Role of TASK2 in the Control of Apoptotic Volume Decrease in Proximal Kidney Cells

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Apoptotic volume decrease (AVD) is prerequisite to apoptotic events that lead to cell death. In a previous study, we demonstrated in kidney proximal cells that the TASK2 channel was involved in the K⁺ efflux that occurred during regulatory volume decrease. The aim of the present study was to determine the role of the TASK2 channel in the regulation of AVD and apoptosis phenomenon. For this purpose renal cells were immortalized from primary cultures of proximal convoluted tubules (PCT) from wild type and TASK2 knock-out mice (5). Apoptosis was induced by staurosporine, cyclosporin A, or tumor necrosis factor α. Cell volume, K⁺ conductance, caspase-3, and intracellular reactive oxygen species (ROS) levels were monitored during AVD. In wild type PCT cells the K⁺ conductance activated during AVD exhibited characteristics of TASK2 currents. In task2−/− PCT cells, AVD and caspase activation were reduced by 59%. Whole cell recordings indicated that large conductance calcium-activated K⁺ currents inhibited by iveriotoxin (BK channels) partially compensated for the deletion of TASK2 K⁺ currents in the task2−/− PCT cells. This result explained the residual AVD measured in these cells. In both cell lines, apoptosis was mediated via intracellular ROS increase. Moreover AVD, K⁺ conductances, and caspase-3 were strongly impaired by ROS scavenger N-acetylcysteine. In conclusion, the main K⁺ channels involved in staurosporine, cyclosporin A, and tumor necrosis factor-α-induced AVD are TASK2 K⁺ channels in proximal wild type cells and iberiotoxin-sensitive BK channels in proximal task2−/− cells. Both K⁺ channels could be activated by ROS production.

Like many epithelial cells, renal cells are capable of regulating their volume in response to variations in external osmotic pressure (1–3). Briefly, cells respond to an increase in medium osmolarity by a process referred to as regulatory volume increase, whereas cells respond to the dilution of external medium by a regulatory volume decrease (RVD)² (4). A variety of transport pathways have been implicated in both processes and result in rapid water flux across the plasma membrane, which causes cells to recuperate their initial volume correspondingly (5). Along the proximal tubule, the cells are submitted to hypotonic shock because water accompanies the transport of ions by membrane co-transport. In response to this osmotic stress, the proximal cells undergo a RVD process that is characterized by an exit of Cl⁻ and K⁺ ions, which finally drives water efflux (6). However, changes in cell volume are not only due to variation in medium osmolarity. It is now well established that the initial process leading toward apoptotic cell death is coupled to normotonic cell shrinkage (7), called apoptotic volume decrease (AVD). The proximal tubule is a major site of agent-induced nephrotoxicity (drugs, heavy metals, hypoxia etc.), which can induce AVD and lead to cell death by apoptosis. It is therefore interesting to understand the mechanisms involved in this phenomenon. As in RVD, the changes in cell volume during AVD are the consequence of an exit of Cl⁻ and K⁺ from the cells, and the question arises as to whether the Cl⁻ and the K⁺ currents are driven by the same type of channels (8). The AVD-induced Cl⁻ channel has been identified as a volume-sensitive outwardly rectifying Cl⁻ channel in both HeLa cells and cardiomyocytes treated with staurosporine (9, 10). This channel shares many properties with the Cl⁻ channel that are induced in RVD in mouse proximal cells (5). The molecular nature of this Cl⁻ channel is not fully elucidated, but the literature data converges toward the conclusion that this Cl⁻ channel type is probably ubiquitously expressed in animal cells (7). By contrast, the molecular identity of the K⁺ channel involved in both AVD and RVD is still under discussion because different candidates have been proposed depending on the tissue under study (8, 11–17). In a previous study, we demonstrated that TASK2 channels were expressed in kidney proximal cells. These channels were involved in the K⁺ and Cl⁻ efflux that occurred during RVD. TASK2 belongs to the family of two pore domains K⁺ channels. Interestingly, Trimarchi et al. (13) have provided evidence that two-pore domain K⁺ channels underlie K⁺ efflux during AVD in mouse embryos. Based on these observations, it was reasonable to postulate that TASK2 K⁺ channels could also be involved in the regulation of AVD and apoptosis in the proximal tubules. Thus, the present study addresses the role of TASK2 in apoptosis induced by staurosporine. In a large variety of cells, staurosporine is known to induce apoptosis through a mitochondria-mediated pathway and to increase oxidative stress by the production of ROS generated by the mitochondria (9, 18). In proximal cells, this mitochondrial mechanism may be the pre-

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2 The abbreviations used are: RVD, regulatory volume decrease; AVD, apoptotic volume decrease; ChTX, charybdotoxin; IbTX, iberiotoxin; STS, staurosporine; CsA, cyclosporin A; TNF-α, tumor necrosis factor-α; ROS, reactive oxygen species; carboxy-H₂DCFDA, (5-and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate; TEA, tetraethylammonium; NAC, N-acetylcysteine.
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dominant mode of inducing apoptosis in the presence of many nephrotoxic agents (19–21). Therefore, the staurosporine-induced apoptosis is a useful model to assess the role of ion channels in controlling apoptosis in renal cells. The present study was conducted on proximal tubule cell lines originating from wild type and task2−/− mice. In wild type mice, we demonstrated that staurosporine-induced AVD was mainly associated with the activity of TASK2 K+ channels. Surprisingly, staurosporine-induced AVD persisted in the task2−/− proximal cell line and could be controlled by a Ca2+-activated K+ channel that is sensitive to iberiotoxin.

