Augmented K\(^+\) currents and mitochondrial membrane depolarization in pulmonary artery myocyte apoptosis

STEFANIE KRICK, OLEKSANDR PLATOSHYN, SHARON S. MC DANIEL, LEWIS J. RUBIN, AND JASON X.-J. YUAN
Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of California School of Medicine, San Diego, California 92103

Received 6 December 2000; accepted in final form 7 May 2001

Augmented K\(^+\) currents and mitochondrial membrane depolarization in pulmonary artery myocyte apoptosis. Am J Physiol Lung Cell Mol Physiol 281: L887–L894, 2001.—The balance between apoptosis and proliferation in pulmonary artery smooth muscle cells (PASMCs) is important in maintaining normal pulmonary vascular structure. Activity of voltage-gated K\(^+\) (K\(_V\)) channels has been demonstrated to regulate cell apoptosis and proliferation. Treatment of PASMCs with staurosporine (ST) induced apoptosis in PASMCs, augmented K\(_V\) current [I\(_{K(V)}\)], and induced mitochondrial membrane depolarization. High K\(^+\) (40 mM) negligibly affected the ST-induced mitochondrial membrane depolarization but inhibited the ST-induced I\(_{K(V)}\) increase and apoptosis. Blockade of K\(_V\) channels with 4-aminopyridine diminished I\(_{K(V)}\) and markedly decreased the ST-mediated apoptosis. Furthermore, the ST-induced apoptosis was preceded by the increase in I\(_{K(V)}\). These results indicate that ST induces PASMC apoptosis by activation of plasmalemmal K\(_V\) channels and mitochondrial membrane depolarization. The increased I\(_{K(V)}\) would result in an apoptotic volume decrease due to a loss of cytosolic K\(^+\) and induce apoptosis. The mitochondrial membrane depolarization would cause cytochrome c release, activate the cytosolic caspases, and induce apoptosis. Inhibition of K\(_V\) channels would thus attenuate PASMC apoptosis.

METHODS AND MATERIALS

Cell preparation. Human PASMCs (Clonetics) were seeded in flasks at a density of 2,500–3,500 cells/cm\(^2\) and incubated in smooth muscle growth medium (Clonetics). The culture medium was changed after 24 h and every 48 h thereafter. Smooth muscle growth medium is composed of smooth muscle basal medium, 5% fetal bovine serum, 0.5 ng/ml of human epidermal growth factor, 2 ng/ml of human fibroblast growth factor, and 5 µg/ml of insulin. The cells were subcultured and plated onto 10- or 25-mm coverslips with trypsin-EDTA buffer (Clonetics) when 70–90% confluence was achieved. The cells at passages 4–6 were used for experimentation.

Electrophysiological measurement. K\(^+\) currents (I\(_{K}\)) were recorded with an Axopatch-1D amplifier and a DigiData 1200 interface (Axon Instruments) with patch-clamp techniques (18, 49). Patch pipettes (2–4 MΩ) were made with a Sutter electrode puller with borosilicate glass tubes and fire polished on a Narishige microforge. Command voltage protocols and data acquisition were performed with pCLAMP soft-
ware. Currents were filtered at 1–2 kHz (–3 dB) and digitized at 2–4 kHz by the amplifier. All experiments were performed at room temperature (22–24°C).

For measurement of whole cell $I_K$, a pipette containing glucose (pH 7.4). For Ca$^{2+}$-free PSS containing 1 mM Ca$^{2+}$, the cells were superfused with Ca$^{2+}$-free PSS (containing 1 mM Ca$^{2+}$). To isolate the optimal Kv current, $[I_{KV}]$, the cells were superfused with 5 mM ATP [which completely blocks ATP-sensitive K$^+$ (KATP) channels (7)].

