Activation of K⁺ channels induces apoptosis in vascular smooth muscle cells

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PULMONARY ARTERIES have a trilamellar structure that is composed of fibroblasts (adventitia), smooth muscle cells (media), and endothelial cells (intima). In pulmonary artery smooth muscle cells (PASMC), there is a natural balance between proliferation and apoptosis under normal conditions (8, 46). Augmentation of proliferation and inhibition of apoptosis in PASMC would lead to pulmonary medial thickening, which is an early vascular lesion in patients with primary pulmonary hypertension (8, 43, 46, 55). Therefore, an imbalance between PASMC proliferation and apoptosis may play a critical role in the development of pulmonary vascular remodeling. Inhibition of PASMC growth and augmentation of cell apoptosis could also serve as therapeutic approaches for patients with pulmonary hypertension (8, 37, 46, 57).

Cytoplasmic K⁺ in excitable and nonexcitable cells plays an important role in maintaining intracellular ion homeostasis to control cell volume (4), regulating cell cycle (7, 9), and inhibiting apoptotic enzymes in the cytosol and nucleus. Cytoplasmic K⁺ concentration is mainly regulated by K⁺ uptake via Na⁺-K⁺-ATPase and K⁺ efflux through K⁺ channels in the plasma membrane. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore that dissipates the H⁺ gradient across the inner membrane of mitochondria, induces apoptosis in many cell types. In rat and human pulmonary artery smooth muscle cells (PASMC), FCCP opened the large-conductance, voltage- and Ca²⁺-sensitive K⁺ (maxi-K) channels, increased K⁺ currents through maxi-K channels [I_{K(Ca)}], and induced apoptosis. Tetraethylammonia (1 mM) and iberiotoxin (100 nM) decreased I_{K(Ca)} by blocking the sarcolemmal maxi-K channels and inhibited the FCCP-induced apoptosis in PASMC cultured in media containing serum and growth factors. Furthermore, inhibition of K⁺ efflux by raising extracellular K⁺ concentration from 5 to 40 mM also attenuated PASMC apoptosis induced by FCCP and the K⁺ ionophore valinomycin. These results suggest that FCCP-mediated apoptosis in PASMC is partially due to an increase of maxi-K channel activity. The resultant K⁺ loss through opened maxi-K channels may serve as a trigger for cell shrinkage and caspase activation, which are major characteristics of apoptosis in pulmonary vascular smooth muscle cells.

mitochondrial membrane potential; cytoplasmic calcium; pulmonary artery smooth muscle cells

Krick, Stefanie, Oleksandr Platoshyn, Michele Sweeney, Hyong Kim, and Jason X.-J. Yuan. Activation of K⁺ channels induces apoptosis in vascular smooth muscle cells. Am J Physiol Cell Physiol 280: C970–C979, 2001.—Intracellular K⁺ plays an important role in controlling the cytoplasmic ion homeostasis for maintaining cell volume and inhibiting apoptotic enzymes in the cytosol and nucleus. Cytoplasmic K⁺ concentration is mainly regulated by K⁺ uptake via Na⁺-K⁺-ATPase and K⁺ efflux through K⁺ channels in the plasma membrane. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore that dissipates the H⁺ gradient across the inner membrane of mitochondria, induces apoptosis in many cell types. In rat and human pulmonary artery smooth muscle cells (PASMC), FCCP opened the large-conductance, voltage- and Ca²⁺-sensitive K⁺ (maxi-K) channels, increased K⁺ currents through maxi-K channels [I_{K(Ca)}], and induced apoptosis. Tetraethylammonia (1 mM) and iberiotoxin (100 nM) decreased I_{K(Ca)} by blocking the sarcolemmal maxi-K channels and inhibited the FCCP-induced apoptosis in PASMC cultured in media containing serum and growth factors. Furthermore, inhibition of K⁺ efflux by raising extracellular K⁺ concentration from 5 to 40 mM also attenuated PASMC apoptosis induced by FCCP and the K⁺ ionophore valinomycin. These results suggest that FCCP-mediated apoptosis in PASMC is partially due to an increase of maxi-K channel activity. The resultant K⁺ loss through opened maxi-K channels may serve as a trigger for cell shrinkage and caspase activation, which are major characteristics of apoptosis in pulmonary vascular smooth muscle cells.
potential in mitochondria ($\Delta \Psi_m$), stimulating Ca$^{2+}$ accumulation in mitochondria (19), and causing oxidative ATP synthesis (23). Therefore, FCCP causes an abolition (i.e., depolarization) of $\Delta \Psi_m$, which subsequently mobilizes Ca$^{2+}$ from mitochondria into the cytosol (11, 12). FCCP induces apoptosis in many cell types (10). In this study, we used patch-clamp techniques and digital imaging fluorescence microscopy to test the hypothesis that FCCP-mediated activation of maxi-K channels contributes to induction of apoptosis in human and animal PASMC.