EXPERIMENTAL PROCEDURES

Transformation of Primary Cultures with pSV3 neo and Culture Protocol—The primary cell culture technique has been described in detail in previous studies (22). Briefly, 10-day-old primary cultures of S1 and S2 segments of proximal tubules from wild type and task2−/− mice were transfected with pSV3 neo using Lipofectin (Invitrogen). After 48 h, selection of the clones was performed by the addition of G418 (500 μg/ml). Culture medium (Dulbecco’s modified Eagle’s medium-F12, Sigma, Saint Quentin Fallavier, France) containing 250 μg/ml G418, 15 mM NaHCO3, 20 mM HEPES (pH 7.4), growth factors (23), and 1% FCS was changed every day. Resistant clones were formed on cultured proximal wild type and task2−/− cells that were exposed to different treatments (STS, CsA, or TNF-α) were rapidly trypsinized (1 times for 45 s), and cell volume measurement was performed just after suspending the cells in Casyton® solution (NaCl isotonic solution).

Electrophysiological Studies—Whole cell currents were performed on cultured proximal wild type and task2−/− cells grown on 35-mm Petri dishes maintained at 37 °C for the duration of the experiments. The ruptured whole cell configuration of the patch-clamp technique was used. Patch pipettes (2- to 4-megaohm resistance) were made from borosilicate capillary tubes (1.5 mm outer diameter, 1.1 mm inner diameter; Fisher Manufacturing) using a two-stage vertical puller (model PP 830, Narishige, Tokyo, Japan). Cells were observed using an inverted microscope; the stage of the microscope was equipped with a water robot micromanipulator (model WR 89, Narishige). The patch pipette was connected via an Ag-AgCl wire to the head stage of a patch amplifier (model VP 500, Biologic). The membrane was ruptured by additional suction to achieve the conventional whole cell configuration. Settings available on the amplifier were used to compensate for cell capacitance. The series resistances were not compensated, but experiments in which the series resistance was higher than 20 megohms were discarded. The offset potentials between both electrodes were zeroed before sealing, and the liquid junction potential was measured experimentally prior to each experiment and corrected accordingly (measured junction potentials were 11.34 ± 0.79 mV for K+ conductance experiments). Solutions were perfused in the extracellular bath using a four-channel glass pipette, with the tip placed as close as possible to the clamped cell. Voltage-clamp commands, data acquisition, and data analysis were controlled via the VP 500 amplifier connected to a computer. The whole cell currents resulting from voltage stimuli were sampled at 2.5 kHz and filtered at 1 kHz.

Different solutions were perfused in the extracellular compartment, and the bathing solutions were held at −50 mV, and 400-ms pulses from −100 to +120 mV were applied in 20-mV increments.

The pipette solution contained (in mM): 100 K-glucanate, 25 KHCO3, 20 KCl, 10 HEPES (pH 7.4 adjusted with 1 N KOH), 5 MgATP, and 0 or 30 EGTA (Pos = 300 millimoles/kg of H2O). To avoid spontaneous activation of volume-sensitive K+ currents, the bath solution was slightly hyperosmotic and contained (in mM): 110 NMDG-Cl, 5 glucose, 5 potassium gluco-

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nate, 1 CaCl$_2$, 1 HEPES (pH 7.4 adjusted with 1 N HCl), and 100 mannitol (Pos = 330–340 millisomole/kg of H$_2$O).

**Measurement of Reactive Oxygen Species (ROS)**—Levels of cellular oxidative stress were measured using the fluorescent probe (5-and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA). Carboxy-H$_2$DCFDA is a cell-permeable indicator for ROS that becomes spontaneously fluorescent when the acetate groups are removed by intracellular esterases and cell oxidation. This probe is trapped mainly in the cytoplasm and is oxidized by several ROS, most notably hydrogen peroxide.

Briefly, proximal wild type and task2$^{-/-}$ cells were incubated in Petri dishes at 37 °C for 30 min in the presence of carboxy-H$_2$DCFDA (10 $\mu$M) and gently washed in serum-free culture medium. Two experimental protocols were developed to measure the fluorescence increase according to the time kinetic leading to ROS increase. For rapid kinetic (less than 1 h), cells were rapidly trypsinized ($\times$10 for 45 s) and incubated in the absence or presence of either STS (1 $\mu$M) or NAC (N-acetylcysteine, 10 mM) or both substances. Variations of fluorescence of the cell suspension were measured every 2 min using a Genius Spectrofluorimeter (SAFAS, Monaco) at 538 nm.

For long time kinetic (experiments performed in the presence of TNF-α), the ROS production was monitored by using fluorescent video microscopy. Briefly, proximal cell lines grown in 35-mm Petri dishes were incubated in the presence of carboxy-H$_2$DCFDA (10 $\mu$M) at 37 °C for 30 min in a humidified atmosphere of 5% CO$_2$, 95% air. Cells were gently washed and incubated in an isotonic serum-free medium containing 30 mM HEPES in the absence or presence of TNF-α. The variation of fluorescence was measured every 15 min (during 24 h) at 538 nm. Data are expressed as the fluorescence ratio $F/F_0$, where $F$ is the absolute fluorescence intensity measured every 15 min and $F_0$ the absolute fluorescence intensity measured at $t = 0$. The mean values of ROS production were obtained from analysis of 18–25 cells in each culture.

**Intracellular Ca$^{2+}$ Measurements**—The intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$])$_i$ was measured in cells grown in Petri dishes and loaded for 45 min at room temperature in the presence of fura 2-AM (2 $\mu$M) and pluronic acid (0.01%). The cells were washed with a NaCl solution containing in mM, 140 NaCl, 5 KCl, 1 MgSO$_4$, 1 CaCl$_2$, 5 glucose, and 20 HEPES (pH 7.4). Cells were successively excited at 350 and 380 nm and the paired images were digitized. Each raw image was the result of an integration of four to five frames averaged four times. The acquisition rate was one image every 10 s. For each monolayer, [Ca$^{2+}$], was monitored in 18–20 random cells. The equation of Grynkiewicz et al. (24) was used to calculate [Ca$^{2+}$]$_i$ from the dual wavelength-to-fluorescence ratio.