**Nuclear morphology determination.** The cells grown on 10-mm coverslips were first washed with PBS (Sigma), fixed in 95% ethanol, and stained with the membrane-permeable and nonspecifically quenched at resting DC microscope. In isolated mitochondria, the relationship between K$^+$ current [K(V)], the cells were superfused with K$^+$-free PSS containing 1 mM tetraethylammonium chloride [TEA; which predominantly blocks Ca$^{2+}$-activated K$^+$ (KCa) channels at doses ≤ 1 mM] (1) and diazyl aided with Ca$^{2+}$-free pipette solutions including 5 mM ATP [which completely blocks ATP-sensitive K$^+$ (KATP) channels] (7).

**RESULTS**

Inhibitory effects of 40 mM K$^+$ and 4-AP on ST-induced apoptosis in human PASMCs. Under control conditions, the cells were examined with a Nikon TE 300 fluorescence microscope, and the cell (nuclear) images were acquired with a high-resolution Solamere fluorescence imaging system. For each coverslip, 5–10 fields (with ~20–25 cells/field) were randomly selected to determine the percentage of apoptotic cells in the total cells based on the morphological characteristics of apoptosis. The cells with clearly defined nuclear breakage, remarkably condensed nuclear fluorescence, and a significantly shrunken cell body and nucleus were defined as apoptotic cells. To quantify apoptosis, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays were also performed with an in situ cell death detection kit (TMR red; Boehringer Mannheim).

**Augmenting effect of ST on Kv channel activity.** Whole cell $I_{KV}$ was isolated in human PASMCs when the contribution of K$^+$ and KATP channels to the current was minimized by removal of extracellular (bath) and intracellular (pipette) Ca$^{2+}$ and by the intracellular addition of 5 mM ATP, which completely blocks KATP channels (7). Extracellular application of 5 mM 4-AP (Fig. 2) or raising the extracellular K$^+$ concentration to 40 mM (data not shown) significantly decreased the currents, suggesting that the currents were mainly generated by K$^+$ efflux through 4-AP-sensitive Kv channels (1, 37, 49). Under these conditions, extracellular application of 0.1 μM ST significantly increased...
The voltage-dependent augmenting effect of ST on \( I_{K(V)} \) and a 2.9-fold increase at 13.7 ± 0.8 mV from 377.4 ± 22.9 to 1,094.7 ± 42.4 pA at +80 mV (P < 0.001; n = 8–14 experiments; Fig. 4A). Furthermore, ST treatment (0.02 µM for 90 min) markedly decelerated the rate of \( I_{K(V)} \) inactivation; the time constants of current inactivation at +80 mV were 46.3 ± 3.9 ms (n = 14 experiments) and 69.6 ± 8.6 ms (P < 0.001; n = 9 experiments) in cells treated with vehicle (DMSO) and 0.02 µM ST, respectively (Fig. 4Ac).

To determine whether the ST-induced increase in \( I_{K(V)} \) preceded its apoptotic effect, we compared the time courses of the ST-induced increase in \( I_{K(V)} \) and apoptosis in human PASMCs treated with 0.02 µM ST for up to 24 h. The ST-induced increase in \( I_{K(V)} \) took place ∼30 min after treatment of the cells with 0.02 µM ST (Fig. 4B). The time course of the ST-induced apoptosis indicated that the percentage of apoptotic nuclei was not significantly increased until 6 h after treatment with 0.02 µM ST; the maximal effect occurred 20 h after treatment with ST (Fig. 4B).

Whole cell \( I_{K(V)} \) (40K) was predominantly due to activation of KV channels (Fig. 3). The ST-mediated activation of KV channels was almost completely blocked by extracellular application of 5 mM 4-AP (Fig. 3B, bottom), suggesting that the ST-induced increase in whole cell \( I_K \) was predominantly due to activation of the 4-AP-sensitive KV channels.

The ST-induced increase in \( I_{K(V)} \) preceded the ST-induced apoptosis. Incubation of human PASMCs in medium containing the low dose (0.02 µM) of ST that was used for the apoptosis experiments (Fig. 1) for 90 min also significantly increased the amplitude of \( I_{K(V)} \) from 13.7 ± 0.8 to 36.7 ± 1.7 pA at −40 mV and from 377.4 ± 22.9 to 1,094.7 ± 42.4 pA at +80 mV (P < 0.001; n = 8–14 experiments; Fig. 4A). Furthermore, ST treatment (0.02 µM for 90 min) markedly decelerated the rate of \( I_{K(V)} \) inactivation; the time constants of current inactivation at +80 mV were 46.3 ± 3.9 ms (n = 14 experiments) and 69.6 ± 8.6 ms (P < 0.001; n = 9 experiments) in cells treated with vehicle (DMSO) and 0.02 µM ST, respectively (Fig. 4Ac).

