Apoptosis recruits two-pore domain potassium channels used for homeostatic volume regulation

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Received 2 August 2001; accepted in final form 31 October 2001

Apoptosis occurs in most cell types, including preimplantation mammalian embryos, and can be triggered by a variety of physiological and pathological stimuli. Independent of the cell type or inducer, this type of orchestrated cell death is nearly always accompanied by cell shrinkage (see Fig. 1A; Refs. 15, 35). Apoptotic volume decreases (AVD) precede other better understood processes of apoptosis such as cytochrome c release, mitochondria membrane potential dissipation, caspase activation, and DNA fragmentation (15, 27, 35, 41). The changes in intracellular ion concentrations accompanying AVD modulate caspase activity and thereby regulate the progression of apoptosis (4, 7, 16, 18). Consequently, the molecules underlying AVD might serve as suitable therapeutic targets for inducing apoptosis in cancer cells or preventing apoptosis in degenerative diseases. Despite its significance, the mechanisms underlying AVD are poorly understood (6, 15, 35, 44). Emerging evidence suggests that potassium release from cells participates in AVD (6, 7, 15, 27, 41, 44); however, the responsible plasma membrane K+ transporter or channel has not been characterized (4, 15, 41). Here we report electrophysiological and pharmacological evidence demonstrating that apoptosis recruits two-pore domain K+ (K_{2P}) channels.

Similarities exist between AVD and regulatory volume decreases (RVD) in response to hypoosmotic shock, predicting that AVD and RVD employ similar mechanisms (15, 35). Hypoosmotic conditions cause cells to swell initially, but in a compensatory action, cells regain their original volume by effluxing K+, Cl−, and organic osmolytes, which results in “osmotically obliged” water loss and RVD (15, 34, 35, 37). K+ can efflux from swollen cells through any of three mechanisms: electroneutral K+−Cl− symporters, an K+−H+ and Cl−−HCO3− antiporter system, or independent K+ channels (35, 44). In particular, calcium-independent, quinine-inhibitable K+ channels participate in K+ efflux during RVD (11, 34, 37). These K+ channels are voltage insensitive and resistant to several K+ channel antagonists such as charybdotoxin (11, 34, 35, 37). Volume decreases accompanying apoptosis might co-opt mechanisms more routinely employed during homeostatic cell volume regulation (as suggested in Refs. 15 and 21).

Interestingly, the emerging family of K_{2P} channels possesses biophysical properties similar to the K+ channels participating in RVD (10, 23, 36). The K_{2P} channels are calcium- and voltage insensitive and are not inhibited by traditional K+ channel antagonists (e.g., charybdotoxin). In addition to being modulated by phospholipids, some K_{2P} channels are sensitive to environmental stresses such as heat and plasma membrane deformations (29, 30, 36). These K_{2P} channels integrate stimuli, adjust membrane conductances accordingly, and therefore are uniquely poised to participate in AVD.

APOTOPSIS OCCURS IN MOST CELL TYPES, INCLUDING PRE-IMPLANTATION MAMMALIAN EMBRYOS, AND CAN BE TRIGGERED BY A VARIETY OF PHYSIOLOGICAL AND PATHOLOGICAL STIMULI. INDEPENDENT OF THE CELL TYPE OR INDUCER, THIS TYPE OF ORCHESTRATED CELL DEATH IS NEARLY ALWAYS ACCOMPANIED BY CELL SHRINKAGE (SEE FIG. 1A; Refs. 15, 35). APOPTOTIC VOLUME DECREASES (AVD) PRECEDE OTHER BETTER UNDERSTOOD PROCESSES OF APOPTOSIS SUCH AS CYTOCHROME C RELEASE, MITOCHONDRIA MEMBRANE POTENTIAL DISSIPATION, CASPASE ACTIVATION, AND DNA FragmentATION (15, 27, 35, 41). THE CHANGES IN INTRACELLULAR ION CONCENTRATIONS ACCOMPANYING AVD MODULATE CASPASE ACTIVITY AND THEREBY REGULATE THE PROGRESSION OF APOPTOSIS (4, 7, 16, 18). CONSEQUENTLY, THE MOLECULES UNDERLYING AVD MIGHT SERVE AS SUITABLE THERAPEUTIC TARGETS FOR INDUCING APOPTOSIS IN CANCER CELLS OR PREVENTING APOPTOSIS IN DEGENERATIVE DISEASES. DESPITE ITS SIGNIFICANCE, THE MECHANISMS UNDERLYING AVD ARE POORLY UNDERSTOOD (6, 15, 35, 44). EMERGING EVIDENCE SUGGESTS THAT POTASSIUM RELEASE FROM CELLS PARTICIPATES IN AVD (6, 7, 15, 27, 41, 44); HOWEVER, THE RESPONSIBLE PLASMA MEMBRANE K+ TRANSPORTER OR CHANNEL HAS NOT BEEN CHARACTERIZED (4, 15, 41). HERE WE REPORT ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL EVIDENCE DEMONSTRATING THAT APOPTOSIS RECRUITs TWO-PORE DOMAIN K+ (K_{2P}) CHANNELS.

