Plasma membrane aquaporin activity can affect the rate of apoptosis but is inhibited after apoptotic volume decrease

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We hypothesize that after the AVD, AQPs become inactive, which halts further water loss and allows K+ concentrations to decrease to levels necessary for apoptotic enzyme activation. Swelling assays on granulosa cells, thymocytes, and CHO-AQP-1 cells revealed that indeed, the shrunken (apoptotic) subpopulation has very low water permeability compared with the normal-sized (nonapoptotic) subpopulation. In thymocytes, AQP-1 is present and was shown to colocalize with the plasma membrane receptor tumor necrosis factor receptor-1 (TNF-R1) both before and after the AVD, which suggests that this protein is not proteolytically cleaved and remains on the cell membrane. Overall, these data indicate that AQP-mediated water loss is important for the AVD and downstream apoptotic events, that the water permeability of the plasma membrane can control the rate of apoptosis, and that inactivation after the AVD may help create the low K+ concentration that is essential in apoptotic cells. Furthermore, inactivation of AQPs after the AVD does not appear to be through degradation or removal from the cell membrane.

Water movement; major intrinsic protein; channel; enzyme

APOPTOSIS IS AN ENDOGENOUS program of controlled cell death that has been implicated in many physiological and pathological processes. Work in recent years has provided considerable insight into the cellular machinery responsible for dismantling a dying cell in preparation for phagocytosis by neighboring cells or resident macrophages. Morphologically, one of the earliest, highly conserved events in apoptosis is water loss and subsequent cell shrinkage. This process has recently been named the apoptotic volume decrease (AVD; Ref. 37). Work on thymocytes (28), granulosa cells (45), and oocytes (45) has shown that the AVD creates an intracellular environment conducive to the activation of important death enzymes such as caspases. Immediately before the AVD, a rapid inhibition of K+ uptake and a significant influx of Na+ occur in the cell; these lead to a sustained depolarization of the plasma membrane (13). This depolarization is caused at least in part by inactivation of the Na+-K+-ATPase and can be inhibited by protein kinase C (13, 24). After depolarization, there is a large efflux of K+ (and to a lesser degree, Na+ and Cl−) that results in lower intracellular concentrations of these ions (7, 8, 14, 19, 28, 42, 60). The decreases in ion levels seem to precede the release of cytochrome c from the mitochondria (37) and are required for the activation of effector caspases and the apoptotic nuclease(s) (28, 45, 51). This efflux of K+ during apoptosis generates an osmotic gradient that draws water out of the cell and thereby causes it to shrink. Despite the integral nature of the AVD to completion of the cell death process, little is known about the mechanism or regulation of water efflux in a dying cell.

In response to an osmotic gradient, water will cross a membrane in two ways: simple diffusion across the hydrophobic phospholipid bilayer or through specific proteinaceous channels called aquaporins (AQPs). Simple diffusion is slow, unregulated, and dictated primarily by the lipid composition of the plasma membrane. In contrast, water movement through AQPs is fast, selective, and subject to rapid cellular regulation (for a review of AQPs, see Refs. 10, 16, 17, 34). Because cell shrinkage also appears to be a rapid phenomenon (8, 14, 28, 30), we hypothesize that AQPs play a role in the movement of water across the plasma membrane in dying cells during the AVD.

AQPs are a subset of the major intrinsic protein (MIP) family of which >80 have been identified in many different organisms ranging from bacteria to humans (44, 47). All members of this family appear to act as transmembrane channel proteins. The conservation of these proteins across evolutionary lines suggests that they play important and very basic roles in cell biology. Currently, 11 mammalian AQPs have been identified, AQP-0 through -10 (1, 26, 44, 47), and we have recently published studies describing a role for AQP-7, -8, and -9 in rat granulosa cells (38).

All members of the AQP family contain six transmembrane segments and two “hemi-channels” that fold together into an hourglass conformation to mediate water movement (for review of AQP structure, see Refs. 10 and 53). Most AQPs possess a cysteine residue on the extracellular side of the membrane between the fifth transmembrane segment and the second hemi-channel. This cysteine residue has been shown to...
bind Hg\(^{2+}\), which sterically blocks the flow of water through the channel. Thus Hg\(^{2+}\) acts as an effective and general inhibitor of most AQPs. In the present study, we utilized both Hg\(^{2+}\) and a cell line that overexpresses AQP-1 to explore a role for these water channels in the AVD of rat granulosa cells and thymocytes and to assess the importance of AQP-mediated water loss in the regulation of the AVD and, therefore, the beginning of the process of cell death.