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observations indicate that the ST-induced enhancement of \( I_{K(V)} \) occurred before the onset of apoptosis in human PASMCs, suggesting that the increased \( I_{K(V)} \) may play a causal role in initiating apoptosis (5, 6, 8, 10, 35, 38, 46, 47).

Effect of ST on R-123 fluorescence. The \( \Delta \Psi_m \) is generated primarily by a proton gradient across the mitochondrial inner membrane (3). Mitochondrial membrane depolarization (less negative in \( \Delta \Psi_m \)) has been demonstrated to induce cytochrome \( c \) release from the mitochondrial intermembrane space to the cytosol (2, 9, 29, 51). Cytochrome \( c \) activates the cytosolic caspases, which are believed to be the final mediator of apoptosis in a variety of cell types (29, 44). Whether ST-mediated apoptosis is due, in part, to \( \Delta \Psi_m \) depolarization was examined by measuring the changes in R-123 fluorescence intensity in human PASMCs.

As shown in Fig. 5, extracellular application of ST (0.02 \( \mu M \)) rapidly increased R-123 fluorescence in human PASMCs. \( \Delta \Psi_m \) is linearly related to the R-123 fluorescence intensity in vitro. The increase in R-123 fluorescence and the decrease in \( \Delta \Psi_m \) indicated that the mitochondrial membrane was depolarized (13, 14). However, increasing the extracellular K\(^+\) concentration from 5 to 40 mM, which decreases the driving force for K\(^+\) efflux across the plasma membrane, had negligible effect on the ST-induced increase in R-123 fluorescence (Fig. 5, B and C). These results indicate that inhibition of K\(^+\) efflux across the plasma membrane (e.g., by reducing the transmembrane K\(^+\) driving force or blocking sarcolemmal K\(^+\) channels) does not interfere with the ST-induced depolarizing effect on \( \Delta \Psi_m \) in PASMCs.

DISCUSSION

The precise control of the balance between PASMC proliferation and apoptosis plays an important role in maintaining normal pulmonary vascular structure and function. An increase in proliferation and a decrease in apoptosis of pulmonary vascular smooth muscle and endothelial cells would lead to pulmonary vascular remodeling and elevation of pulmonary vascular resistance and arterial pressure as observed in patients with pulmonary hypertension (9, 15, 16, 40, 43).

The observations from this study demonstrated that apoptosis induced by ST is linked to mitochondrial membrane depolarization and activation of sarcolemmal \( K_V \) channels in human PASMCs. The \( \Delta \Psi_m \) depolarization would trigger the release of cytochrome \( c \) that activates caspases in the cytosol and induces apoptosis (20, 29, 51). The increase in \( I_{K(V)} \) would result in a loss of cytosolic K\(^+\), which relieves the tonic suppression of K\(^+\) on the caspases and nucleases and therefore induces DNA degradation and apoptosis (6, 80).