SIMILARITIES EXIST BETWEEN AVD AND REGULATORY VOLUME DECREASES (RVD) IN RESPONSE TO HYPOOSMOTIC SHOCK, PREDICTING THAT AVD AND RVD EMPLOY SIMILAR MECHANISMS (15, 35). HYPOOSMOTIC CONDITIONS CAUSE CELLS TO SWELL INITIALLY, BUT IN A COMPENSATORY ACTION, CELLS REGAIN THEIR ORIGINAL VOLUME BY EFFLUXING K+, Cl−, AND ORGANIC OSMOLyTES, WHICH RESULTS IN “OSMOTICALLY OBLIGED” WATER LOSS AND RVD (15, 34, 35, 37). K+ CAN EFFLUX FROM SWOLLEN CELLS THROUGH ANY OF THREE MECHANISMS: ELECTRONEUTRAL K+−Cl− SYMPORTERS, AN K+−H+ AND Cl−−HCO3− ANTIPORTER SYSTEM, OR INDEPENDENT K+ CHANNELS (35, 44). IN PARTICULAR, CALCIUM-INDEPENDENT, QUININE-INHIBITABLE K+ CHANNELS PARTICIPATE IN K+ EFFLUX DURING RVD (11, 34, 37). THESE K+ CHANNELS ARE VOLTAGE INSENSITIVE AND RESISTANT TO SEVERAL K+ CHANNEL ANTAGONISTS SUCH AS CHARYBDOTOXIN (11, 34, 35, 37). VOLUME DECREASES ACCOMPANYING APOPTOSIS MIGHT CO-OPT MECHANISMS MORE ROUTINELY EMPLOYED DURING HOMEOSTATIC CELL VOLUME REGULATION (AS SUGGESTED IN Refs. 15 AND 21).

INTERESTINGLY, THE EMERGING FAMILY OF K_{2P} CHANNELS POSSESSES BIOPHYSICAL PROPERTIES SIMILAR TO THE K+ CHANNELS PARTICIPATING IN RVD (10, 23, 36). THE K_{2P} CHANNELS ARE CALCIUM- AND VOLTAGE INSENSITIVE AND ARE NOT INHIBITED BY TRADITIONAL K+ CHANNEL ANTAGONISTS (E.G., CHARYBDOTOXIN). IN ADDITION TO BEING MODULATED BY PHOSPHOLIPIDS, SOME K_{2P} CHANNELS ARE SENSITIVE TO ENVIRONMENTAL STRESSES SUCH AS HEAT AND PLASMA MEMBRANE DEFORMATIONS (29, 30, 36). THESE K_{2P} CHANNELS INTEGRATE STIMULI, ADJUST MEMBRANE CONDUCTANCES ACCORDINGLY, AND THEREFORE ARE UNIQUELY POISED TO PARTICIPATE IN AVD.
Mouse one-cell embryos (zygotes) exhibit robust RVD (19, 37) and AVD (see Fig. 1; Refs. 25, 26, 41) and provide an excellent system for analyzing K⁺ channels underlying volume changes, because the volume of these large (80-μM diameter) cells can be reliably monitored optically and zygotes are amenable to electrophysiological techniques (19, 31, 41). Oxidative stress (200 μM H₂O₂) evokes rapid AVD and K⁺ efflux from zygotes (41). The K⁺ efflux associated with AVD can be noninvasively quantified by self-referencing a potassium-selective electrode within the diffusive boundary layer and thereby comparing the concentration of K⁺ ([K⁺]) in medium nearby embryos to that remote from embryos (for a description of the self-referencing electrode technique see Refs. 40 and 41). Using self-referencing technology we demonstrate here that the K⁺ channels responsible for K⁺ efflux during AVD have pharmacological properties similar to those known to participate in RVD and consistent with K₂P channels, suggesting that apoptosis co-opts existing mechanisms for homeostatic cell volume control.