**Chemical Compounds**—STS (Sigma) and CsA (Sigma) were prepared in Me$_2$SO and used at final concentrations of 1 and 25 $\mu$M, respectively. TNF-α (Sigma) was prepared in distilled water and used at a final concentration of 0.5 ng/ml. Cloflium was prepared at 10 mM in a solution containing 50% Me$_2$SO, 50% water. Cloflium and CsA were a gift from Dr. Barhanin (UMR CNRS 6097). Stock solutions of xanthine (50 mM) and xanthine oxidase (50 milliunits ml$^{-1}$) were prepared and kept at +5 °C and −20 °C, respectively. The fluorescent probe carboxy-H$_2$DCFDA (Molecular Probes) was used at 10 $\mu$M (stock solution at 10 mM in Me$_2$SO). Fura 2-AM (Molecular Probes) was dissolved at 3 mM in Me$_2$SO and added to the loading solution at a final concentration of 2 $\mu$M, along with 0.01% pluronic acid. NAC (10 mM), tetraethylammonium (TEA, 1 mM), charybdotoxin (ChTX, 10 nm), and iberiotoxin (IbTX, 100 nm) were obtained from Sigma.

**RESULTS**

**Induction of Caspase-3 Activation and Chromatin Condensation by STS in Wild Type and task2$^{-/-}$ Proximal Cell Lines**—Previous experiments performed on proximal cell lines have established a crucial role for the TASK2 K$^+$ channel in the regulatory volume decrease during hypotonic shock. Furthermore, a specific cell volume decrease (AVD) is generally observed during apoptosis process. In the present study, the involvement of TASK2 channels during chemically induced apoptosis was investigated. For this purpose, proximal cell lines from wild type and task2$^{-/-}$ mice were exposed to STS (1 $\mu$M) for 6 h, and the caspase-3 activity was determined.

As illustrated in Fig. 1A, STS exposure induced a strong increase in caspase-3 activity in wild type cells. Interestingly, this increase was ~2-fold higher than that observed in task2$^{-/-}$ cells (Fig. 1A). This suggests that TASK2 channels were involved in the STS-induced apoptotic process. The STS-induced caspase-3 activation was significantly inhibited by cloflium, high [K$^+$]$_o$ or external acidic pH confirming the involvement of TASK2 (these effectors have already been shown to inhibit TASK2 K$^+$ currents). In these cells, the potent blocker of Ca$^{2+}$-dependent K$^+$ channels, IbTX, did not affect the STS-induced caspase-3 activation. Surprisingly, the addition of STS still enhanced the level of caspase-3 in task2$^{-/-}$ cells. As expected, this moderate increase was not modified by the addition of cloflium or by the acidification of the external pH. However, the application of IbTX or high [K$^+$]$_o$, abolished STS-induced caspase-3 activation. These results suggested that, in task2$^{-/-}$ cells, the STS-induced caspase-3 activation could be driven by an IbTX-sensitive K$^+$ channel.

To verify these observations, the apoptosis phenomenon was also assessed on the basis of morphological criteria. Wild type and task2$^{-/-}$ cell lines were stained with Hoescht-33258 and propidium iodide to selectively distinguish between apoptotic and necrotic cells. Under control conditions, the nuclei of wild type cells excluded propidium iodide and exhibited a normal morphology with Hoescht-33258 diffusely labeling the normal chromatin. In sharp contrast, after STS exposure (1 $\mu$M, 8 h), Hoescht-33258 staining revealed that several cells exhibited very intense staining of condensed and fragmented chromatin and were not stained with propidium iodide, indicating preservation of the plasma membrane integrity. The condensation and fragmentation of DNA clearly show that STS induced apoptosis. Less than 8.9±1.2% of the total cells exhibited propidium iodide-labeled nuclei. These morphological characteristics could also be observed independently with orcein staining: control cells (without STS) did not exhibit chromatin condensation. By contrast, a dense and thin crown of nuclear coloration, typical of chromatin condensation, could be observed after STS exposure.

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Hoescht-33258 and orcein staining (data not shown) on STS-treated task2/−/− cells revealed a significant decrease of condensed and fragmented chromatin figures as compared with wild type cells. Based on these morphological criteria, the number of apoptotic cells was determined in both cell lines. Fig. 1B shows the percentage of apoptotic cells determined after 4, 6, or 8 h in the presence STS (1 μM). In both cell lines, the percentage of apoptotic cells increased significantly with the time of incubation. At each time (4, 6, and 8 h), the percentage of apoptotic cells was higher in wild type than in task2/−/− cell lines. At 8 h, the percentage of apoptotic cells reached 33.4% in the wild type cells revealed a significant decrease of con-

STS-induced AVD in Proximal Cell Lines—To check whether the STS-induced apoptotic process was related to an AVD phenomenon, the time course of relative cell volume variation during STS treatment was measured in proximal cell lines from wild type and task2/−/− mice. In both cell lines, cell shrinkage started as early as 1 h after STS exposure (1 μM, Fig. 2A). Six h after application of STS, the relative mean cell volume decreased by 34.2 ± 2.6% in wild type cells but only by 20.2 ± 4.5% in task2/−/− cell lines. The STS-mediated AVD was then studied in the presence of clofilium, ChTX, high [K+]i, or IbTX. As illustrated in Fig. 2B, in wild type cell lines, the STS-induced AVD was strongly inhibited by clofilium, high [K+]i, but not by ChTX or IbTX. Moreover, AVD was completely blocked by the acidification of the external solution (Fig. 2B). In task2/−/− cell lines, the moderate STS-induced AVD was inhibited by ChTX, IbTX, or high [K+]i, and was insensitive to clofilium or external acidic pH (Fig. 2C).