METHODS

Cell preparation. Rat PASMC were prepared from pulmonary arteries of Sprague-Dawley rats (150–200 g) (62, 63). The isolated pulmonary arteries were incubated for 20 min in Hanks' balanced salt solution containing 1.5 mg/ml collagenase (Worthington). Adventitia and endothelium were carefully removed after the incubation. The remaining smooth muscles were then digested with 1.5 mg/ml collagenase and 0.5 mg/ml elastase (Sigma Chemical) at 37°C. The cells were plated onto 25-mm coverslips and incubated in DMEM containing 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO$_2$ in air at 37°C. Human PASMC (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 medium was changed after 24 h and every 48 h thereafter. Smooth muscle growth medium is composed of smooth muscle basal medium, 5% FBS, 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 $\mu$g/ml insulin. Cells were subcultured or plated onto 25-mm coverslips using trypsin-EDTA buffer (Clonetics) when 70–90% confluence was achieved. The cells at passages 4–6 were used for experimentation.

Electrophysiological measurement. Whole cell and single-channel K$^+$ currents ($I_{K}$) were recorded on an Axopatch-1D amplifier and a DigiData 1200 interface (Axon Instruments) using patch-clamp techniques (20, 62). Patch pipettes (2–4 MΩ) were fabricated on a Sutter electrode puller using borosilicate glass tubes and fire-polished on a Narishige microforge. Command voltage protocols and data acquisition were performed using pCLAMP software (Axon Instruments). Currents were filtered at 1–2 kHz (−3 dB) and digitized at 2–4 kHz using the amplifier. In experiments with cell-attached patches, a gigaohm seal was achieved using fire-polished glass electrodes filled with a high-K$^+$ (135 mM) solution. The bath solution was the standard physiological salt solution (PSS) with 4.7 mM KCl. Under these conditions, the actual patch membrane potential was unknown; however, it was assumed that the patch membrane potential is equal to the difference between the pipette command potential and the actual resting membrane potential (which is about −40 mV in the cell preparation used in this study) (62, 63). Thus voltages are expressed as pipette (or applied command) potentials. All experiments were performed at room temperature (22–24°C).

Immunocytochemistry. The cells, grown on 10-mm coverslips, were first washed with PBS (Sigma Chemical) and then fixed in 95% ethanol and stained with the membrane-permeable nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical). DAPI (5 $\mu$M) was dissolved in an antibody buffer containing 500 mM NaCl, 20 $\mu$M NaN$_3$, 10 $\mu$M MgCl$_2$, and 20 $\mu$M Tris-HCl (pH 7.4). The blue fluorescence emitted at 461 nm was used to visualize the cell nuclei. The DAPI-stained cells were examined with a fluorescence microscope (model TE 300, Nikon), and the cell (nuclear) images were acquired using a high-resolution fluorescence imaging system (Solamere).

For each coverslip, 5–10 fields (~20–25 cells/field) were randomly selected to determine the percentage of apoptotic cells in total cells on the basis of the morphological characteristics of apoptosis: cell (nuclear) shrinkage, nuclear condensation, and nuclear breakage. The cells with clearly defined nuclear breakage, remarkably condensed nuclear fluorescence, and significantly shrunken cell body and nucleus were defined as apoptotic cells. The relative cross-sectional nuclear area of the DAPI-stained cells (on the basis of the area of pixels) was measured using the NIH Imaging software. To quantify apoptosis, TdT-mediated dUTP nick end labeling assays were also performed with the In Situ Cell Death Detection Kit (TMR Red, Boehringer Mannheim); the nuclear morphology was examined by labeling with DAPI.