METHODS

Animals, embryo collection and culture. Female B6C3F1 mice (6 wk old) were purchased from Charles River Laboratory (Boston, MA) and subjected to a 14:10-h light-dark cycle for at least 1 wk before use. Animals were cared for according to procedures approved by the Marine Biological Laboratory and Women and Infants Hospital Animal Care Committees. Zygotes were collected 21–22 h after injection of human chorionic gonadotropin from pregnant mare serum gonadotropin-primed female mice mated with males. After cumulus removal, zygotes were cultured at 37°C in humidified air (7% CO₂) in modified potassium simplex optimized medium (KSOM) supplemented with nonessential amino acids and 2.5 mM HEPES. The zona pellucida (ZP) was removed mechanically after mild treatment with pronase. No differences in physiological signals were observed between ZP-intact and ZP-free zygotes.

Self-referencing potassium electrode technique and image acquisition. The self-referencing system used to monitor [K⁺] near embryos and oocytes was identical to that previously described (40, 41). Physiological measurements were conducted at 37°C in covered glass-bottom petri dishes coated with poly-l-lysine (MatTek, Ashland, MA) with 4 ml of HEPES-buffered KSOM (HKSOM) containing reduced NaHCO₃ (4 mM) and elevated HEPES (14 mM). Potassium-selective electrodes (tip diameter of 3–5 μm) were fabricated using K⁺ ionophore I-cocktail B (Fluka, Milwaukee, WI) and backfilled with 100 mM KCl. All electrodes were calibrated and confirmed to be Nernstian before use (38, 39, 41). A silver/silver chloride reference electrode completed the circuit in solution by way of a 3 M NaCl-3% agar bridge. During recording, the potassium-selective electrode was oscillated in a dampened square wave parallel to the electrode axis over a distance of 10 μm with a frequency of 0.3 Hz. The near position of this oscillation was 5 μm from the ZP, or the plasma membrane in cases where the ZP was removed. Data acquisition and manipulation were performed as described previously (40, 41). The hardware and software controlling electrode movements, signal amplification, and data acquisition were designed and constructed by the BioCurrents Research Center at the Marine Biological Laboratory (Woods Hole, MA; www.mbl.edu/BioCurrents).

The morphometric features of zygotes were analyzed from digital images captured with a microscope-mounted Cohu analog video camera (Cohu, San Diego, CA) and a personal computer running Metamorph Software (Universal Imaging, West Chester, PA). Morphometric measurements were obtained from images with Metamorph. All physiology and morphometric data were processed in Excel (Microsoft, Seattle, WA) and Sigma Plot (SPSS Science, Chicago, IL). Data from individual embryos were interpolated and averaged using a program employing the interpolation function of MatLab software (MatLab, Cambridge, MA). Interpolation between data points gathered from any individual embryos allowed calculation of the average and standard deviation from a group of zygotes from which data were gathered at different times relative to the pharmacological treatment. All data are presented as means ± SD, and statistical comparisons (ANOVA) were done in Excel with statistical significance defined as P < 0.05.

Pharmacology. Table 1 lists the pharmacological agents and concentrations used to determine the K⁺ channel subtype recruited during apoptosis. These concentrations used are consistent with those that effectively modulate K⁺ channels in other cell types. Stock solutions of agents were prepared in solvents (water, DMSO, or EtOH) and diluted in media to the working concentration on the day of use. The concentration of solvents did not exceed 0.1%, and control solvent applications were conducted on the same batches of embryos. Self-referencing technology was used to monitor [K⁺] near embryos before and during a 40-min exposure to each pharmacological agent. To test whether agents modulated H₂O₂-induced K⁺ efflux, zygotes were pretreated with each K⁺ channel inhibitor for 10–15 min followed by addition of 200 μM H₂O₂. For caged calcium experiments, zygotes were preloaded with 1-(4,5-dimethoxy-2-nitrophenoxy)-1,2-diaminonaphthy-V,N,N’,N”-tetraacetic acid, tetra(acetoxy-methyl) ester (DMNPE-DTAM; Molecular Probes, Eugene, OR) with standard procedures and calcium was uncaged by photolysis with 368-nm light (14, 32). Various patterns of photolysis were tested including continual light exposure for 10 s and pulsed exposure (5–10 pulses of 200- to 500-ms duration at 1 Hz) (32). Data were analyzed by ANOVA (Excel), and statistical significance was defined as P < 0.05.

