Apoptosis repressor with caspase domain inhibits cardiomyocyte apoptosis by reducing K⁺ currents

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Apoptosis repressor with caspase domain inhibits cardiomyocyte apoptosis by reducing K⁺ currents. Am J Physiol Cell Physiol 284: C1405–C1410, 2003. First published January 29, 2003; 10.1152/ajpcell.00279.2002.—Cell shrinkage is an early prerequisite in programmed cell death, and cytoplasmic K⁺ is a dominant cation that controls intracellular ion homeostasis and cell volume. Blockade of K⁺ channels inhibits apoptotic cell shrinkage and attenuates apoptosis. We examined whether apoptotic repressor with caspase recruitment domain (ARC), an antiapoptotic protein, inhibits cardiomyocyte apoptosis by reducing K⁺ efflux through voltage-gated K⁺ (Kv) channels. In heart-derived H9c2 cells, whole cell Kv currents (I_{K(V)}) were isolated by using Ca²⁺-free extracellular (bath) solution and including 5 mM ATP and 10 mM EGTA in the intracellular (pipette) solution. Extracellular application of 5 mM 4-aminopyridine (4-AP), a blocker of Kv channels, reversibly reduced I_{K(V)} by 50–60% in H9c2 cells. The remaining currents during 4-AP treatment may be generated by K⁺ efflux through 4-AP-insensitive K⁺ channels. Overexpression of ARC in heart-derived H9c2 cells significantly decreased I_{K(V)}, whereas treatment with staurosporine, a potent apoptosis inducer, enhanced I_{K(V)} in wild-type cells. The staurosporine-induced increase in I_{K(V)} was significantly suppressed and the staurosporine-mediated apoptosis was markedly inhibited in cells overexpressing ARC compared with cells transfected with the control neomycin vector. These results suggest that the antiapoptotic effect of ARC is, in part, due to inhibition of Kv channels in cardiomyocytes.

Apoptotic volume decrease; potassium channels; cardiac myocytes

IN THE CARDIOVASCULAR SYSTEM, apoptosis plays a critical role not only in the physiological processes of homeostasis and development but also in the pathogenesis of certain diseases such as heart failure (9). Apoptosis is regulated via protein-protein interactions that link effector signals to activation of cellular proteases. One of the interaction motifs involves the caspase recruitment domain (CARD). Apoptosis repressor with caspase recruitment domain (ARC) is a cardiac and skeletal muscle CARD-containing protein that binds to the initiator caspase-2/8 and attenuates death receptor-induced apoptosis (11, 17). Overexpression of ARC in cardiomyocytes has been demonstrated to inhibit hypoxia/ischemia-induced apoptosis by blocking cytochrome c release (7) and to prevent oxidant stress-mediated cell death by preserving mitochondrial integrity and function (14).

A critical feature of apoptosis is early cell shrinkage (2, 3, 13, 18, 19). Cell volume is primarily controlled by intracellular ion homeostasis, and cytoplasmic K⁺ is a dominant cation that contributes to the regulation of cell volume in many cell types (12). Apoptotic cell shrinkage has been demonstrated to result, at least in part, from perturbations in the normally high intracellular K⁺ concentration ([K⁺]; ~150 mM) as a result of increased K⁺ efflux or loss through sarcolemmal K⁺ channels. A high concentration of cytosolic K⁺ is not only required for maintaining cytoplasmic ion homeostasis but necessary to preserve normal cell volume and also critical for suppressing the activity of caspases and nucleases (3, 13). Indeed, blockade of sarcolemmal K⁺ channels inhibits the apoptotic volume decrease and attenuates apoptosis in many cell types (1–6, 8, 13, 15, 18–20). This study was therefore designed to test the hypothesis that ARC suppresses apoptosis in heart-derived H9c2 cells, in part by inhibiting K⁺ channels in the plasma membrane.