Activation of caspasas and apoptotic nucleases are dependent on a total decrease in ionic strength of the cytoplasm that is brought about primarily by a loss of intracellular K\(^+\) concentration ([K\(^+\)]\(_i\)). Logically, for this to occur, ion efflux must be significantly greater than water loss. Thus we hypothesize that after the AVD, AQPs are inactivated, which makes the plasma membrane significantly less permeable to water. Inactivation of these water channels coupled with the continued efflux of ions would reduce the ionic strength of the cytoplasm to levels conducive to the activity of apoptotic enzymes. Accordingly, we have also examined the water permeability characteristics of normal and shrunken (apoptotic) cells within the same population as well as the regulation of AQP-1 in thymocytes after the AVD.

Materials and Methods

Animals and cell cultures. All rats were raised in-house and provided with food and water ad libitum. Immature 21- to 23-day-old female Sprague-Dawley rats were injected subcutaneously with 10 IU of pregnant mare serum gonadotropin (PMSG, kindly provided by Dr. A. F. Parlow and the National Hormone and Pituitary Program at NIDDK). Animals were killed 48 h later by CO\(_2\) asphyxiation and the ovaries were removed and cleaned of surrounding fascia. Granulosa cells were then harvested into McCoy's 5A medium by needle puncture as previously described (45). The thymus was also removed with a cell line that overexpresses AQP-1 to explore a role that was isotypically controlled normal and shrunken (apoptotic) cells within the same population as well as the regulation of AQP-1 in thymocytes after the AVD.

Debris, chromatin, and membranes were pelleted by ultracentrifugation (100,000 \(g\) for 30 min at 4°C, and the resultant supernatant was collected and analyzed for protein content via Bradford assay (15). To assess nuclease activity (45), 5 \(\mu\)g of lyase protein was incubated with 5 \(\mu\)l of linearized plasmid (Stratagene, La Jolla, CA) that was linearized with the Smal restriction enzyme) without or with 50 \(\mu\)M HgCl\(_2\) or 50 mM EDTA in a total volume of 10 \(\mu\)l that contained 50 mM Tris-HCl, 1 mM MgCl\(_2\), and 1 mM CaCl\(_2\). Samples were incubated at 37°C for 1.5 h and then at 55°C for 1 h with 20 \(\mu\)g of proteinase K. Samples were then electrophoresed on a 1% agarose gel (1.5 h at 80 V), stained with ethidium bromide, and visualized by UV transillumination.

Caspase assay. Caspase-3-like protease activity was measured using a fluorometric assay as previously described (14, 28). Briefly, cytoplasmic extracts were prepared by resuspending granulosa cells in 10 mM MgCl\(_2\) with 0.25% Nonidet P-40. Debris was pelleted at 100,000 \(g\) for 30 min and supernatants were placed on ice. Next, 10–50 \(\mu\)l of extract (measured by Bradford assay; Ref. 15) was preincubated in solution that contained 50 mM HEPES (pH 7.5), 10 mM dithiothreitol, 10% sucrose, and 0.1% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS) \(\pm\) treatments with 200 \(\mu\)M of the noncompetitive inhibitor DEVD-fmk (Asp-Glu-Val-Asp-fluoromethylketone; Kamiya Biomedical, Seattle, WA). Parallel samples were prepared without inhibitor. DEVD-af substrate (Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; Kamiya Biomed) was then added to all tubes (200 \(\mu\)l final concentration). Samples were incubated for 5 min at 30°C, and fluorescence at 505 nm was measured (excitation, 400 nm) on a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Samples were incubated an additional 1 h and fluorescence was again measured. A standard curve of fluorescence vs. free 7-amino-4-trifluoromethylcoumarin (afc) was then used to calculate the specific activity of caspase-3-like enzymes in each sample.