Fig. 3. Augmenting effect of ST on whole cell \( I_{K(V)} \) in human PASMCs. Aa: families of superimposed currents averaged from 14 cells before (control) and during 15-min application of 0.1 \( \mu M \) ST in the absence and presence of 5 mM 4-AP. The currents were elicited by test potentials ranging from −40 to +80 mV (holding potential −70 mV). Leakage and capacitive currents were subtracted. Ab: composite I-V curves obtained from PASMCs before (control (Cont)) and during ST application in the absence and presence of 4-AP. Data are means ± SE; \( n = 14 \) experiments. Control I-V curve is significantly different from the I-V curve during ST application (\( P < 0.001 \)) by Student-Newman-Keuls test. Summarized data show the amplitude of \( I_{K(V)} \) at −40 mV (Ac) before and during application of 0.1 \( \mu M \) ST, the ST-induced percent increase in \( I_{K(V)} \) at various test potentials (Ad), and the time constant (\( \tau_{\text{inact}} \)) of current inactivation at +80 mV (Ae). Data are means ± SE; \( n = 14 \) experiments. *** \( P < 0.001 \) vs. Cont. B: representative time courses of the effect of ST on \( I_{K(V)} \), in the presence of tetrathylammonium chloride (TEA); Top: changes in the amplitude of \( I_{K(V)} \) at +80 mV before, during, and after application of 0.1 \( \mu M \) ST. 4-AP (5 mM) was applied to the cells when ST-induced increase in \( I_{K(V)} \) reached the plateau. Bottom: changes in the amplitude of \( I_{K(V)} \) at +80 mV before, during, and after application of ST in solutions containing 5 or 40 mM K\(^+\).
Furthermore, the K\textsuperscript{+} loss would also lead to cell shrinkage (the apoptotic volume decrease), an early characteristic commonly used to identify apoptotic cells (5, 6, 10, 35, 46). Inhibition of K\textsubscript{V} efflux through KV channels with 40 mM K\textsuperscript{+} or 4-AP, a potent blocker of KV channels, negligibly affected the ST-induced DC\textsubscript{m} depolarization but significantly attenuated the ST-induced apoptosis. These results suggest that ST mediates apoptosis in human PASMCs, at least in part, by two independent mechanisms: 1) DC\textsubscript{m} depolarization and 2) activation of KV channels in the plasma membrane.

KV channels are ubiquitously expressed in both excitable and nonexcitable cells (27, 37) and play important roles in the regulation of excitability, neurotransmitter release, muscle contraction, and cell volume, proliferation, and differentiation (12, 27, 39, 49). At the molecular level, KV channels are homomeric and heteromeric tetramers composed of the pore-forming \( \alpha \)-subunits and the regulatory \( \beta \)-subunits (27, 37). The \( \alpha \)-subunit is composed of six membrane-spanning segments termed S1 through S6, with a protein kinase C

![Fig. 6. Schematic diagram illustrating the possible mechanisms involved in ST-induced apoptosis in PASMCs. Cyto-c, cytochrome c; \(-\), inhibition.](http://ajplung.physiology.org/)
(PKC) binding site located intracellularly between the S4 and S5 segments (27). The PKC-mediated phosphorylation of the α-subunits inhibits the K⁺ currents generated by K⁺ efflux through Kv channels encoded by Kv1.1, Kv2.1, Kv4.1, or Kv4.2 (4), whereas PKC-induced phosphorylation of the Kvβ1.2 and Kvβ1.3 subunits is necessary for the inactivation of Kv channels (31, 32, 34). Therefore, inhibition of PKC would increase Kv channel activity (4, 31, 32, 34), promote K⁺ efflux, and induce a loss of cytosolic K⁺. ST is a non-specific inhibitor of PKC (17, 30), and the ST-induced increase in IK(V) and apoptosis in human PASMCs may be, at least in part, due to the inhibition of PKC.

As mentioned earlier, cell shrinkage (the apoptotic volume decrease) due to the alterations in cell volume and the loss of intracellular K⁺ is an early requisite feature for the activation of the caspases and other cytosolic, proapoptotic enzymes (5, 6, 10, 22, 23, 35, 46). Indeed, our observations indicated that ST-induced apoptosis was accompanied by a significant increase in caspase-3 concentration, and the decrease in K⁺ concentration in the assay buffer significantly reduced caspase-3 activity (data not shown). These results suggest that blockade of Kv channels may attenuate PASMC apoptosis by diminishing the apoptotic volume decrease and caspase activity via maintaining a high concentration of cytosolic K⁺.