STS Activates Two Different Types of K+ Currents in Wild Type and task2/−/− Proximal Cell Lines—The above experiments suggested the involvement of the TASK2 channel in the AVD process in wild type proximal cells. Whole cell experiments were then performed to further analyze the nature of the K+ conductance triggered by STS exposure. Fig. 3A illustrates the K+ currents recorded in wild type proximal cells before the addition of STS, the voltage step protocol elicited small outwardly rectifying currents. The corresponding I/V curve (Fig. 3B) indicated a reversal potential of −71.5 ± 5 mV and a maximal slope conductance (calculated between +80 and +120 mV) of 3.3 ± 0.7 nS (n = 15). STS exposure induced a large increase of the outward currents, with a maximal slope conductance of 20.3 ± 1.4 nS (n = 15) without significant modification of the reversal potential (−74.3 ± 3 mV). To determine the possible role of cytosolic Ca2+ in the development of STS-induced currents, experiments were performed using a pipette solution containing the Ca2+ chelating agent EGTA (30 mM) to greatly reduce the intracellular free Ca2+ concentration. In 100% of the cells tested, the STS-induced conductance was not modified. These STS-activated K+ currents were inhibited by clofilium (10 μM, maximal slope conductance 3.5 ± 0.8 nS, n = 15). Fig. 3C showed the mean currents recorded at +100 mV under different experimental conditions; the STS-induced currents exhibited no sensitivity to TEA, IbTX, or ChTX but were strongly reduced in the presence of clofilium or when perfusing an acidic external bath solution (pH 6). These characteristics tightly correspond to TASK2 K+ conductance.

Whole cell experiments were also performed in the task2/−/− cell line to better characterize the IbTX-sensitive K+ permeability involved in the residual AVD described in Fig. 2. A–C. Fig. 3D illustrates the K+ currents recorded in task2/−/− proximal cells before and after STS exposure. Without STS, the volt-
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FIGURE 2. Effects of clofilium, ChTX, IbTX, high [K⁺]e, and external acidic pH on the time course of STS-induced AVD in proximal cell lines from wild type and task2⁻/⁻ mice. A, cell volume was measured by an electronic sizing technique with a CASY 1 cell counter (SCHA´RFE SYSTEM). For control conditions, wild type (open hexagons) and task2⁻/⁻ cells (closed squares) were suspended in caseyn® solution (NaCl isotonic solution), and relative volume was measured every hour (for each time point, cells are isolated by trypsinization a few minutes before measuring). Cell volume measurements were also performed in the presence of STS (1 μM). Values are expressed as the percent of cell volume variation measured during STS treatment in wild type (WT) and task2⁻/⁻ (task2⁻/⁻) cell lines. Values are mean ± S.E. of 7 different experiments. *p < 0.05; **p < 0.005; ***p < 0.001, Student’s t-test. B, effect of clofilium, ChTX, IbTX, and external acidic pH on STS-induced AVD in proximal cell line from wild type mice. Experiments were performed in the presence of STS (1 μM, closed circles) supplemented with clofilium (10 μM, closed triangles pointing up), charybdotoxin (10 nM, closed stars), iberiotoxin (100 nM, closed rhombus), high [K⁺]e (125 mM, open rhombus), or high [K⁺]e and external acidic pH (closed triangles pointing down). Values are mean ± S.E. of 9 different experiments. C, effect of clofilium, ChTX, IbTX, and external acidic pH on STS-induced AVD in proximal cell line from task2⁻/⁻ mice. Experiments were performed in the presence of STS (1 μM, closed circles) supplemented with clofilium (10 μM, closed triangles pointing up), charybdotoxin (10 nM, closed stars), iberiotoxin (100 nM, closed rhombus), high [K⁺]e (125 mM, open rhombus) or with a medium adjusted to an acidic pH (closed triangles pointing down). Values are mean ± S.E. of 7 different experiments.

STS-induced ROS Production in Wild Type and task2⁻/⁻ Proximal Cell Lines—It has been already proposed that ROS production induced by STS could be an early process to induce AVD (9). To test this possibility, wild type and task2⁻/⁻ cell lines were loaded with a membrane-permeable fluorescent probe (carboxy-H2DCFDA) to determine the intracellular level of ROS. In wild type cells (Fig. 4A), the intracellular level of ROS rapidly increased with time after STS addition. STS-induced ROS production (ROS production at 30 min 1.48 ± 0.62 relative intensity, n = 6) was completely inhibited by the ROS scavenger NAC (Fig. 4A). NAC alone did not significantly affect the ROS level in the absence of STS (data not shown). In the task2⁻/⁻ cell line (Fig. 4C), STS exposure induced a smaller increase in ROS production, compared with wild type cells (1315 ± 36 pA, n = 15).

Fig. 3G further illustrates this difference by showing the distribution of the STS-induced current recorded in wild type and task2⁻/⁻ cell lines. The individual records were arranged in 5 arbitrary groups according to the maximal current recorded at +100 mV in the presence of STS. In wild type cells, the STS-induced conductance measured at +100 mV were 1103.8 ± 55 pA (n = 27) and only 658.3 ± 65 pA in task2⁻/⁻ cells (n = 28). The difference was statistically significant (p ≤ 0.0001 by t test). All together, in the wild type cell line, 93% of the STS-induced currents are between 800 and 1800 pA. In sharp contrast, in the task2⁻/⁻ cell line only 57% of the records are in this range of currents. Moreover 12 records are moderately stimulated by STS in the task2⁻/⁻ cell line (current below 800 pA) as compared with only 2 records in the wild type cell line.