METHODS AND MATERIALS

Cell culture and DNA transfection. The embryonic rat heart-derived myogenic cell line H9c2 (7) was obtained from the American Type Culture Collection. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Subconfluent H9c2 cells were transfected with 10 μg of pcDNA3-Neo or pcDNA3-ARC-Flag with lipofectamine. After 48 h, the transfected cells were replated in 10% FBS-DMEM supplemented with G418 sulfate (500 μg/ml). Colonies derived from single cells were picked and expanded as stably transfected cell lines. The control and ARC-transfected H9c2 cells were plated on 25-mm coverslips in 10% FBS-DMEM and cultured in a 37°C, 5% CO₂ humidified incubator for 3–5 days before experimentation. Transfection of two clones of ARC, termed ARC-5 and ARC-24, was determined by Western blot analysis with an

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antibody against a glutathione S-transferase (GST)-ARC fusion protein (~33 kDa) and an anti-Flag antibody for epitope-flagged ARC (Flag-ARC) (7).

Electrophysiological measurements. Whole cell K+ currents (I_K) were recorded with an Axopatch-1D amplifier and a DigiData 1200 interface (Axon Instruments) by patch clamp techniques (8). Patch pipettes (2–4 MΩ) were fabricated on a Sutter electrode puller with borosilicate glass tubes and were fire polished on a Narishige microforge. The average resistance of the electrodes used for recording whole cell I_K was 3.6 ± 0.1 and 3.7 ± 0.1 MΩ in control (Neo) cells and ARC-transfected cells, respectively. Step-pulse protocols and data acquisition were performed with pCLAMP software. Currents were filtered at 1–2 kHz (~3 dB) and digitized at 2–4 kHz with an Axopatch-1D amplifier. Membrane capacitance (C_m) and series resistance (R_s) were compensated with the patch-clamp amplifier.

The bath solution for recording whole cell I_K, contained (in mM) 141 NaCl, 4.7 KCl, 3.0 MgCl_2, 1 EGTA, 10 HEPES, and 10 glucose (pH 7.4). The internal (pipette) solution for recording I_K, contained (in mM) 125 KCl, 4 MgCl_2, 10 HEPES, 10 EGTA, and 5 Na_2ATP (pH 7.2). 4-Aminopyridine (4-AP; Sigma) was directly dissolved in the bath solutions on the day of use. The pH value of the solution was checked after addition of the drug and readjusted to 7.4. Staurosporine was prepared as a 1 mM stock solution in DMSO, which was then diluted in the culture media or bath solutions to the desired concentration. In I_K measurement experiments, the same amount of DMSO used for dissolving staurosporine was added to control solutions (<0.03%). Vehicle (DMSO) alone had negligible effects on I_K in H9c2 cells. All experiments were performed at room temperature (22–24°C).

Measurement of cell death. Apoptosis was detected and quantified with 4',6-diamidino-2-phenylindole (DAPI; Sigma) staining and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay. The cells, grown on 10-mm coverslips, were first washed with PBS (Sigma) and then fixed in 95% ethanol and stained with the membrane-permeant nucleic acid stain DAPI. DAPI (5 μM) was dissolved in an antibody buffer containing 500 mM NaCl, 20 μM Na_3[V], 10 μM MgCl_2, and 20 μM 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 Nikon TE 300 fluorescence microscope, and the cell nuclear images were acquired with a high-resolution Solamare fluorescent imaging system. For each coverslip, 5–10 fields (with ~20–25 cells in each field) were randomly selected to determine the percentage of apoptotic cells in total cells on the basis of the morphological characteristics of apoptosis. Cells with clearly defined nuclear breakage, remarkably condensed nuclear fluorescence, and significantly shrunken cell body and nucleus were defined as apoptotic cells. To quantify apoptosis, TUNEL assays were also performed with the In Situ Cell Death Detection Kit (TMR Red; Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s protocol.

Western blot analysis. Cells were washed in cold PBS, scraped in 0.3 ml of lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, and 30 μg/ml aprotinin), and incubated on ice for 30 min. The cell lysates were sonicated and centrifuged at 12,000 rpm for 10 min, and the insoluble fraction was discarded. The protein samples separated on 12% SDS-PAGE were then transferred onto nitrocellulose membranes by electroblotting as previously described (7, 8). Expression of ARC and epitope-tagged ARC (ARC-Flag) in the transfected stable clones was analyzed by using monoclonal antibodies against ARC and Flag (7).