Measurement of rhodamine fluorescence. The cells, grown on 25-mm coverslips, were loaded with rhodamine 123 (R123, Molecular Probes) by incubation with 10 $\mu$g/ml for 30 min at 37°C (11, 12). R123 is taken up selectively by mitochondria (29, 30), and its uptake is dependent on $\Delta \Psi_m$. R123 fluorescence was excited at 488 nm and measured at 530 nm using a GEN IV charge-coupled device camera connected to a microscope (model TE 300, Nikon). In isolated mitochondria, the relationship between R123 fluorescence and $\Delta \Psi_m$ is linear (13). The R123 fluorescence, which is quenched at resting $\Delta \Psi_m$, increases with mitochondrial membrane depolarization (11, 12). The R123 fluorescence signals were stored in a Macintosh computer and analyzed using QVD software (Solamere). The percent change of the R123 fluorescence from the baseline level is used for comparison between responses.

Measurement of [Ca$^{2+}$]$_i$. The cells were loaded with fura 2-AM (3 $\mu$M) for 30 min at 24°C under an atmosphere of 5% CO$_2$/95% air. The fura 2-loaded cells were then superfused with PSS for 20 min at 32°C to wash away extracellular fura 2-AM and to allow sufficient time for intracellular esterases to cleave cytosolic fura 2-AM into the active fura 2. Fura 2 fluorescence (510-nm emission, 360- and 380-nm excitation) from the cells and background was measured using a charge-coupled device camera connected to a Nikon microscope. The fluorescence signals were collected continuously and stored in an IBM-compatible computer for later analysis. The 360- to 380-nm excitation ratio of the fluorescent images were then calculated and calibrated to express [Ca$^{2+}$]$_i$. (18, 62).

Reagents and solutions. For measuring whole cell $I_{K}$ and [Ca$^{2+}$]$_i$, a coverslip containing the cells was positioned in a recording chamber (~0.75 ml) and superfused (2–3 ml/min) with the standard extracellular (bath) PSS. The PSS contained (in mM) 141 NaCl, 4.7 KCl, 1.8 CaCl$_2$, 1.2 MgCl$_2$, 10 HEPES, and 10 glucose (pH 7.4). In Ca$^{2+}$-free PSS, CaCl$_2$ was replaced by equimolar MgCl$_2$ and 1 mM EGTA was added to chelate residual Ca$^{2+}$. The pipette (internal) solution for recording whole cell $I_{K}$ contained (in mM) 135 KCl, 4 MgCl$_2$, 10 HEPES, 10 EGTA, and 5 Na$_2$ATP (pH 7.2). For single-channel $I_{K}$ recording in cell-attached patches, the pipette (external) solution contained (in mM) 135 KCl, 4 MgCl$_2$, 10 HEPES, and 10 EGTA (pH 7.4).

FCCP (Sigma Chemical) and valinomycin (Sigma Chemical) were prepared as 20 mM stock solutions in DMSO. Aliquots of the stock solutions were diluted 1:1,000–4,000 into PSS (for electrophysiological and fluorescent experiments) or 10% FBS-DMEM (for immunocytochemical experiments). Similar dilutions of DMSO (0.017–0.05%), alone, were used as vehicle control in PSS or the culture media. Tetraethylammonium (TEA; Sigma Chemical) and iberiotoxin (IBTX; Sigma Chemical) were directly dissolved into
PSS or culture media on the day of use. The pH values of all solutions were checked after addition of the drugs and readjusted to 7.4. In high-K⁺ (25 or 40 mM) solution or culture medium, NaCl in PSS and in the customized DMEM (Medi- aTech) was replaced, mole-for-mole, by KCl to maintain the solution’s osmolarity.

Statistics. The composite data are expressed as means ± SE. Statistical analysis was performed using paired or unpaired Student’s t-test or ANOVA and post hoc tests (Student Newman-Keuls) where appropriate. Differences were considered to be significant when P < 0.05.

RESULTS

Effects of FCCP on R123 fluorescence in PASMC.ΔΨₘ is primarily generated by a proton gradient across the mitochondrial inner membrane (3, 19, 23). Changes in ΔΨₘ were determined in human PASMC loaded with R123 (11, 12); mitochondrial depolarization increases R123 fluorescence. In human PASMC, FCCP significantly increased R123 fluorescence (i.e., depolarized ΔΨₘ, Fig. 1A). Increasing extracellular K⁺ concentration from 5 to 25 mM, which decreases the driving force for K⁺ efflux, and extracellular application of 1 mM TEA or 100 nM IBTX, which blocks maxi-K channels, negligibly affected the R123 fluorescence (Fig. 1). Furthermore, pretreatment of the cells with 25 mM K⁺, 1 mM TEA, or 100 nM IBTX had little effect on the FCCP-induced increases in R123 fluorescence (Fig. 1B). These results indicate that inhibition of K⁺ efflux across the plasma membrane, as a result of reduced K⁺ driving force or blocked maxi-K channels, does not interfere with the depolarizing effect of FCCP on ΔΨₘ in PASMC.

