stable manner. Importantly, the knockdown of TRPP2 in renal epithelial cells had a converse effect. Finally, our results on cytosolic Ca^{2+} signalling are supported by the direct measurement of $[\text{Ca}^{2+}]$ in the ER and mitochondria, using organelle-targeted genetically encoded Ca^{2+} sensors.

Processes that disrupt the delicate balance between the rates of cell proliferation and apoptosis have been reported to result in cyst formation (Boletta *et al*, 2000) and are most likely to have an important function in other disease manifestations, such as vascular aneurysms. Apoptosis is a pathological feature of PKD, and there is ample evidence that apoptosis has a central function in cyst formation. Tubular epithelial cell apoptosis is increased early on in animal models of PKD and in kidneys from humans with ADPKD (Woo, 1995; Kip *et al*, 2005; Tao *et al*, 2005; Bukanov *et al*, 2006; Starremans *et al*, 2008). Increased rates of apoptosis have been reported in *Pkd1*- and *Pkd2*-deficient mice (Kip *et al*, 2005; Starremans *et al*, 2008). Whereas apoptosis is virtually absent in mature wild-type kidneys, the degree of apoptosis in *Pkd1*-deficient animals ranged from a small number of cells undergoing apoptosis (Piontek *et al*, 2007) to many apoptotic cells in cysts and tubules (Starremans *et al*, 2008), depending on the study. Induction of apoptosis results in cyst formation in tubular epithelial cells in 3D collagen culture, and cystogenesis in this system is inhibited by overexpression of Bcl-2 (Lin *et al*, 1999). Moreover, a recent study showed that caspase inhibition or ablation of caspase 3 reduces apoptosis in renal epithelia and attenuates cyst formation as well as kidney failure in PKD animal models (Tao *et al*, 2005, 2008).

Although the link between apoptosis and PKD is well established, the function of PKD proteins in apoptosis regulation is poorly understood. Our results show that TRPP2 is an antiapoptotic cation channel in the ER and provide a molecular mechanism for this function. These data also support the concept that channel localization is critical for its cellular function, as Ca^{2+} influx through cation channels in the plasma membrane can promote cell death. Thus, the tight regulation of TRPP2 ER localization by adapter proteins, such as PACS-1/2 and PIGEA-14 (Hidaka *et al*, 2004; Köttgen *et al*, 2005; Köttgen and Walz, 2005), may determine the sensitivity of tubular epithelial cells to apoptotic stimuli and regulate the balance between cell death and proliferation. This process may have an important function in the homeostasis of the developing and mature nephron. Polycystin-1, the other protein mutated in ADPKD, forms a heteromeric complex with TRPP2 and has also been shown to protect cells from apoptosis (Boletta *et al*, 2000; Boca *et al*, 2006). The identification of ligands for polycystin-1 will be essential to address the physiological regulation and the functional interaction between polycystin-1 and TRPP2 in ER calcium homeostasis and antiapoptotic signalling pathways.

Materials and methods

Materials

TRPP2 constructs were described previously (Köttgen *et al*, 2005). The plasmid pLXSN was kindly provided by D Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). The plasmids pMD-G and pMD-gp were a kind gift of R Mulligan. The retroviral transfer vector pLXSN-TRPP2 and the psGEM-TRPP2 vector for cRNA synthesis were generated using standard cloning techniques. For electrophysiology and Ca^{2+} imaging experiments, untagged TRPP2 constructs were used. Yellow Cameleons were kindly provided by A Miyawaki (YC4_{ER}) and N Demaurex (YC4.1_{mito}) (Miyawaki *et al*, 1997; Arnaudeau *et al*, 2001). For knockdown experiments, canine TRPP2-targeting and control shRNAs (sequences available upon request) were cloned into the pLVTH vector (Addgene plasmid 12262). Chemicals were obtained from Fluka, Sigma, or Alexis Biochemicals.

Cell culture, transfection, virus production, and transduction

HeLa, HEK-293, and MDCK cells were grown at 37°C in DMEM supplemented with 10% heat-inactivated fetal bovine serum. Cells were transfected with Eugene 6 or calcium phosphate, and experiments were performed 2–5 days after transfection. The retrovirus was produced by co-transfection of HEK-293 cells with three plasmids (pMD-G, pMD-gp, and a retroviral transfer vector). Cells were infected in the presence of 8 µg/ml polybrene and selected with 500 µg/ml geneticin. MDCK cells expressing tetracycline repressor tTR-KRAB and the shRNA cassette were generated by a lentiviral gene transfer, as described previously (Wiznerowicz and Trono, 2003).