Statistical analysis. The composite data are expressed as means ± SE. Statistical analysis was performed with the 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

Electrophysiological and pharmacological properties of Kv currents in H9c2 cells. Whether Kv channels are functionally expressed in H9c2 cells was first examined with a whole cell configuration of patch clamp techniques. Depolarizing the cells from a holding potential of ~70 mV to a series of test potentials ranging from −40 to +80 mV in 20-mV increments elicited outward K+ currents. Extracellular application of 5 mM 4-AP, a relative selective blocker of Kv channels, significantly decreased the currents (Fig. 1, A and B). The inhibitory effect of 4-AP was reversible, because when the drug was washed out the currents returned to the control level (Fig. 1, A and B). The activation threshold of the 4-AP-sensitive Kv channels was more negative than ~−45 mV as shown by the current-voltage (I-V) relationship curve (Fig. 1C). As shown by the time course in Fig. 1D, the half-inhibition of 4-AP (5 mM) took place ~30–40 s after treatment. The kinetic analysis of the Kv currents indicated that the current was activated rapidly and inactivated very slowly in H9c2 cells. The time constants for activation (τ_act) and inactivation (τ_inact) of the 4-AP-insensitive Kv currents were much greater than those of the 4-AP-sensitive Kv currents (Fig. 1, E and F), suggesting that the 4-AP-sensitive Kv channels activate and inactivate faster than the 4-AP-insensitive Kv channels. These results indicate that H9c2 cells functionally express multiple Kv channels with different biophysical (e.g., activation and inactivation kinetics) and pharmacological (e.g., sensitivity to 4-AP) properties.

Overexpression of ARC decreases whole cell I_K(V). In H9c2 cells stably transfected with ARC-5 and ARC-24, the protein levels of ARC-5 and ARC-24, determined with a monoclonal antibody specifically against ARC, were ~20 times greater than those in Neo cells (Fig. 2A). Expression of epitope-tagged ARC (Flag-ARC) in H9c2 cells stably transfected with ARC-5 and ARC-24 was determined with a monoclonal anti-Flag antibody as a control. These results indicate that the protein level of ARC in the ARC-transfected cells was significantly greater than that in control (Neo) cells.

Overexpression of ARC significantly decreased the current density of whole cell I_K(V) compared with the Neo cells (Fig. 2B). At ~40 mV, the average current density was 2.5 pA/pF in Neo cells (n = 58), 0.8 pA/pF (P < 0.001) in cells expressing ARC-5 (n = 41), and 0.9 pA/pF in cells expressing ARC-24 (n = 17). At +80 mV, the average current density was 162.6, 66.5 (~59% decrease, P < 0.001), and 72.4 (~55% decrease, P < 0.001) pA/pF in Neo cells and cells transfected with ARC-5 and ARC-24, respectively (Fig. 2C). C_m was comparable in Neo cells (22 ± 1 pF; n = 90) and...
ARC-transfected cells (23 ± 1 pF; n = 84), suggesting that the ARC-induced reduction of $I_{KV}$ is not caused by changes of cell size or cell surface area. In addition to decreasing the amplitude and current density of $I_{KV}$ (Fig. 2C, a and b), overexpression of ARC-24 also significantly increased the $\tau_{act}$ for $I_{KV}$ at +80 mV (Fig. 2Cc). These results suggest that ARC protein may, directly or indirectly, interfere with Kv channel proteins to decrease the function of the channels.

Overexpression of ARC inhibits staurosporine-induced increase in $I_{KV}$. In control H9c2 (Neo) cells, treatment with staurosporine (0.02 μM for 30 min), a potent apoptosis inducer, significantly increased the current amplitude and density of $I_{KV}$ (Fig. 3, A and B, left). Overexpression of ARC, in addition to reducing $I_{KV}$, markedly inhibited the staurosporine-mediated increase in $I_{KV}$ (Fig. 3, A and B, right). At +80 mV, extracellular application of 0.02 μM staurosporine for 30 min increased the current amplitude and density of $I_{KV}$ from 3,900 ± 12 pA and 160 ± 12 pApF to 6,000 ± 45 pA (by ~54%) and 250 ± 12 pApF (by ~56%) in control (Neo) cells, whereas staurosporine increased $I_{KV}$ from 2,000 ± 34 pA and 80 ± 23 pApF to 2,500 ± 23 pA (by ~25%) and 102 ± 21 pApF (by ~27%) in ARC-transfected cells (Fig. 3, C and D). These results indicate that ARC proteins also prevent Kv channels from being activated by the apoptosis inducer staurosporine.