Maintaining a negative ΔΨₘ induces Ca²⁺ accumulation in mitochondria (11, 12, 19). The FCCP-induced ΔΨₘ depolarization in PASMC (Fig. 1) would therefore mobilize Ca²⁺ from mitochondria to the cytosol. Indeed, extracellular application of FCCP reversibly increased [Ca²⁺]ᵢ in rat PASMC in the presence (by 181 ± 14 nM, n = 28) or absence (by 134 ± 12 nM, n = 25) of extracellular Ca²⁺. Pretreatment of the cells with 10 μM cyclopiazonic acid (CPA) did not abolish the FCCP-induced rise in [Ca²⁺]ᵢ in human PASMC bathed in Ca²⁺-free solution, suggesting that FCCP releases Ca²⁺ from mitochondria (data not shown).

FCCP increases the large-conductance Iₖ(Ca) in rat PASMC. A large-amplitude single-channel Iₖ was observed in cell-attached membrane patches (with symmetrical K⁺ gradient) of rat PASMC during sustained depolarization to positive potentials (Fig. 2A). Slope conductance of the channels responsible for the current, determined by current-voltage relationships obtained from 12 cells, ranged from 200 to 225 pS (218 ± 8 pS). This is consistent with the slope conductance (200–250 pS) of the large-conductance maxi-K channels that have been identified and characterized in vascular smooth muscle cells (5, 41, 42, 44, 53). Thus this large-amplitude Iₖ in rat PASMC was actually Iₖ(Ca), resulting from K⁺ efflux through the large-conductance maxi-K channels.

Fig. 1. Effects of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), 25 mM K⁺, tetraethylammonia (TEA), and iberiotoxin (IBTX) on mitochondrial membrane potential (ΔΨₘ) in pulmonary artery smooth muscle cells (PASMC). A, top: pseudocolor images showing rhodamine 123 (R123) fluorescence, used to estimate the mitochondrial membrane potential (ΔΨₘ). A, bottom: magnified images showing the peripheral area of the cell before (a) and after (b) treatment with FCCP. B, bottom: representative R123 fluorescence recorded in PASMC before and during application of 5 μM FCCP. Magnified images show the peripheral area of the cell before (a) and (b) treatment with FCCP. A, bottom: representative R123 fluorescence recorded in PASMC before and during application of FCCP, 25 mM K⁺ (25K), 1 mM TEA, and 100 nM IBTX. B: summarized data showing FCCP-induced relative changes of R123 fluorescence in the absence (control) and presence of 25 mM K⁺, TEA, or IBTX in rat and human PASMC. Values are means ± SE, with number of cells in parentheses.

Extracellular application of 5 μM FCCP for 1–2 min significantly increased single-channel Iₖ(Ca); the steady-state open probability (Nₚₒ) was increased ninefold (from 0.06798 to 0.71057; Fig. 2B). This augmentation was negligibly influenced by pretreatment of the cells with the ATP-sensitive K⁺ channel blocker glibenclamide (6, 42); Nₚₒ was increased 17-fold (from 0.00685 to 0.12665) in the presence of 5 μM glibenclamide (Fig. 3A). In PASMC, extracellular application
of FCCP reversibly induced membrane depolarization (from $-46 \pm 1.6$ to $-29.4 \pm 2.2$ mV). Therefore, the FCCP-induced activation of maxi-K channels was likely due to the synergistic effects of 1) a rise in $\left[\text{Ca}^{2+}\right]_c$ due to $\text{Ca}^{2+}$ release from mitochondria and 2) a plasma membrane depolarization. This is why the current amplitudes shown in Fig. 2 were increased during FCCP application.

In the excised (outside-out) membrane patches, however, extracellular application of 5 μM FCCP had a negligible effect on single-channel $I_{K_{\text{Ca}}}$. $N_{P_o}$ was slightly increased 0.1-fold (from 0.28367 to 0.30083; Fig. 3B). These results suggest that the FCCP-induced increase in $I_K$ resulted primarily from activation of maxi-K channels as a result of $\text{Ca}^{2+}$ release from mitochondria, rather than from activation of ATP-sensitive $K^+$ channels as a result of inhibited ATP synthesis (6, 42).