Ca^{2+} imaging

HeLa and HEK 293 cells were plated on 30-mm glass coverslips and mounted in a perfusion chamber on the stage of an inverted microscope (Zeiss, Axiovert 200M, Fluor × 40/1.3 oil-immersion objective). Cells were incubated with Fura-2 AM (Molecular Probes; 2 µM) for 30 min at room temperature and then continuously superfused with modified Ringer solution (composition in mM: 145 NaCl, 0.4 KH₂PO₄, 1.6 K₂HPO₄, 1 MgCl₂, 1.3 Ca^{2+} -gluconate, 5 glucose; pH 7.4) for 10–20 min. Drugs and agonists were applied in the nominal absence of Ca^{2+} . Ca^{2+} -free solution (Ca^{2+} -gluconate was replaced by 5 mM EGTA) was perfused 30 s before triggering Ca^{2+} release from intracellular stores. Fura-2 was alternately excited at 340 and 380 nm, and the fluorescence filtered at 510 nm was collected and recorded at 1 Hz using a CCD-based imaging system (Cool-SNAP fx, Roper Scientific Inc.) running Metafluor software (Molecular Devices). For every experiment, signals of 5–10 cells were recorded and the mean of these signals was referred to as one independent experiment. The calibration of the Fura-2 ratio (340/380 nm) was performed as described by Grynkiewicz *et al* (1985). Fura-2 imaging with MDCK cells conditionally expressing shRNA and GFP was performed essentially in the same way. However, in this case, only the cells with predefined intermediate and comparable intensities of GFP signal were included in the analysis (5–19 cells per measurement).

The refilling of intracellular stores with Ca^{2+} and its leak were analysed in permeabilized MDCK cells. The cells were loaded with 10 µM Mag-Fura-2 AM (Biotium) for 1 h at 37°C in the modified Ringer solution. Loaded cells were perfused shortly with the intracellular buffer (ICB; 125 mM KCl, 19 mM NaCl, 10 mM HEPES, 1 mM EGTA, pH 7.3) and permeabilized with 15 µg/ml digitonin in ICB for 2–3 min. This procedure removed the cytosolic fraction of Mag-Fura-2 and most of the GFP fluorescence. The cells were subsequently washed with ICB for 13 min and with ICB containing 200 nM free Ca^{2+} for 2 min. A concentration of 1.5 mM ATP (sodium salt) or 30 µM CPA was applied in ICB containing 200 nM free Ca^{2+} and 1.4 mM MgSO₄. At the end of each experiment, the cells were perfused with 2 µM ionomycin in ICB to calculate the minimal 340/380 ratio (R_0). The maximal rates of the ER Ca^{2+} loading and leak were estimated from the derivative of R/R_0 calculated over a time span of 6 or 10 s, respectively.

The method for dual-emission ratio imaging of $[\text{Ca}^{2+}]$ with the use of cameleons was derived from the review by Miyawaki *et al* (1997). Cells were excited at 440 ± 10 nm using an excitation filter and a 455DRLP dichroic mirror (Chroma). Fluorescence emission from the cameleons was imaged at two emission wavelengths using

a filterwheel (Ludl Electronic Products) to alternately change the two emission filters (Chroma, 480DF15 and 535DF25). Changes in fluorescence ratio $R = (\text{fluorescence intensity at } 535 \text{ nm} - \text{background intensity at } 535 \text{ nm}) / (\text{fluorescence intensity at } 475 \text{ nm} - \text{background intensity at } 475 \text{ nm})$ were calibrated as described previously (Arnaudeau *et al*, 2001), using the equation

$$[Ca^{2+}] = K_d [(R - R_{\min}) / (R_{\max} - R)]^{(1/n)}$$

where $R_{\min}$ and $R_{\max}$ are the ratios obtained in the absence of Ca^{2+} and at saturating $[Ca^{2+}]$, respectively. K_d is the apparent dissociation constant and n is the Hill coefficient of the Ca^{2+} calibrations curves that have been obtained *in situ* (Arnaudeau *et al*, 2001).

Apoptosis assays

Apoptosis was induced in serum-starved MDCK cells with C_2 -ceramide or actinomycin D. Drug solvents, DMSO or ethanol (EtOH), were used for control treatments. The caspase 3-like activity in cell lysates was measured in the presence of 60 μM fluorogenic substrate Ac-DEVD-AMC. The fluorescence increase was monitored for 45 min in the GeminiXS microplate reader (Molecular Devices) at excitation/emission wavelengths of 380/460 nm. Note that the activities in cells treated with either apoptotic inducer should not be compared to each other, as they were measured on different units of the GeminiXS reader. The amount of nucleosomes in the cytoplasmic fraction of apoptotic cells was

measured using Cell Death Detection ELISA Plus kit (Roche) according to the manufacturer's instructions, except that the lysis buffer without BSA was used to measure the total protein level in the lysates.

Statistical analysis

Data are presented as original recordings or as mean values $\pm$ s.e.m. (n = number of experiments, unless otherwise stated). Unpaired and paired Student's *t*-test as applicable was used for statistical analysis. A *P*-value of <0.05 was accepted to indicate statistical significance (marked with an asterisk).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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