As shown in Fig. 1, extracellular application of 5 mM 4-AP was unable to block all outward Kv currents in Neo H9c2 cells. To determine whether ARC proteins inhibit 4-AP-sensitive $I_{KV}$, we measured current amplitude and density of $I_{KV}$ in the absence and presence of 4-AP in Neo and ARC-5-transfected cells. Extracellular application of 5 mM 4-AP reduced the current density of $I_{KV}$ by 39.3 ± 15.2% at −40 mV and by 67.6 ± 2.6% at +80 mV in Neo cells, whereas 4-AP decreased the current density by 14.1 ± 1.9% at −40 mV and by 28.4 ± 2.8% at +80 mV in ARC-5-transfected cells (P < 0.001 vs. Neo cells; Fig. 4). These results indicate that most of the Kv current component enhanced by ST is 4-AP-sensitive $I_{KV}$ and that over-

Fig. 1. Inhibitory effect of 4-aminopyridine (4-AP) on whole cell voltage-gated K+ current ($I_{KV}$) in control (Neo) H9c2 cells. A: representative superimposed currents, elicited by test potentials ranging from −40 to +80 mV (with holding potential of −70 mV), recorded in Neo cells before (control), during (4-AP), and after (washout) extracellular application of 5 mM 4-AP. The 4-AP-sensitive currents (bottom) were obtained by subtracting the currents recorded during application of 4-AP from the currents recorded under control conditions. B: summarized current-voltage ($I$-$V$) relationship curves (means ± SE; n = 12) are presented for Neo cells in control, 4-AP, and washout conditions. C: $I$-$V$ curve for the 4-AP-sensitive currents (means ± SE). D: time course of the 4-AP-mediated inhibitory effect on $I_{KV}$ at +80 mV in a Neo cell. Two representative superimposed currents (elicited by test potentials from −40 to +80 mV in 10-mV increments) recorded at the time indicated by arrows are shown above the time-course curve. The vertical and horizontal bars indicate 500 pA and 50 ms, respectively. $I_{max}$, normalized currents. E and F: kinetic analysis of activation ($E$) and inactivation ($F$) of 4-AP-sensitive and -insensitive Kv currents. Top: normalized currents at +80 mV (averaged from 17 cells) showing the activation ($E$) and inactivation ($F$) kinetics of the 4-AP-sensitive $I_{KV}$, (currents measured during application of 5 mM 4-AP) and the 4-AP-sensitive $I_{KV}$, (currents obtained by subtracting the currents recorded during 4-AP treatment from currents recorded under control conditions). Bottom: summarized data (means ± SE; n = 17) showing the time constants for activation ($\tau_{act}$) and inactivation ($\tau_{inact}$) of the 4-AP-insensitive and 4-AP-sensitive currents at +80 mV. *P < 0.05, **P < 0.001 vs. 4-AP insensitive.
expression of ARC in H9c2 cells significantly inhibits the 4-AP-sensitive $I_{K(V)}$.

**Overexpression of ARC inhibits staurosporine-induced apoptosis in H9c2 cells.** Activation of $K^+$ channels has been associated with the early apoptotic volume decrease in cells undergoing apoptosis, whereas pharmacological blockade of $K^+$ channels inhibits the apoptotic volume decrease and apoptosis in neurons, lymphocytes, vascular smooth muscle cells, and tumor cell lines (1–6, 8, 13, 15, 18–20). The next set of experiments was designed to determine whether the ARC-mediated inhibition of Kv channels associates with an increase in cell survival when cells are challenged with staurosporine.

In Neo H9c2 cells, ~5% of cells underwent apoptosis under control conditions. Treatment with 0.02 μM staurosporine markedly increased the percentage of cells undergoing apoptosis (to 50 ± 8%; $n = 12$; $P < 0.001$) (Fig. 5). However, staurosporine-induced apoptosis was significantly inhibited in cells stably transfected with ARC (Fig. 5B). These results suggest that overexpression of ARC enhances cardiomyocyte survival by inhibiting Kv channel activity and/or by preventing the channels from being activated by apoptosis inducers.