**Fig. 2.** Single-channel $\text{Ca}^{2+}$-sensitive $K^+$ current [$I_{K(\text{Ca})}$] in cell-attached membrane patches of rat PASMC in symmetrical $K^+$ gradient (A) and effect of FCCP on $I_{K(\text{Ca})}$ (B). Representative current traces were recorded from a PASMC membrane patch at different membrane potentials of $+50$ to $+120$ mV (left). Composite current-voltage relationship ($I-V$ curve) indicates that the slope conductance of this channel is $218 \pm 8$ pS ($n = 8$; right). B: unitary current recordings (top) and all-points amplitude histograms (bottom) from a cell-attached membrane patch of PASMC before (control), during (FCCP), and after (washout) 3-min application of 5 μM FCCP. The patch membrane potential was held at $+70$ mV. Horizontal lines denote the current level when the channel is closed. $N_{P_o}$, open channel probability.

**Fig. 3.** Effects of FCCP on single-channel $I_{K(\text{Ca})}$ in a cell-attached patch of PASMC pretreated with glibenclamide (A) and in an outside-out patch of PASMC (B). A: single-channel recording (top) and amplitude histograms (bottom) from a cell-attached membrane patch before (control), during (FCCP), and after (washout) 3-min application of 5 μM FCCP in the presence of 4 μM glibenclamide. Patch membrane potential was held at $+70$ mV. B: single-channel recording (top) and amplitude histograms (bottom) from an outside-out membrane patch before (control) and during (FCCP) application (for 2 min) of 5 μM FCCP. Patch membrane potential was held at $+60$ mV.
FCCP increases whole cell $I_{\text{K(Ca)}}$ in human PASMC. Extracellular application of IBTX (100 nM) and TEA (1 mM), blockers of maxi-K channels (2, 42), significantly decreased whole cell $I_K$ in human PASMC (Fig. 4A, a and b). Consistent with the single-channel results in rat PASMC (Figs. 2 and 3A), application of 5 μM FCCP reversibly increased whole cell $I_K$ in human PASMC (Fig. 4Ac). The IBTX-sensitive, TEA-sensitive, and FCCP-activated components of whole cell $I_K$ were activated at approximately ~40 mV (Fig. 4B) and show marked outward rectification at potentials more positive than +40 mV (Fig. 4C). The kinetics of the IBTX-sensitive, TEA-sensitive, and FCCP-activated components of whole cell $I_K$ are very similar to those of the noisy $I_{\text{K(Ca)}}$ observed in vascular smooth muscle cells (2, 17). Furthermore, FCCP rapidly decreased membrane input resistance at a holding potential of 0 mV; only K$^+$ channels were active under these conditions (the calculated equilibrium potentials for K$^+$, Cl$^-$, Na$^+$, and Ca$^{2+}$ were −84, −1.5, +66, and +122 mV, respectively).

Inhibitory effect of IBTX on FCCP-induced increase in $I_{\text{K(Ca)}}$ in human PASMC. In the absence of IBTX in the pipette solution, extracellular application of 5 μM FCCP significantly increased the activity of maxi-K channels in cell-attached membrane patches; $NP_\text{o}$ was increased 67-fold (from 0.00234 to 0.15999; Fig. 5A). Inclusion of 100 nM IBTX in the pipette solution significantly decreased the activity of maxi-K channels; averaged $NP_\text{o}$ values were 0.0257 ± 0.0212 and 0.00044 ± 0.00006 in the absence and presence of IBTX, respectively. Furthermore, the FCCP-induced increase in single-channel $I_{\text{K(Ca)}}$ was almost abolished when 100 nM IBTX was included in the pipette solution (Fig. 5B). These results suggest that the FCCP-induced increase in $I_K$ was mainly due to activation of the IBTX-sensitive maxi-K channels.

Inhibitory effect of 40 mM K$^+$ or TEA on FCCP-induced apoptosis in PASMC. Treatment of rat or human PASMC with FCCP (5–15 μM for 20 h) induced cell (nuclear) shrinkage, nuclear condensation, nuclear breakage, and apoptotic bodies in 15–40% of the cells, while <3% of the untreated control cells showed these apoptotic characteristics (Fig. 6, A and B). Increasing extracellular [K$^+$] from 5 to 40 mM, which attenuates $I_K$ by reducing the K$^+$ driving force, decreased the FCCP-induced apoptosis by ~30% in rat PASMC (from 32 ± 5 to 22 ± 5%, $P < 0.001$) and ~47% in human PASMC (from 38 ± 6 to 18 ± 5%, $P < 0.001$; Fig. 6C). Furthermore, treatment of the cells with 1 mM TEA or 100 nM IBTX, which blocks maxi-K channels, also significantly inhibited the FCCP-induced apoptosis in rat and human PASMC (Fig. 6C).