STS exposure significantly increased the outward currents, which reached a maximal conductance of 15.6 ± 2.3 nS (Erev = −70.2 ± 2.5 mV, n = 16) without significant change of the reversal potential. These STS-induced K⁺ conductances were inhibited by the addition of ChTX or IbTX to the external solution. Experiments were also performed using pipette solution containing Ca²⁺ chelating agent EGTA (30 mM) to greatly reduce the intracellular free Ca²⁺ concentration. In 100% of the cells tested, the STS-induced conductance was completely blocked (n = 5). As shown in Fig. 3F, the mean STS-induced K⁺ currents (measured at +100 mV) exhibited a different pharmacological profile as compared with wild type cells; they were inhibited by TEA, IbTX, and ChTX but remained insensitive to clofilium or per-
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FIGURE 3. STS induces activation of whole cell K\(^+\) currents in proximal cell lines from wild type and task2\(^{-/-}\) mice. A–C, whole cell K\(^+\) currents from wild type proximal cells were measured before (control) and 1 h after STS exposure (1 \(\mu\)M) in the absence or presence of iberiotoxin (100 nM) or clofilium (10 \(\mu\)M). The pipettes were filled with Ca\(^{2+}\) (0 mM EGTA) or Ca\(^{2+}\)-free (30 mM EGTA) pipette solutions as indicated. Membrane voltage was held at −50 mV and stepped to test potential from −100 to +120 mV in 20-mV increments. The figures show the whole cell recordings (A) and the corresponding current-voltage (I–V) relationships (B, C, histogram of K\(^+\) current values recorded in wild type proximal cell lines at +100 mV in the presence of TEA (1 mM), ChTX, IbTX, and clofilium or at an external acidic pH. Values are mean ± S.E. of 8 cells from 8 different monolayers. *** \(p < 0.001\), Student’s t test. D–F, whole cell K\(^+\) currents from task2\(^{-/-}\) proximal cells were measured before (control) and 1 h after STS exposure (1 \(\mu\)M) in the absence or presence of clofilium (10 \(\mu\)M) and iberiotoxin (100 nM). The pipettes were filled with Ca\(^{2+}\) (0 mM EGTA) or Ca\(^{2+}\)-free (30 mM EGTA) pipette solutions as indicated. The figures show the whole cell recordings (D) and the corresponding current-voltage (I–V) relationships (E, F, histogram of K\(^+\) current values recorded at +100 mV in the presence of TEA, ChTX, IbTX, clofilium, or at an external acidic pH from task2\(^{-/-}\) proximal cell lines. Values are mean ± S.E. of 8 cells from 8 different monolayers. *** \(p < 0.001\), Student’s t test. G, current amplitude distribution of individual whole cell records measured after STS exposure in proximal cell lines from wild type (closed bars) and task2\(^{-/-}\) (open bars) mice. The individual records (n) are distributed according to the maximal current measured at +100 mV. 5 arbitrary classes of currents were defined as indicated. This histogram was constructed with 28 and 27 records obtained from wild type and task2\(^{-/-}\) proximal cell line, respectively.

The data obtained using the whole cell technique have clearly demonstrated that, in the wild type cell line, the STS-induced K\(^+\) currents were Ca\(^{2+}\) insensitive; by contrast, in the task2\(^{-/-}\) cell line, the STS-induced K\(^+\) current was Ca\(^{2+}\) dependent. The above experiments suggested the involvement of Ca\(^{2+}\) in the AVD phenomenon in the task2\(^{-/-}\) cell line. To confirm this hypothesis, wild type and task2\(^{-/-}\) cells were loaded with Fura 2-AM and maintained in NaCl buffer to determine the intracellular level of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)). In the wild type cell line (Fig. 4B), STS induced a sustained elevation of [Ca\(^{2+}\)]\(_i\). The STS-induced Ca\(^{2+}\) increase was completely inhibited by NAC (Fig. 4B). NAC alone did not significantly affect the [Ca\(^{2+}\)]\(_i\) in the absence of STS (data not shown). Similarly in the task2\(^{-/-}\) cell line (Fig. 4D), STS exposure induced also a rapid increase of [Ca\(^{2+}\)]\(_i\), with the same sensitivity to NAC. In conclusion, both cell lines exhibited identical Ca\(^{2+}\) production kinetics when exposed to STS.

STS-induced AVD and Caspase-3 Activation Are Mediated by ROS—The effect of NAC on relative volume changes was then tested in wild type and task2\(^{-/-}\) cell lines. As illustrated in Fig. 5, A and C, addition of NAC completely abolished the STS-induced AVD in both cell lines. The putative inhibitory effect
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FIGURE 4. ROS production and intracellular Ca\(^{2+}\) increase during STS exposure in proximal cell lines from wild type and task2\(^{-/-}\) mice. A and C, STS-induced ROS production in wild type (A) and task2\(^{-/-}\) (C) cell lines. Cells were incubated for 30 min in carboxy-H2DCFDA (10 \(\mu\)M) and washed in serum-free culture medium. After trypsinization, isolated loaded cells were incubated in the absence (closed circles) or presence (open circles) of STS (1 \(\mu\)M) or STS + NAC (10 mM, closed triangles). The emitted fluorescence intensity was measured every 2 min at 538 nm. Values are expressed as the relative variations of the fluorescence intensity \(\pm\) S.E. of 17 different experiments. Wild type and task2\(^{-/-}\) maximal ROS levels are statistically different with \(p < 0.05\) at 25 min and \(p < 0.005\) at 30 min (Students t-test). B and D, STS-induced [Ca\(^{2+}\)]\(_i\) increase in wild type (B) and task2\(^{-/-}\) (D) cell lines. Wild type (B) and task2\(^{-/-}\) (D) cell lines grown on Petri dishes were loaded for 45 min at room temperature with fura 2-AM (2 \(\mu\)M) and pluronic acid (0.01%). Addition of STS (1 \(\mu\)M) in the absence (closed circles) or presence (open circles) of NAC (10 mM) is indicated by the arrow. Values are mean \(\pm\) S.E. of 20 –30 cells from 6 different monolayers (~120–180 cells were analyzed per cell line).