**DISCUSSION**

Apoptotic volume decrease resulting from loss of intracellular ions and water is an early prerequisite for...
apoptosis and usually occurs before cytochrome c release, activation of caspases, and DNA fragmentation (3, 5, 10, 13, 19). The apoptotic volume decrease is not inhibited by caspase inhibitors but is significantly inhibited by blockade of K\textsubscript{\textit{Ca}} channels, suggesting that the apoptotic cell shrinkage is coupled to the activity of K\textsubscript{\textit{Ca}} channels (1–6, 8, 13, 15, 18–20). The inhibitory effect of K\textsuperscript{+} channel blockers on apoptosis observed in neurons (4, 20), lymphoid cells (2, 3, 10, 13), thymocytes (5, 6), smooth muscle cells (8), hepatoma cells (15), neutrophils (1), and epithelial cells (13) further supports the contention that K\textsuperscript{+} efflux through sarcolemmal K\textsubscript{\textit{Ca}} channels plays an important role in apoptosis.

In the current study using heart-derived H9c2 cells, we observed that 1) 4-AP-sensitive Kv channels were expressed in these cells, and the corresponding \( I_{\text{K}^{\text{V}}} \) was enhanced by staurosporine, a proapoptotic agent; 2) overexpression of ARC decreased \( I_{\text{K}^{\text{V}}} \) density and inhibited the staurosporine-induced \( I_{\text{K}^{\text{V}}} \) increase; and 3) overexpression of ARC increased cell survival in cells exposed to proapoptotic staurosporine treatment. These results suggest that inhibition of K\text{v} channel activity may serve as an additional mechanism by which ARC attenuates cardiomyocyte apoptosis.

H9c2 myocytes express multiple Kv channel subunits as shown by RT-PCR experiments (data not shown). It is possible that ARC, in addition to inhibiting 4-AP-sensitive Kv channels, may also inhibit 4-AP-insensitive Kv channels and other types of K\textsuperscript{+} channels that are sensitive or insensitive to 4-AP. In ARC-transfected H9c2 cells, the current amplitude and current density of \( I_{\text{K}^{\text{V}}} \) were much lower, whereas the time constant for current activation was much higher, than those in Neo cells. These results suggest that ARC...
proteins may 1) downregulate Kv channel expression, thereby reducing the total number of Kv channels available for generating whole cell $I_{K(V)}$; 2) interact directly or indirectly with the channel protein, thereby affecting channel gating; and 3) interact directly with the channel pore, thereby blocking channel conduction.

In addition to controlling cell volume, cytoplasmic K$^+$ has been shown to suppress the activity of caspases and nucleases (10). In lymphocytes, for example, treatment with staurosporine decreased intracellular [K$^+$] from 140 to 55 mM whereas a decrease in [K$^+$] in the assay buffer from 150 to 80 mM caused a 2.4-fold increase in DNA degradation in isolated nuclei (5, 10). Thus the ARC-mediated inhibition of Kv channels would contribute to maintain a high cytoplasmic [K$^+$], which may further the direct inhibitory effect of ARC on the apical initiator caspases (e.g., caspase-2 and -8) and indirectly inhibit the executioner caspase (e.g., caspase-3) (10, 11).

In summary, multiple mechanisms are involved in the antiapoptotic effect of ARC on cardiomyocytes: 1) inhibition of caspase activation (11); 2) blockade of hypoxia/ischemia-induced cytochrome c release (7); 3) prevention of H$_2$O$_2$-mediated loss of membrane integrity and disruption of the mitochondrial membrane potential (14); 4) blockade of sarcolemmal Kv channels, which possibly contributes to inhibition of the apoptotic cell shrinkage; and 5) inhibition of proapoptotic agent-mediated activation of Kv channels. The precise mechanisms by which ARC blocks Kv channels remain unclear. The ARC-mediated inhibition of staurosporine-induced increase in $I_{K(V)}$ may be due to its blocking effect on cytochrome $c$ release (7), because cytoplasmic dialysis of cytochrome $c$ increases $I_{K(V)}$ in vascular smooth muscle cells (16). Whether and how ARC directly interacts with the Kv channel $\alpha$- and $\beta$-subunits to block the channel conduction requires further investigation.

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