In the absence of TEA, treatment of the cells with FCCP decreased the cross-sectional area of nuclei by ~57% in rat PASMC ($n = 61$, $P < 0.001$) and by ~50% in human PASMC ($n = 42$, $P < 0.001$). In the presence of 1 mM TEA, however, FCCP only decreased the nuclear areas by ~28% in rat PASMC and ~25% in human PASMC, indicating that TEA significantly inhibited the FCCP-mediated nuclear shrinkage. The
inhibitory effects of 40 mM K\textsuperscript{+}, TEA, and IBTX on the FCCP-induced apoptosis in PASMC were also observed using TdT-mediated dUTP nick end label staining (data not shown).

**Inhibitory effect of 40 mM K\textsuperscript{+} on valinomycin-induced apoptosis.** The transmembrane K\textsuperscript{+} efflux is determined by the K\textsuperscript{+} electrochemical gradient (driving force) and the K\textsuperscript{+} permeability. Valinomycin is a K\textsuperscript{+} ionophore that increases K\textsuperscript{+} efflux and induces apoptosis in variety of cell types (16, 25), including rat and human PASMC (Fig. 7A). Increasing extracellular K\textsuperscript{+} from 5 to 40 mM, which decreases the K\textsuperscript{+} electrochemical gradient, significantly inhibited the valinomycin-induced apoptosis in rat (from 88 to 63%, \(P < 0.001\)) and human (from 81 to 63%, \(P < 0.001\)) PASMC (Fig. 7B). These results suggest that FCCP-mediated apoptosis in PASMC is inhibited by 40 mM K\textsuperscript{+}, TEA, and IBTX on the FCCP-induced apoptosis in PASMC were also observed using TdT-mediated dUTP nick end label staining (data not shown).

**DISCUSSION**

FCCP induced apoptosis with characteristic cell shrinkage, nuclear condensation, and breakage in PASMC. In rat and human PASMC, FCCP depolarized \(\Delta \Psi_m\), mobilized Ca\textsuperscript{2+} from the mitochondria to the cytosol, activated maxi-K channels, increased \(I_{K(Ca)}\), and induced apoptosis. Blockade of the maxi-K channels by IBTX and TEA or decrease of K\textsuperscript{+} efflux by reducing the K\textsuperscript{+} driving force significantly inhibited the FCCP-induced PASMC apoptosis. These results suggest that FCCP-mediated apoptosis in PASMC is

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**Fig. 5.** Inhibitory effect of IBTX on FCCP-induced increase in single-channel \(I_{K(Ca)}\) in human PASMC. A and B: unitary current recordings (top) and amplitude histograms (bottom) from cell-attached membrane patches before (control), during (FCCP), and after (washout) application of 5 \(\mu M\) FCCP in the absence (-IBTX, A) and presence (+IBTX, B) of 100 nM IBTX in the pipette (extracellular) solution. Patch membrane potential was held at +70 mV. Horizontal lines denote the current level when the channel is closed.

**Fig. 6.** Inhibitory effects of 40 mM K\textsuperscript{+}, IBTX, and TEA on FCCP-induced apoptosis in PASMC. A: 4’,6’-diamidino-2-phenylindole (DAPI)-stained nuclei of rat (a) and human (b) PASMC cultured in media with and without 15 \(\mu M\) FCCP for 20 h. B: dose-response curves (means \(\pm SE\)) showing the percentage of apoptotic cells before and after treatment with 5, 10, and 15 \(\mu M\) of FCCP (for 20 h). C: summarized data showing FCCP-induced apoptosis in rat (left) and human (right) PASMC treated with 40 mM K\textsuperscript{+} (40K), 100 nM IBTX, and 1 mM TEA, respectively. Values are means \(\pm SE\), with number of experiments in parentheses. ***\(P < 0.001\) (Student-Newman-Keuls test) vs. FCCP.
partially due to activation of maxi-K channels in the plasma membrane. The resultant K\(^+\) loss through opened K\(^+\) channels may be a trigger for apoptosis in pulmonary vascular smooth muscle cells (Fig. 8).