Calcium imaging. The ratiometric fluorescent dye fura 2-AM was used to measure intracellular Ca²⁺ concentration ([Ca²⁺]). Zygotes were incubated with 2.5 μM fura 2-AM (Molecular Probes) in KSOM for 30 min at 37°C and then washed, and calcium imaging was conducted at excitation wavelengths of 334/380 nm and measured at an emission wavelength of 520 nm with the Attoloum fluor imaging system. Free calcium measurements were taken every 15 s for 10 min before application of H₂O₂ and for 40 min subsequently. No transient rise in calcium was observed.

RESULTS

Treatment of one-cell mouse zygotes with 200 μM H₂O₂ evoked rapid AVD and K⁺ efflux (Fig. 1). During the first 25–35 min of H₂O₂ exposure, dying zygotes shrunk to ~70% of their original cross-sectional area, which equated to a >40% decline in cell volume (Fig. 1). Concurrently, the [K⁺] near embryos gradually increased to a peak [K⁺] of 1.6 ± 0.5 μM above that of the bulk media (Fig. 1B). By using the Fick equation (40), this change in [K⁺] can be equated to an efflux of 0.4 pmol of K⁺·embryo⁻¹·min⁻¹. K⁺ efflux ceased with

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Extended $\text{H}_2\text{O}_2$ exposure, and $[\text{K}^+]$ near embryos returned to homeostatic levels by 40–60 min (Fig. 1B).

Previously, the broad-spectrum $\text{K}^+$ channel blocker tetraethylammonium (TEA) was shown to inhibit $\text{H}_2\text{O}_2$-induced $\text{K}^+$ efflux from zygotes, suggesting that the $\text{K}^+$ effluxed through $\text{K}^+$ channels in the plasma membrane (41). To delineate the specific class of $\text{K}^+$ channel participating in AVD we used a series of pharmacological agents that target $\text{K}^+$ channel subtypes (Table 1). Agonists and antagonists to $\text{ATP}$-sensitive, voltage-sensitive, and calcium-activated $\text{K}^+$ channels failed to modulate AVD associated $\text{K}^+$ efflux (Table 1), suggesting that apoptosis was not recruiting these channel types. The constancy of $[\text{Ca}^{2+}]$ during the first 40 min of $\text{H}_2\text{O}_2$ exposure when $\text{K}^+$ efflux occurred ($75 \pm 14 \text{nM}$ 10 min before $\text{H}_2\text{O}_2$, $94 \pm 14 \text{nM}$ after $\text{H}_2\text{O}_2$; $n = 9$) and the failure of uncaged calcium, strontium, and thapsigargin to modulate AVD (Table 1) indicated that the $\text{K}^+$ channels underlying AVD were calcium independent. These pharmacological properties are consistent with those of $\text{K}_\text{ATP}$ channels (10, 23, 36).

$\text{K}_\text{ATP}$ channels typically set membrane leak conductances as they allow $\text{K}^+$ to move down its electrochemical gradient (23). To maintain high intracellular $[\text{K}^+]$, these leak conductances are counterbalanced by $\text{ATP}$ase activity (e.g., $\text{Na}^+\text{K}^+\text{ATP}$ase), such that at homeostasis there is no net $\text{K}^+$ efflux or $\text{K}^+$ influx. Block of the $\text{Na}^+\text{K}^+\text{ATP}$ase with a cocktail of 50 $\mu\text{M}$ strophantidin and 1 $\mu\text{M}$ ouabain (2, 3, 43) uncovered the resting leak potassium conductance indicative of the presence of $\text{K}_\text{ATP}$ channels. On strophantidin-ouabain exposure, a slight but statistically significant $\text{K}^+$ efflux increased $[\text{K}^+]$ near embryos ($0.13 \pm 0.06 \mu\text{M}$ $[\text{K}^+]$ above background; $n = 9$, $P < 0.003$). This cocktail, however, did not inhibit $\text{H}_2\text{O}_2$-induced $\text{K}^+$ efflux ($1.4 \pm 0.3 \mu\text{M}$ $[\text{K}^+]$ above background).