Due to the outwardly directed electrochemical gradient for K⁺, opening of the plasmalemmal K⁺ channels would promote efflux or loss of cytosolic K⁺ and induce the apoptotic volume decrease and apoptosis. There are at least four types of K⁺ channels in vascular smooth muscle cells: 1) Kv channels, 2) KCa channels, 3) KATP channels, and 4) inward rectifier K⁺ channels (27, 37). In neurons, serum deprivation- and ST-mediated apoptosis is associated with an early enhancement of the delayed rectifier IK(V), which leads to a net loss in cytosolic K⁺ and induces the apoptotic volume decrease. Pharmacological blockade of Kv channels or attenuation of IK(V) with high extracellular K⁺ both inhibit the apoptotic volume decrease and apoptosis (8, 46, 47). In Jurkat T cells, Fas/Fas ligand-mediated apoptosis is accompanied by a cytosolic K⁺ loss due to an increased K⁺ efflux (17). More recently, activation of KCa and Kv channels has been shown to participate in pathways leading to cell apoptosis mediated by tumor necrosis factor (38), ultraviolet radiation (45), and carboxylic cyanide p-trifluoromethoxyphenylhydrazone (28). These observations suggest that all K⁺ channels expressed in the plasma membrane may be involved in the apoptotic volume decrease and apoptosis.

Our study also showed that ST depolarizes ΔΨm, which would trigger the release of cytochrome c and subsequently activate cytosolic caspases, such as caspase-3, and induce apoptosis (2, 3, 6, 17, 29, 30, 44). This ΔΨm-mediated apoptosis appears to be independent of cytosolic K⁺ loss because decreasing the K⁺ driving force by perfusing the cells with a 40 mM K⁺-containing solution did not affect the ST-induced ΔΨm depolarization. It remains to be investigated how ST mediates mitochondrial membrane depolarization in human PASMCs, 2) whether the ST-induced activation of Kv channels interacts with the ST-mediated ΔΨm depolarization in initiating apoptosis, and 3) whether ST induces ΔΨm depolarization by affecting K⁺ channels in the mitochondrial inner membrane. Nevertheless, our observations imply that ST-induced apoptosis may involve multiple cellular pathways in human PASMCs. Recently, it has been found that the inner mitochondrial membrane contains multiple K⁺ channels such as KATP and KCa (11, 24, 36, 41, 42); therefore, it is possible that the ST-induced mitochondrial membrane depolarization is due to activation of the K⁺ channels in the mitochondrial membrane (11).

The function and expression of Kv channels have been demonstrated to be inhibited in PASMCs from patients with primary pulmonary hypertension (48, 50). The decreased activity of the Kv channels is reported to be responsible, at least in part, for pulmonary vasoconstriction and PASMC proliferation (16, 39, 48, 50). As shown in this study, inhibition of Kv channel activity by pharmacological blockade of Kv channels attenuated ST-induced apoptosis in PASMCs. These results direct us to speculate that the inhibited Kv channel function and expression in PASMCs from patients with primary pulmonary hypertension may also contribute to enhance pulmonary vascular medial thickening via inhibition of PASMC apoptosis.

Pulmonary vasoconstriction and vascular smooth muscle proliferation both contribute to the elevated pulmonary vascular resistance and arterial pressure in patients with pulmonary hypertension (16, 40, 43). Durmowicz and Stenmark (15) recently provided evidence that a lack of apoptosis plays a role in the vascular remodeling associated with chronic pulmonary hypertension. Our study demonstrated that ST-mediated activation of Kv channels exerts an antiproliferative effect on human PASMCs by inducing apoptosis. The cellular mechanisms involved in the ST-induced apoptosis include 1) mitochondrial membrane depolarization, 2) activation of cytosolic caspase-3, and 3) activation of Kv channels in the plasma membrane (Fig. 6). By developing drugs specifically targeted to Kv channels located in the pulmonary vasculature, we may find an effective alternative treatment for patients suffering from pulmonary hypertension.

We thank Ying Zhao for technical assistance.

This work was supported by National Heart, Lung and Blood Institute Grants HL-54043 and HL-64945 (to J. X.-J. Yuan).

S. Krick is an Ambassadorsial Scholar of Rotary International. J. X.-J. Yuan is an Established Investigator of the American Heart Association.

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