**Involvement of K\(^+\) efflux through sarcolemmal K\(^+\) channels in apoptosis.** K\(^+\) is the predominant cation in the cytosol. Maintenance of a high [K\(^+\)] in the cytoplasm (140–150 mM) is essential for governing cell excitability (42), 2) setting resting \(E_m\) (42), 3) regulating apoptotic enzyme activity (24), and 4) controlling cell volume (4). Cytoplasmic K\(^+\) at normal concentration (~140 mM) decreases apoptotic DNA fragmentation and caspase-3-like protease activation (24). Decrease in [K\(^+\)]\(_c\), due to elevated K\(^+\) efflux through opened K\(^+\) channels, results in cell shrinkage (4, 16, 25) and reduces the inhibitory effect of cytoplasmic K\(^+\) on caspase-3-like protease and the internucleosomal DNA cleavage nuclease (24). Caspases and nucleases are major inducers of apoptosis (52). Therefore, an increase in K\(^+\) efflux, partially due to activated sarcolemmal K\(^+\) channels, is necessary for the initiation of apoptosis (24, 60, 61). The observations in PASMC from the present study are consistent with the results observed in neurons and lymphocytes (24, 60, 61): blockade of K\(^+\) channels by TEA or decreasing the K\(^+\) driving force by raising extracellular K\(^+\) significantly attenuated the apoptosis.

It is unknown whether the apoptosis induced by increasing K\(^+\) channel activity depends on the time course of K\(^+\) efflux. A transient (or short-term) increase in K\(^+\) efflux or cytosolic K\(^+\) loss would relieve its tonic suppression on caspase activity and thus trigger the caspase-mediated apoptosis (e.g., in the presence of apoptosis inducers). Because apoptosis is an irreversible process, apoptosis may occur any time when K\(^+\) efflux is increased.

In in vivo experiments, PASMC apoptosis has been observed in hypertrophied pulmonary arteries (8). Apoptosis can take place in different cell cycle phases; therefore, increasing K\(^+\) efflux should be able to work on an already modified pulmonary vascular wall. However, whether apoptosis induced by increased K\(^+\) efflux only occurs in the cells that contribute to hypertrophy, but not in the normally controlled cells, is unknown. Further study is needed to define whether apoptosis induced by increasing K\(^+\) efflux depends on cell phenotype.

**Activation of maxi-K channels by FCCP-induced Ca\(^{2+}\) release.** In vascular smooth muscle cells including PASMC, the large-conductance maxi-K channels are regulated by cytoplasmic Ca\(^{2+}\) and \(E_m\) (5, 42, 53). A localized rise in [Ca\(^{2+}\)]\(_c\), due to metabolic inhibition-mediated Ca\(^{2+}\) mobilization from mitochondria and
the S/ER, activates maxi-K channels and increases $I_{K(Ca)}$ (39, 41, 63). FCCP is a proton ionophore that 1) depolarizes $\Delta \Psi_m$ by dissipating the $H^+$ gradient across the inner membrane of mitochondria (11, 12, 19, 2) releases $Ca^{2+}$ from the mitochondria into the cytosol (11, 12, 19, 36, 64), and 3) inhibits ATP production by uncoupling oxidative phosphorylation (23). There are numerous close contacts between the mitochondria and S/ER (47), suggesting that these two organelles may coordinate with each other in releasing $Ca^{2+}$ to or sequestering $Ca^{2+}$ from the cytosol.

In the presence of other uncouplers (e.g., antimycin, rotenone, and cyanide) and mitochondrial ATPase inhibitors (e.g., oligomycin), FCCP is still able to increase $[Ca^{2+}]_c$ in the absence of extracellular $Ca^{2+}$ (11, 12, 28). In PASMC, pretreatment of the cells with thapsigargin or CPA attenuated, but did not abolish, the FCCP-induced increase in $[Ca^{2+}]_c$ in the absence of extracellular $Ca^{2+}$ (11, 12, 15, 63). These results suggest that FCCP releases $Ca^{2+}$ from multiple intracellular stores (e.g., mitochondria and S/ER) (28, 34, 63). $Ca^{2+}$ sparks, caused by the coordinated opening of $Ca^{2+}$ release channels in the S/ER, activate maxi-K channels and increase whole cell $I_{K(Ca)}$ (27, 41). Taken together, these results suggest that maxi-K channels can be efficiently opened by $Ca^{2+}$ release from intracellular organelles in vascular smooth muscle cells.