Some $\text{K}^+$ channels underlying RVD are quinine sensitive (12, 37), and quinine (1 $\mu\text{M}$) reduced both AVD and $\text{K}^+$ efflux from zygotes exposed to $\text{H}_2\text{O}_2$ (Fig. 2; Table 1). After pretreatment with quinine, $\text{H}_2\text{O}_2$ evoked only modest shrinkage of zygotes to 93% of their original cross-sectional area (Fig. 2, $A$ and $B$). $[\text{K}^+]$ of the medium near quinine/$\text{H}_2\text{O}_2$-treated embryos increased to only 0.1 $\mu\text{M}$ above background compared with 1.6 $\mu\text{M}$ in nearby control $\text{H}_2\text{O}_2$-treated zygotes (Fig. 2C; Table 1), indicating an attenuated $\text{H}_2\text{O}_2$-induced $\text{K}^+$ efflux. Likewise, the Cl– channel antagonists 5-nitro-(3-phenylpropylamino)benzoic acid (NPPB) and DIDS, known to inhibit RVD, also reduced $\text{H}_2\text{O}_2$-evoked $\text{K}^+$ efflux (Table 1), presumably by feedback.

### Table 1. Responses to potassium channel agonists and antagonists

<table>
<thead>
<tr>
<th>Agent</th>
<th>Conc</th>
<th>n</th>
<th>Δ[K⁺] μM</th>
<th>P</th>
<th>Δ[K⁺] μM</th>
<th>P</th>
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<tr>
<td><strong>Evoked K⁺ Efflux</strong></td>
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<td><strong>ATP-sensitive K⁺ channels</strong></td>
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<td>Agonist</td>
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<tr>
<td>Diazoxide</td>
<td>1 μM</td>
<td>4</td>
<td>0.06 ± 0.14</td>
<td>NS</td>
<td>1.5 ± 0.3</td>
<td>NS</td>
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<tr>
<td>Pinacidil</td>
<td>250 μM</td>
<td>5</td>
<td>0.05 ± 0.10</td>
<td>NS</td>
<td>0.10 ± 0.14</td>
<td>NS</td>
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<tr>
<td>ADP</td>
<td>500 μM</td>
<td>5</td>
<td>0.03 ± 0.08</td>
<td>NS</td>
<td>0.10 ± 0.14</td>
<td>NS</td>
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<td><strong>Voltage-sensitive K⁺ channels</strong></td>
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<td>Antagonist</td>
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<tr>
<td>Dendrotoxin</td>
<td>150 nM</td>
<td>7</td>
<td>1.5 ± 0.3</td>
<td>NS</td>
<td>1.5 ± 0.3</td>
<td>NS</td>
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<tr>
<td><strong>Large-conductance, Ca²⁺-activated K⁺ channels</strong></td>
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<td>Agonist</td>
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<td>Caged Ca²⁺</td>
<td>5–25 μM</td>
<td>6</td>
<td>0.08 ± 0.18</td>
<td>NS</td>
<td>1.6 ± 0.4</td>
<td>NS</td>
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<tr>
<td>Strontium</td>
<td>10 μM</td>
<td>6</td>
<td>0.09 ± 0.08</td>
<td>NS</td>
<td>0.07 ± 0.08</td>
<td>NS</td>
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<tr>
<td>Thapsigargin</td>
<td>2 μM</td>
<td>4</td>
<td>0.03 ± 0.12</td>
<td>NS</td>
<td>0.12 ± 0.12</td>
<td>NS</td>
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<tr>
<td>Antagonist</td>
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<tr>
<td>Charybdotoxin</td>
<td>1 μM</td>
<td>4</td>
<td>1.8 ± 0.8</td>
<td>NS</td>
<td>1.8 ± 0.8</td>
<td>NS</td>
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<tr>
<td>Iberiotoxin</td>
<td>100 μM</td>
<td>8</td>
<td>1.9 ± 0.7</td>
<td>NS</td>
<td>1.9 ± 0.7</td>
<td>NS</td>
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<tr>
<td>Paxilline</td>
<td>10 μM</td>
<td>4</td>
<td>1.4 ± 0.8</td>
<td>NS</td>
<td>1.4 ± 0.8</td>
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<td><strong>K₂P channels</strong></td>
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<tr>
<td>Quinine</td>
<td>1 mM</td>
<td>6</td>
<td>0.05 ± 0.08</td>
<td>NS</td>
<td>0.1 ± 0.2</td>
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<tr>
<td>NPPB</td>
<td>100 μM</td>
<td>9</td>
<td>0.05 ± 0.08</td>
<td>NS</td>
<td>0.07 ± 0.08</td>
<td>NS</td>
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<tr>
<td>DIDS</td>
<td>100 μM</td>
<td>9</td>
<td>0.05 ± 0.08</td>
<td>NS</td>
<td>0.1 ± 0.2</td>
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<td>Agonist</td>
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<tr>
<td>AA</td>
<td>10 μM</td>
<td>6</td>
<td>0.05 ± 0.04</td>
<td>NS</td>
<td>1.7 ± 0.8</td>
<td>NS</td>
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<tr>
<td>Modulator</td>
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<tr>
<td>Phorbol ester</td>
<td>20 nM</td>
<td>9</td>
<td>0.05 ± 0.06</td>
<td>NS</td>
<td>0.3 ± 0.1</td>
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<tr>
<td><strong>Control (H₂O₂ alone)</strong></td>
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<td>9</td>
<td>0.05 ± 0.04</td>
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<td>1.6 ± 0.5</td>
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</table>