of NAC on STS-induced K\(^+\) conductances (1-h STS exposure) was then tested in whole cell experiments in both cell lines. In wild type cells (Fig. 5B), the STS-induced K\(^+\) currents (1190 ± 71 pA measured at +100 mV) were strongly reduced in the presence of NAC (252 ± 89 pA). Similarly, the STS-induced K\(^+\) currents in task2\(^{-/-}\) cells (1190 ± 71 pA measured at +100 mV) were also largely reduced after NAC exposure (252 ± 89 pA, Fig. 5D). This indicates that ROS generation is an upstream signal in STS-induced apoptosis, independent of the nature of K\(^+\) channels involved in the AVD process (TASK2 or IbTX-sensitive K\(^+\) channels). To confirm this hypothesis, STS-induced caspase-3 activation was measured in the presence of NAC (Fig. 5E). NAC markedly inhibited STS-induced caspase-3 activation in both cell lines.

STS-induced K\(^+\) Currents Are Activated by ROS Production—Whole cell experiments were performed to test the effect of oxidative stress (500 \(\mu\)M H\(_2\)O\(_2\) or xanthine (50 mM) and xanthine oxidase (50 milliunits ml\(^{-1}\)), referred to as X/X\(_{ox}\)) on the K\(^+\) currents in wild type and task2\(^{-/-}\) cell lines. As illustrated in Fig. 6A, in the wild type cell line, addition of H\(_2\)O\(_2\) to the bath solution induced a rapid increase (<15 min) of the K\(^+\) currents. These currents were significantly inhibited by clofiliarum and insensitive to IbTX (not shown), suggesting an activation of TASK2 channels by H\(_2\)O\(_2\). Experiments were also performed using pipette solutions containing 30 mM EGTA to greatly reduce the intracellular free Ca\(^{2+}\) concentration. In 100% of the cells tested, the H\(_2\)O\(_2\)-induced conductance was completely insensitive to intracellular Ca\(^{2+}\) scavenging (10 mM, Fig. 6B). Conversely, in the task2\(^{-/-}\) cell line, the addition of H\(_2\)O\(_2\) also increased K\(^+\) currents, which were largely blocked by IbTX, insensitive to clofiliarum (Fig. 6C), and completely blocked by 30 mM EGTA (Fig. 6D).

CsA-induced AVD and caspase-3 activation are mediated by ROS. To confirm the difference obtained between wild type and task2\(^{-/-}\) cell lines in STS-induced AVD and caspase-3 activity, experiments were performed by replacing the apoptotic inducer STS with CsA (25 \(\mu\)M). CsA significantly increased caspase-3 activity in both cell lines (Fig. 7A). However, the level of caspase-3 activity remained lower in the task2\(^{-/-}\) cell line as compared with the wild type cell line. In the wild type cell line, CsA-induced caspase-3 activation was inhibited by clofiliarum (10 \(\mu\)M), extracellular pH, high [K\(^+\)]\(_o\), or NAC (10 mM). By contrast, the moderate CsA-induced caspase-3 activation in task2\(^{-/-}\) cells remained insensitive to clofiliarum (10 \(\mu\)M) but was completely abolished by IbTX (100 mM), high [K\(^+\)]\(_o\), or NAC (10 mM).

The measurements of the CsA-induced volume change confirmed these observations. In the wild type cell line, IbTX had no effect, whereas clofiliarum abolished the CsA-induced volume decrease (Fig. 7B). In the task2\(^{-/-}\) cell line, the opposite sensitivity was observed: clofiliarum did not modify, whereas IbTX blocked the CsA-induced volume decrease (Fig. 7C). In both cell lines, NAC or high [K\(^+\)]\(_o\) completely inhibited the CsA-induced volume decrease. To confirm that CsA-induced ROS production could be an early process to induce AVD, wild type and task2\(^{-/-}\) cells were loaded with a membrane-permeable fluorescent probe, carboxy-H2DCFDA, to determine the intracellular level of ROS.

In wild type cells (Fig. 7D), the intracellular level of ROS rapidly increased with time after CsA addition (ROS production at 30 min = 1.44 ± 0.05 relative intensity, \(n = 6\)). CsA-induced ROS production was completely inhibited by the ROS scavenger NAC (Fig. 7D). In the task2\(^{-/-}\) cell line (Fig. 7E), CsA exposure induced a smaller increase of ROS production (ROS production at 30 min = 1.34 ± 0.02 relative intensity, \(n = 6\)).
insensitive to IbTX. task2−/− cells exhibited lower caspase-3 activity and higher relative cell volume, which were inhibited by iberiotoxin and NAC, respectively. In these cells clofilium did not modify caspase 3 activity and the relative volume change induced by TNF-α.

Fig. 8C shows the ROS increase induced by TNF-α. This increase remained smaller in the task2−/− cell line than in the wild type cell line but was completely abolished by NAC in both cell lines (data not given).