We previously reported that preincubation of PASMC with 1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid (BAPTA)-AM (20 $\mu$M for 60 min) completely abolishes the FCCP-induced increases in $[Ca^{2+}]_c$ and whole cell $I_{K(Ca)}$ in PASMC (63). BAPTA-AM is a membrane-permeable $Ca^{2+}$ chelator that enters not only the cytosol but also intracellular stores. Thus, after hydrolysis of the acetoxymethyl ester by intracellular esterases, BAPTA is able to buffer $Ca^{2+}$ in cytosol and stores, including mitochondria and sarcoplasmic reticulum. As shown in Fig. 3B, FCCP negligibly affected single-channel $I_{K(Ca)}$ in outside-out patches. These observations suggest that FCCP is not a direct activator of the maxi-K channel protein.

TEA is a nonselective blocker of $K^+$ channels in vascular smooth muscle cells. Therefore, the TEA-mediated inhibitory effect on the FCCP-induced apoptosis is potentially due to its blockade of maxi-K and Kv channels in rat and human PASMC. Indeed, our preliminary observations (data not shown) and those from other investigators (60, 61) demonstrated that activation of the TEA-sensitive $K_V$ channels also contributes to induce apoptosis in PASMC and neurons.

Other possible mechanisms involved in apoptosis mediated by FCCP or $K^+$ channel activation. Mitochondrial intermembrane space contains several proteins that are liberated through the outer membrane to participate in initiation of apoptosis (35, 50). Release of cytochrome $c$ to the cytosol (31, 59) and translocation of the apoptosis-inducing factor to the nucleus (50) initiate the apoptotic cascade. A direct relationship between $\Delta \Psi_m$ depolarization and the release of cytochrome $c$ (and apoptosis-inducing factors) has been demonstrated to play an important role in apoptosis (10, 22, 54). However, whether $\Delta \Psi_m$ depolarization is required for apoptosis is still unclear (14, 32).

Mitochondrial $K^+$ channels, which are regulated by ATP, have been identified in the inner membrane of mitochondria (26, 48). The $K^+$ electrochemical gradient across the mitochondrial inner membrane favors $K^+$ flux from the mitochondrial intermembrane space into the mitochondrial matrix. Activation of mitochondrial $K^+$ channels would thus lead to matrix swelling, outer membrane breakdown, release of cytochrome $c$, and loss of mitochondrial function (3). Many $K^+$-permeable channels have been described in the inner membrane of mitochondria (1, 40), but whether maxi-K (and Kv) channels are also distributed in the mitochondrial inner membrane and participate in the regulation of cytochrome $c$ release and mitochondrial function is unknown.

Maintaining sufficient $Ca^{2+}$ in the S/ER (21, 33) and mitochondria (64) has been demonstrated to be essential for cell survival. Indeed, depletion of $Ca^{2+}$ from the S/ER and mitochondria results in growth arrest (49) and induces apoptosis (21, 64). In addition, Bcl-2 represses apoptosis by regulating the S/ER $Ca^{2+}$ (33). Therefore, in addition to activation of maxi-K channels, the FCCP-induced apoptosis may also be due to 1) direct release of $Ca^{2+}$ from the S/ER and mitochondria, 2) inhibition of oxidative ATP production (23), and 3) dephosphorylation of BAD induced by $Ca^{2+}$-activated calcineurin (58).

Summary and conclusion. The results from this study suggest that FCCP-induced apoptosis in rat and human PASMC is partially due to activation of maxi-K channels in the plasma membrane. FCCP depolarizes $\Delta \Psi_m$ and releases $Ca^{2+}$ from mitochondria to the cytosol. The local rise in $[Ca^{2+}]_c$ activates maxi-K channels and increases $I_{K(Ca)}$. The resultant $K^+$ loss due to elevated $K^+$ efflux may play an important role in the onset of apoptosis in pulmonary vascular smooth muscle cells (Fig. 8). Activation of maxi-K channels by $Ca^{2+}$ sparks due to $Ca^{2+}$ release from intracellular organelles also triggers vasodilation. Thus development of drugs directed at activation of K$^+$ channels in PASMC would be potentially a useful therapeutic approach for treatment of pulmonary hypertension that is characterized by sustained vasoconstriction and excessive vascular medial hypertrophy.

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