Values are means ± SD of $n$ experiments. $\text{K}^+$ efflux evoked by agent alone is expressed as the difference in $\text{K}^+$ concentration ($[\text{K}^+]$) between the media near embryos and that away from embryos. Modulation of $\text{K}^+$ efflux evoked by $\text{H}_2\text{O}_2$ is expressed as the difference in $[\text{K}^+]$ between the media near embryos and that away from embryos. Values are compared with control values at bottom of table to determine significance. AA, arachidonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid. S (bold) = $P < 0.02$; NS = $P > 0.05$. 

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between these interrelated ion systems. Likewise, clofilium tosylate and NPPB strongly inhibited AVD (NPPB/\text{H11005}) 99.7% and clofilium/\text{H11006} 5.1% and clofilium/\text{H11007} 95.0% and clofilium/\text{H11008} 3.4% change in cross-sectional area, compared with 70% in control zygotes).

The cytoskeleton, particularly actin filaments, is disrupted by H$_2$O$_2$ (8, 42) and cleaved by apoptotic enzymes (caspases) (9, 13, 33) and modulates the activity of some K$^+$ channels (28). However, K$_{2P}$ channels are not modulated by the cytoskeleton (23, 30, 36), and inhibition of caspases does not block AVD (5, 7, 16, 27). Direct disassembly of microfilaments in zygotes with cytochalasin D (2 g/ml) did not evoke K$^+$ efflux (0.07 ± 0.08 μM [K$^+$] above background), attenuate H$_2$O$_2$-induced K$^+$ efflux (1.4 ± 0.2 μM [K$^+$] above background), or prevent AVD (73% original cross-sectional area) (Fig. 3C; Table 1). Apoptosis-associated membrane blebbing was blocked by cytochalasin D (Fig. 3A), as reported in other cells (17). These results demonstrate that the K$^+$ channel underlying AVD is not modulated by the actin cytoskeleton, compatible with the properties of K$_{2p}$ channels.

Although ATP and mercury modulate cell volume changes, these agents did not inhibit K$^+$ efflux associated with apoptosis. Application of 5 mM ATP effectively reduced RVD in zygotes (19, 37). Similarly, ATP attenuated AVD but uncoupled K$^+$ efflux from AVD (Fig. 3, A and B). Zygotes preincubated with 5 mM ATP followed by H$_2$O$_2$ shrunk to only 87% their original cross-sectional area compared with ~70% in control zygotes (Fig. 3B). Nevertheless, K$^+$ efflux (1.5 ± 0.2

Fig. 1. Apoptotic volume decrease (AVD) and K$^+$ efflux during apoptosis in mouse embryos treated with 200 μM H$_2$O$_2$. A: images of a zygote before (left) and 35 min after (right) treatment with H$_2$O$_2$ showing AVD and membrane blebbing. B: treatment of zygotes with H$_2$O$_2$-induced K$^+$ efflux (solid lines) coincident with AVD (dotted lines). The middle solid and dotted lines are the interpolated mean of 9 embryos, and the outer 2 lines (2 dotted and 2 solid) are SD. These lines demarcate a band in which most of the data is located. K$^+$ efflux from zygotes increased the concentration of K$^+$ [K$^+$] in the medium 5 μm from embryos relative to that 15 μm away (background). Cell shrinkage was determined from images obtained at an equatorial focal plane and is presented as the mean cross-sectional area relative to the original cross-sectional area before H$_2$O$_2$ application.