DISCUSSION

In previous studies, we demonstrated that TASK2 K+ channels participate in the K+ efflux during RVD in mouse proximal cells in primary cultures (25). However, it is well documented that the cell volume decrease also accompanies apoptosis (known as AVD) (7). Apoptotic cell shrinkage is an early prerequisite event for apoptosis and precedes caspase-3 activation during the apoptotic process (7). This volume change results from a loss of cytosolic ions (e.g. K+ and Cl−) and water in response to apoptosis inducers. Generally, most ion efflux occurs via K+ and Cl− channels (26–28). Because similarities exist between RVD in response to osmotic shock and AVD, it has been postulated that the Cl− and K+ channels involved in both processes could be the same (5). Therefore we investigate whether the TASK2 channels could play a crucial role in apoptosis. For this purpose, we have developed immortalized cell lines from primary cultures of proximal tubules obtained from wild type and task2+/− mice. In wild type cells, the present findings indicate that the K+ channels involved in STS-induced AVD exhibit properties consistent with the TASK2 channels. Thus, the K+ channel activated by STS is decreased by clofilium application or external pH acidification and is Ca2+ independent. The observation that clofilium and acidic pH also block STS-induced AVD and caspase-3 activation clearly indicates that the activity of the TASK2 channel is related to apoptosis in proximal tubule cells. To further implicate TASK2 in STS-induced AVD, experiments were performed in proximal cells from task2−/− mice. In these cells, STS-induced AVD and caspase-3 activity were significantly reduced. Nevertheless, in the absence of TASK2, the cells partially maintained their apoptotic capability. Interestingly, the addition of IbTX completely inhibits AVD and caspase-3 activation, suggesting that Ca2+−activated K+ channels are involved in these phenomena. Moreover, the biophysical properties of the K+ currents (i.e. blockade by chTX or IbTX and dependence on internal Ca2+) underlying AVD and apoptosis in task2−/− proximal cells confirm the implication of Ca2+−sensitive large conductance K+ channels (BK channels). However, the relation between the activation of K+ channels/AVD in both cell lines and the downstream activation of caspase-3 remains unclear. Hughes et al. (29) suggest that the intracellular level of K+ directly regulates the apoptotic process by controlling the activity of death enzymes. They demonstrated that apoptosis is enhanced under conditions where the [K+]i is diminished and that apoptosis is inhibited when K+ efflux is prevented. Therefore, enhanced K+ efflux is an essential mediator not only of early apoptotic cell shrinkage but also of downstream caspase-3 activation and DNA fragmentation.
Role of TASK2 in the Control of Apoptotic Volume Decrease

Many studies have demonstrated that an increase in K⁺ channel activity is involved in the cell volume changes during apoptosis. Different classes of K⁺ channels could be involved in apoptosis (11). In excitable cells, STS enhances voltage-gated K⁺ channels (Kv) (14, 30), BK channels (31, 32), Kir (7, 28), and Kᵦᵣ (33) channels. Members of the two pore domain channels family, such as TREK (13) and TALK (34), are also involved in hydrogen peroxide-induced apoptosis. TASK2 belongs to this last family and is mainly expressed in the proximal tubule. Therefore it is not surprising that, in addition to participating in RVD, it might also participate in AVD. Interestingly, TASK2 channels are reported to be activated by superoxide donors (34). It is well known that STS induces the mitochondrial apoptotic pathway via ROS generation (35). In the present study, the ROS scavenger NAC blocks STS-induced AVD, TASK2 currents, and finally decreases caspase-3 activity. It is therefore probable that the activation of TASK2 is mediated by ROS production. This conclusion is further supported by the observation that H₂O₂ and xanthine oxidase-induced K⁺ currents exhibit biophysical features of TASK2 currents in wild type cells. Interestingly the observation that STS-induced ROS production was insensitive to K⁺ channel inhibitors indicates that ROS generation was upstream of AVD and caspase-3 activation.

However, the mechanism of activation of TASK2 channels by ROS remains ambiguous. One hypothesis suggests a direct effect of ROS on the cell surface membrane. ROS are known to induce the lipid peroxidation of membrane rafts (36), thus, it is also possible that ROS could regulate raft formation through a combination of cytoskeleton modification, raft-associated proteins, and signal activation leading to TASK2 modulation. Another hypothesis proposes that some amino acid residues (histidine, tryptophan, cysteine, and methionine) of the channel might form disulfide bonds in the presence of ROS and then modulate the channel biophysical properties (34, 37, 38).

Surprisingly, STS-induced AVD and apoptosis are also observed in cells lacking TASK2. In these cells, a calcium-activated large conductance K⁺ channel (BK channel) seems to play a major role in the AVD process. Although the apoptotic cascade appears less efficient than in wild type cells, BK channels qualitatively play the same role as TASK2. Analyzing the distribution of the K⁺ current amplitudes shows that the frequency of STS-induced currents was identical in both cell lines. However, the mean amplitude of the STS-induced K⁺ currents in task2⁻/⁻ cells was lower than the amplitude measured in wild type cells. Consequently the cells expressing TASK2 K⁺ channels increased further their K⁺ conductance in response to STS than the cells expressing BK channels. We can assume that the reduction of cell volume is correlated to the ion conductances of the cell membrane (mainly K⁺ and Cl⁻ in the case of AVD) and is limited by the ion with the lowest conductance. This assumption would explain why task2⁻/⁻ cells exhibited a reduced AVD.

A question becomes what signal is responsible for the BK channel activation. As observed in wild type cells, the application of NAC completely inhibited STS-induced AVD, BK currents, and caspase-3 activation. Moreover, H₂O₂ strongly stimulated the K⁺ currents. As expected for BK currents, H₂O₂ stimulation was abolished by ChTX and IbTX and depends on cytoplasmic calcium. However, conflicting results are reported in the literature concerning the effect of H₂O₂ on the BK channel activity. For some authors, H₂O₂ blocked the channel (39),
whereas for others, H$_2$O$_2$ activated the channel (40). The mechanism underlying BK channel regulation by H$_2$O$_2$ is complex (41). It could depend on H$_2$O$_2$ concentration and on the cell side applied (inside or outside the cell). Interestingly, the smaller mean current amplitude recorded in task2$^{-/-}$ cells could also be related to a lower ROS increase. This lower ROS production and the smaller K$^+$ conductance (see above) could explain why STS-induced AVD was reduced in these cells. It remains that we have no argument to explain why the task2$^{-/-}$ cells exhibited a smaller increase in ROS in the presence of an apoptotic inducer.