Mitochondrial membrane potential analysis. Granulosa cells obtained by day-2 PMSG-treated rats were cultured at 10\(^6\) cells/0.5 ml of serum-free McCoy's 5A medium in 5-m1 polystyrene round-bottom tubes with or without 50 \(\mu\)M HgCl\(_2\) for 48 h. After this incubation, the mitochondrial membrane-potential-sensitive dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR) was added to a final concentration of 10 \(\mu\)M. Cells were incubated for 30 min in the dark and then analyzed by flow cytometry. Cells were first analyzed on a dot plot of forward vs. side scatter and gates were drawn to exclude cell aggregates and debris (data not shown). Gated cell populations were then compared on a dot plot of FL-1 vs. FL-2 (585 nm) fluorescence. Cells with normal mitochondrial membrane potential, JC-1 forms aggregates and displays a high FL-2 fluorescence. Loss of mitochondrial membrane potential causes the aggregates to dissociate into monomers and...
AQUaporins and apoptosis

RESULTS

Inhibition of AQP2 blocks AVD and apoptosis. To assess the role of AQP2 in the AVD, apoptosis was induced in granulosa cells by culturing for 48 h in serum-free medium in the presence or absence of increasing concentrations of HgCl2. After culture, cells were analyzed by flow cytometry for changes in size. As shown in Fig. 1, Hg2+ inhibits cell shrinkage in a dose-dependent manner with maximal inhibition detected at 50 μM. Concentrations of Hg2+ >50 μM had no additional effect (data not shown). Thus 50 μM HgCl2 was used in all subsequent experiments.

To address the significance of AQP2 to the overall death process, we have analyzed three common characteristics that occur in most models of cell death: DNA degradation, caspase-3 activation, and mitochondrial membrane permeability change (ΔΨ). DNA degradation was assessed by flow cytometry. Figure 2A illustrates typical DNA histograms obtained with granulosa cells incubated in the presence or absence of HgCl2 (50 μM) and defines the gates used to score cells with degraded (hypodiploid; M1) or normal (diploid to tetraploid; M2) amounts of DNA. As shown quantitatively in Fig. 2B, in the absence of HgCl2 there was a significant increase in the proportion of the population that displayed a hypodiploid amount of DNA over the control (t = 0). The decreased water movement blocks the appearance of this apoptotic population in a dose-dependent manner down to levels seen in the nonapoptotic cells (t = 0). This suggests that the inhibition of water movement through AQP2 can block downstream activation of DNA degradation.

Analysis of the biochemical properties of apoptotic nucleases has revealed them to be sensitive to the ionic composition of the buffer in which they are analyzed (29, 31). To control for the possibility that HgCl2 may directly inhibit the apoptotic nuclease activity in dying granulosa cells, nucleases were extracted from granulosa cells incubated as above (in 0 μM HgCl2), and the extracts were analyzed for the ability to degrade an isolated pUC18 plasmid in the presence or absence of 50 μM HgCl2. As shown in Fig. 2C, incubation with extract from dying granulosa cells degraded the plasmid into smaller fragments that generated a smear on an ethidium bromide-stained agarose gel (control lane). Inclusion of 50 μM HgCl2 in the assay had no effect on the nuclease activity of the extracts,
of apoptosis. One prominent feature of the intrinsic pathway is utilized in this study, is known to engage the intrinsic pathway studied in this manner. Inhibition of AVD and apoptosis is not specific to cell type or stimulus, we expanded these studies to examine apoptosis of thymocytes and to thapsigargin and C8-ceramide. To better detect the enhancement of apoptosis in response to these which suggests that the effects seen in Fig. 2B were mediated by inhibition of AQPs and not by direct effects of HgCl2 on nuclease as a control, the right lane in Fig. 2C demonstrates that this nuclease activity, which is Ca2+/Mg2+ dependent, is inhibited by EDTA.

Caspase-3 is a central effector caspase that is implicated in many different models of cell death. As shown in Fig. 3, caspase-3-like enzyme activity was significantly enhanced in dying granulosa cells (control) compared with a freshly isolated population (t = 0). The appearance of caspase-3-like activity in these cells could be completely blocked by inclusion of 50 μM HgCl2 in the culture medium (in vivo samples). To assess the possibility that HgCl2 was exerting a direct inhibitory effect on this enzyme, preparations of active caspase-3-like enzymes were prepared from dying granulosa cells and assayed in the presence or absence of 50 μM HgCl2 or 50 mM EDTA as described. Results are from a representative experiment repeated two additional times.