Fig. 2. Quinine attenuated AVD and K$^+$ efflux from zygotes treated with 200 μM H$_2$O$_2$. A: zygotes treated with H$_2$O$_2$ in the presence of 1 mM quinine did not exhibit appreciable AVD or membrane blebbing, whereas control zygotes underwent AVD and membrane blebbing. Quinine reduced AVD (B) and K$^+$ efflux (C) evoked by H$_2$O$_2$. In B and C, the middle solid and dotted lines are the interpolated mean and the outer 2 lines represent the interpolated SD (n = 8 for quinine + H$_2$O$_2$; 9 for H$_2$O$_2$ alone).
μM [K⁺] above background) and membrane blebbing occurred (Fig. 3B). Similarly, the mercurial compound (30 μM HgCl₂) known to block aquaporins (20) also failed to inhibit H₂O₂-induced K⁺ efflux (1.8 ± 0.3 μM [K⁺] above background), but remarkably, this K⁺ efflux was associated with cell swelling rather than AVD (Fig. 4). The time course of HgCl₂/H₂O₂-induced cell swelling was similar to that of H₂O₂-induced AVD (Fig. 4B), suggesting that independent channels move water and ions across the plasma membrane during apoptosis. Separate water and ion mechanisms also are likely to be employed during RVD, because RVD is similarly inhibited by ATP (37). The uncoupling of ion fluxes

**Fig. 3.** ATP inhibited AVD, cytochalasin D prevented membrane blebbing, and phorbol 12-myristate 13-acetate (PMA) reduced AVD and K⁺ efflux. **A:** zygotes treated with H₂O₂ in the presence of ATP blebbed but failed to shrink, whereas those treated in the presence of cytochalasin D shrunk but did not bleb. In contrast, PMA attenuated H₂O₂-induced shrinkage and prevented blebbing. **B:** zygotes treated with H₂O₂ in the presence of ATP effluxed K⁺ (solid lines) despite reduced shrinkage (dotted lines). “Slight shrinkage and K⁺ efflux evoked by ATP alone. C:** zygotes treated with H₂O₂ in the presence of cytochalasin D effluxed K⁺ (solid lines) and shrunk (dotted lines) despite failure to bleb. **D:** zygotes treated with H₂O₂ in the presence of PMA failed to efflux appreciable K⁺ (solid lines) and shrunk negligibly (dotted lines). For B–D, the middle solid and dotted lines are the interpolated mean and the outer 2 lines represent the interpolated SD (n = 9 for HgCl₂ + H₂O₂, 6 for HgCl₂, 9 for H₂O₂).

**Fig. 4.** Zygotes treated with H₂O₂ in the presence of mercury swelled rather than shrunk (A). In the presence of HgCl₂, H₂O₂ induced a dramatic increase in the cross-sectional area of zygotes (solid lines; B). Control zygotes exposed to mercury but not treated with H₂O₂ did not swell or shrink (dotted lines; B), whereas zygotes treated with H₂O₂ alone shrunk (dashed lines; B). For each data set (solid, dotted, and dashed), the middle line is the interpolated mean and the outer 2 lines represent the interpolated SD (n = 9 for HgCl₂ + H₂O₂, 6 for HgCl₂, 9 for H₂O₂).
from volume changes challenges the direct role of ion fluxes in eliciting osmotically obliged water loss during RVD and AVD. Regardless, these results showed that the K⁺ channels underlying AVD are not modulated by ATP or mercury, in agreement with their identity as K₂P channels.

Few pharmacological tools are available to directly probe K₂P channels, but some of the six K₂P channel family members are activated by arachidonic acid (AA) (10, 23, 30, 36). AA (10 μM) failed to evoke K⁺ efflux, potentiate H₂O₂-induced K⁺ efflux, or attenuate AVD in mouse zygotes (Table 1). Several members of the K₂P channel family are inhibited by protein kinase C (PKC) (23, 30, 45), and PKC activators suppress apoptosis and reduce RVD (16). Activating PKC with the phorbol ester phorbol 12-myristate 13-acetate (PMA) effectively inhibited both AVD and K⁺ efflux from zygotes induced by oxidative stress (Fig. 3, A and D). In the presence of PMA, H₂O₂ exposure evoked less shrinkage to only 88% of the zygote’s original volume (Fig. 3, A and D) and significantly reduced K⁺ efflux such that [K⁺] near embryos rose to only 0.3 μM above background (Fig. 3D, Table 1). These observations suggest that a specific subtype of K₂P channel, modulated by PKC but not AA, underlies AVD.