In the present study, ROS production is accompanied by a parallel increase in cytosolic Ca$^{2+}$ in wild type and task2$^{-/-}$ proximal cells. This increase is probably due to ROS-induced mitochondrial damage (9). Therefore, Ca$^{2+}$ could activate the BK channels in task2$^{-/-}$ cells. Surprisingly, in wild type cells, Ca$^{2+}$ increase does not modify TASK2 channel activity and induce any significant K$^+$ conductance through BK channels. This raises the problem of the participation of BK channels in cell volume control in wild type proximal cells. Although BK channels have been found to be involved in RVD in primary cultures of rabbit proximal tubule (42), they do not participate in RVD in primary cultures of mouse proximal tubules (25). In this species, RVD and AVD are mainly initiated by TASK2 K$^+$ channels (22, 43). In fact, preliminary studies performed in wild type proximal cells indicate that, in the absence of EGTA, the addition of STS never triggered BK conductance, whereas the addition of ionomycin induced BK currents in less than 5% of the whole cells recorded. Thus, it is probable that the involvement of BK channels in the AVD process would remain very small when TASK2 K$^+$ channels are present.

STS is a useful apoptosis inducer in most mammalian cells, and we have also studied the effect of another apoptotic agent that has a more specific renal effect. CsA is one of the most widely used immunosuppressive drugs for organ transplantation, but its efficacy is limited by its nephrotoxicity. Notably, several groups have identified tubular apoptosis during CsA treatment both in vitro and in vivo (44–46). In the cell models used in the present study, acute CsA application induced AVD and caspase-3 activation probably by a mechanism related to ROS production. Although complementary experiments are required to further characterize the K$^+$ channels involved in CsA-induced apoptosis, the pharmacology of both AVD and caspase-3 activity clearly suggests that TASK2 K$^+$ and BK

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3 S. L’Hoste, M. Poet, C. Duranton, R. Belfodil, H. Barriere, I. Rubera, M. Tauc, C. Poujeol, J. Barhanin, and P. Poujeol, unpublished data.
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channels control apoptosis in wild type and task2⁰⁻/⁻ cells, respectively.

Staurosporine and CsA have severe toxic effects on renal cells and mediated mainly a mitochondrial apoptosis. It was therefore interesting to check the role of TASK2 K⁺ channels in apoptosis mediated by the membrane TNF-α death receptor. TNF-α is an inflammatory cytokine that induces programmed cell death in a variety of tissues (47). Experimental evidence supports several mechanisms to account for such effects, including activation of caspase-3 and generation of free radicals (48–50). As observed for STS and CsA, TNF-α induced an increase in the ROS production associated with AVD and caspase-3 stimulation in both lines. However, the action of TNF-α was delayed with a maximal effect reached after 24 h of incubation. These different effects of TNF-α were strongly reduced in task2⁰⁻/⁻ cells. The complementary experiments performed by using clofilium and iberiotoxin indicated that TASK2 K⁺ channels were involved in TNF-α-induced apoptosis in wild type cells, whereas BK channels were rather involved in task2⁰⁻/⁻ cells. Finally the three apoptotic agents used in the present study probably increased apoptosis via the same mechanisms including ROS production, activation of K⁺ channels, AVD, and caspase-3 increase. If it is relatively well established that STS and CsA stimulate the mitochondrial apoptosis pathway, the nature of apoptosis induced by TNF-α is more complex. In rat hepatoma cells, TNF-α activated K⁺ and Cl⁻ channels and induced apoptotic processes (28) but it is not clear whether ROS are involved in this phenomenon. In the kidney, involvement of ROS in apoptosis is controversial because it has been proposed that ROS formation could be associated with apoptosis but would not be the causal agent (20, 51, 52). In a very recent study performed in human proximal tubules, it was demonstrated that albumin induced apoptosis through the mitochondrial pathway independently of ROS production (21).

Concerning death receptors, several authors described that either Fas receptor or TNF receptor-mediated apoptosis was prevented by various antioxidants including NAC (48, 53). These observations support the essential role of ROS even in death receptor-mediated apoptosis.

In conclusion, the originality of the model proposed here is based on the central role of TASK2 K⁺ channels in the control of cell volume during hypotonic shock (RVD) (22, 25, 43) or apoptosis phenomenon (AVD) in proximal tubule cells. This study shows that in proximal cell lines, there is a clear interplay between STS-induced apoptosis, AVD, ROS production, and K⁺ channels. Moreover the results demonstrate that BK channels can contribute to the K⁺ efflux involved in AVD in proximal cells in the absence of TASK2 channels. For the moment we do not know the mechanism of this compensation. Recently, an up-regulation of a BK channel in response to high-K⁺ diet has been demonstrated in Romk⁻⁻/⁻ mice (54). The cell function addressed in this work is clearly different from that of the present study, but their data indicate that the function usually mediated by a dedicated channel could be maintained by BK channels in the absence of the former. Thus BK channel can be up-regulated in task2⁰⁻/⁻ cells to maintain the function (AVD) that is controlled by the TASK2 channel in wild type cells. Phenotypic analysis of the task2⁰⁻/⁻ mice has already revealed the
role of TASK2 K⁺ channels in bicarbonate handling along the proximal tubule (55). Concerning apoptosis, preliminary in situ experiments using the TUNEL technique in renal slices show that the basal apoptosis level was identical in wild type and task2⁻/⁻ mice (data not given). However, we are now undertaking ischemia/reperfusion experiments in which we measure apoptosis and necrosis balance in both mice strains. I/R is well known to induce mitochondrial apoptosis and it will be of interest to determine whether the renal function of wild type mice during the reperfusion could be better preserved than that of task2⁻/⁻ mice.

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