Growth factor withdrawal-induced apoptosis, such as that utilized in this study, is known to engage the intrinsic pathway of apoptosis. One prominent feature of the intrinsic pathway is that the mitochondrial membranes undergo ΔΨ that leads to release of cytochrome c and eventual activation of caspase-3. We next analyzed the effect inhibition of AQPs had on the ΔΨ values in dying granulosa cells using the mitochondrial membrane potential dye JC-1. As shown in Fig. 4, there is a significant increase in the percentage of the population that has undergone ΔΨ after 48 h in culture relative to the freshly isolated population (t = 0). In contrast, 50 μM HgCl2 completely blocked this depolarization event. This suggests that AQP-mediated water loss is required for apoptotic mitochondrial changes.

Inhibition of AVD and apoptosis is not specific to cell type or signal. The results thus far have been obtained with granulosa cells undergoing growth factor withdrawal. To ensure that these effects were not specific to this cell type or stimulus, we expanded these studies to examine apoptosis of thymocytes and in response to thapsigargin and C8-ceramide. To better detect the enhancement of apoptosis in response to these

![Fig. 2. HgCl2 blocks the activation of nuclease activity in apoptotic granulosa cells. A: DNA histograms show the DNA content of granulosa cells cultured for 48 h in the absence or presence of 50 μM HgCl2. Cells gated in the M1 are considered hypodiploid. B: quantitative analysis of the percentage of the population that contained hypodiploid DNA in freshly isolated cells (t = 0) or cells incubated with increasing concentrations of HgCl2 for 48 h (means ± SE of 4 separate experiments); *P < 0.05 compared with cells cultured in the absence of HgCl2. Measurements from freshly isolated cells (t = 0) are included for comparison and are not assessed for statistical significance. C: direct effects of HgCl2 (50 μM) on the apoptotic nuclease. Nuclease activity was extracted from granulosa cells as described in MATERIALS AND METHODS. Linearized pUC18 plasmid was then incubated in the absence (no extract) and presence (control) of extract, or extract plus 50 μM HgCl2 or 50 mM EDTA as described. Results are from a representative experiment repeated two additional times.](http://ajpcell.physiology.org/)

![Fig. 3. HgCl2 blocks the activation of caspase-3-like activity in dying granulosa cells. For in vivo studies, granulosa cells were cultured for 48 h (to induce apoptosis) in the absence or presence of 50 μM HgCl2. Extracts were then prepared and analyzed for caspase-3-like activity as described in MATERIALS AND METHODS. For in vitro studies, granulosa cells were cultured for 48 h (no HgCl2) and then extracts were prepared. Extracts were then tested for caspase-3-like enzyme activity in the absence or presence of 50 μM HgCl2. All results are means ± SE of three separate experiments; *P < 0.5 compared to in vivo controls. Measurements from freshly isolated cells (t = 0) are included for comparison and are not assessed for statistical significance. AFC, 7-amino-4-trifluoromethylcoumarin.](http://ajpcell.physiology.org/)

![Fig. 4. HgCl2 blocks the change in mitochondrial membrane potential in dying granulosa cells. A: contour plots of FL-1 vs. FL-2 fluorescence from a representative experiment. Cells in the R4 region were considered depolarized. B: quantitative analysis of the percentage of the population that contained depolarized mitochondria in freshly isolated cells (t = 0); cells incubated for 48 h as in Figs. 2, 3, and 4 (control); or cells incubated 48 h with 50 μM HgCl2; *P < 0.05 compared with cells cultured in the absence of HgCl2. Measurements from freshly isolated cells (t = 0) are included for comparison and are not assessed for statistical significance.](http://ajpcell.physiology.org/)
agents, the incubation times were reduced to 24 h for both granulosa cells and thymocytes. As shown in Fig. 5, thapsigargin and C6-ceramide stimulated cell shrinkage and DNA degradation in both granulosa cells and thymocytes above growth factor withdrawal. HgCl2 attenuated the effects of these agents with the same efficiency as growth factor withdrawal. Thus the ability to block apoptosis through the inhibition of AQP's is neither cell type nor signal specific.