**DISCUSSION**

We demonstrate here that the channel responsible for K⁺ efflux during apoptosis has pharmacological and electrical characteristics similar to those known to participate in RVD and consistent with K₂P channels, suggesting that apoptosis recruits existing mechanisms for homeostatic cell volume control. These K₂P channels are not modulated by ATP, AA, calcium, the actin cytoskeleton, or transplasma membrane voltage, and therefore AVD can occur independent of other cellular signaling cascades. This independence could prevent apoptosis from being erroneously triggered in healthy cells or mistakenly attenuated in cells destined to die.

The K⁺ channels underlying AVD exhibit properties consistent with K₂P channels. Both K₂P channels and those participating in apoptosis are calcium- and voltage independent, resistant to several K⁺ channel antagonists such as charybdotoxin, dendrotoxin, and iberiotoxin, not modulated by the actin cytoskeleton, and inhibited by PKC (10, 23, 30, 36, 45). Although specific pharmacological tools are not presently available for K₂P channels, both K₂P channels and the K⁺ channels underlying AVD are blocked by quinine derivatives (10, 23). One member of the K₂P channel family, TRAAK, is activated by AA (10, 23, 30, 36); however, AA failed to evoke K⁺ efflux or potentiate H₂O₂-induced K⁺ efflux or AVD (Table 1). The biophysical properties of the K₂P channel underlying AVD most closely resemble those of the AA-insensitive K₂P channel family member, TREK (23). Interestingly, TREK is nearly ubiquitously expressed and might provide a common mechanism by which apoptosis coordinates AVD in many cell types.

The K₂P channel we describe here as participating in AVD exhibits biophysical properties remarkably similar to those of the K⁺ channel involved in RVD. Both AVD and RVD recruit a channel that is calcium- and voltage insensitive and inhibited by quinine derivatives. We propose that apoptosis is co-opting K⁺ channels more typically regulating homeostatic cell volume (15, 21). By employing these channels, a new cell volume set point is acquired by physiologically agreeable means. In support of the convergence of AVD and RVD mechanisms, osmotic changes can induce apoptosis (15, 21, 35).

In addition to participating in AVD and RVD, the K₂P channel we characterize here might also participate in cell cycle regulation. Osmotic changes alter the cell cycle through yet unidentified mechanisms, and early stages of apoptosis are frequently accompanied by cell cycle arrest (1, 22, 24). When populations of somatic cells are treated with apoptotic agents, not all of the cells respond (7, 15, 18), which may reflect differences in cell cycle progression. In zygotes, Day et al. (9a, 9b) described a K⁺ channel that is tightly regulated by the cell cycle and is calcium- and charybdotoxin insensitive. Although we did not notice any relationship between the cell cycle and modulation of H₂O₂-induced AVD or K⁺ efflux, present studies are underway to investigate whether the K₂P channel we characterize here links AVD, RVD, and cell cycle regulation progression.

None of the pharmacological agents that reduced K⁺ efflux evoked by H₂O₂ fully rescued embryo development (data not shown). H₂O₂ acts independently on several pathways (DNA damage, mitochondrial damage), only one of which is K₂P channels and AVD. Because quinine only inhibits K⁺ efflux and AVD, it is not surprising that we were unable to completely rescue embryos from H₂O₂ insult with quinine alone. Nonetheless, the inhibition of K⁺ efflux by quinine establishes a quinine-sensitive process as underlying AVD, one piece of evidence consistent with participation of K₂P channels in apoptotic K⁺ efflux and AVD (23).

In summary, we demonstrate that the K⁺ channel responsible for K⁺ efflux during apoptosis in zygotes shares biophysical features with K₂P channels. Furthermore, we suggest that apoptosis co-opts K₂P channels usually responsible for homeostatic regulation of cell volume.

We thank Kasia Hammer, Richard Sanger, and Jane McLaughlin for assistance with the self-referencing technique and pharmacology and Gaudenz Danuser for interpolation support.

This work was supported by the Lalor Foundation (D. L. Keefe and J. R. Trimarchi) and National Center for Research Resources Grant P41-RR-01395 (P. J. S. Smith).

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