HgCl2 was used in this study to inhibit the water movement through AQPs. Previous studies have suggested that HgCl2 may directly influence signaling pathways including the MAPK pathway (35, 59). To ensure that HgCl2 was blocking apoptosis only by inhibition of AQPs and not inadvertently by activating MAPK or through the well-known phosphatidylinositol 3-kinase (PI3-K) survival pathway, we analyzed the ability of inhibitors of these pathways to attenuate the effects of HgCl2. As shown in Fig. 6, inhibition of MAPK kinase with PD-098,059 (3, 20, 43) or PI3-K with wortmannin (4, 46) or LY-294002 (54) in granulosa cells undergoing growth factor withdrawal enhanced cell death slightly, which indicates a

Fig. 5. HgCl2 blocks the AVD and DNA degradation in granulosa cells and thymocytes in response to growth factor withdrawal, thapsigargin, and C6-ceramide. A: effects on granulosa cells. B: results with thymocytes. Cells were cultured for 24 h in serum-free medium in the absence or presence of 2 μM thapsigargin or 50 μM C6-ceramide as indicated and were then fixed in ethanol and analyzed by flow cytometry for forward light scattering (size) and DNA content. Gates were drawn similar to those depicted in Figs. 1 and 2 to determine the proportion of the population that was shrunken or contained a hypodiploid amount of DNA; *P < 0.05 compared with cells cultured in absence of HgCl2. Measurements from freshly isolated cells (t = 0) are included for comparison and are not assessed for statistical significance.

Fig. 6. Inhibitors of MAPK kinase (PD-098,059) and phosphatidylinositol 3-kinase (PI3-K; wortmannin and LY-294002) do not affect the ability of HgCl2 to block the AVD and DNA degradation in granulosa cells and thymocytes in response to growth factor withdrawal. A: effects on granulosa cells. B: results with thymocytes. Cells were cultured for 24 h in serum-free medium in the absence or presence of 2 μM thapsigargin or 50 μM C6-ceramide as indicated and were then fixed in ethanol and analyzed by flow cytometry for forward light scattering (size) and DNA content. Gates were drawn similar to those depicted in Figs. 1 and 2 to determine the proportion of the population that was shrunken or contained a hypodiploid amount of DNA; *P < 0.05 compared with cells cultured in absence of HgCl2. Measurements from freshly isolated cells (t = 0) are included for comparison and are not assessed for statistical significance.
nominal level of activity in the cells. Thymocyte apoptosis was not significantly affected by these agents, which indicates very low basal activity levels of these survival pathways in these cells. Importantly, these compounds did not influence the ability of HgCl₂ to block cell shrinkage or DNA degradation in either cell type. This suggests that HgCl₂ is not significantly activating these pathways, and thus inhibition of apoptosis seen with the use of HgCl₂ is most likely through the ability of this compound to inhibit AQP function.

Overexpression of AQP-1 enhances AVD and apoptosis. The results from Figs. 1–6 suggest that inhibition of plasma membrane permeability may influence the ability of a cell to undergo the AVD and initiate the apoptotic cascade. To determine whether increasing the plasma membrane water permeability may enhance the rate of apoptosis in an appropriately stimulated cell, we examined CHO cells transfected with an AQP-1 expression vector (CHO-AQP-1) or an empty vector (CHO-vector). Expression levels of AQP-1 in these cells have been previously characterized by Ma et al. (36), and our lab has confirmed these results by Western blot analysis (data not shown). To assess plasma membrane water permeability, cell size was measured by flow cytometry before and after 2-min exposure to medium diluted to 170 mosM. Figure 7A depicts overlay histograms of the relative cell size (forward scatter) from these two measurements in these two cell lines (0 and 2 min are shown). CHO-vector cells displayed distinct swelling in this time frame, although the magnitude of this swelling was greatly enhanced in the CHO-AQP-1 cells, which indicates (as expected) increased water permeability in the CHO-AQP-1 cells. We next determined whether this increased water permeability in CHO-AQP-1 cells would increase the number of cells in the population that undergo apoptosis (apoptosis detected through cell shrinkage and DNA degradation). As shown in Fig. 7B, at the beginning of the experiment (t = 0), CHO-AQP-1 cells displayed a slightly higher although not significantly different level of apoptosis (both cell shrinkage and DNA degradation) than CHO-vector cells. This difference was greatly magnified after 48 h of growth factor withdrawal. This indicates that increasing the permeability of the plasma membrane enhances the rate of cell death.

Water permeability is significantly decreased in apoptotic cells after AVD. Water loss after the AVD must be attenuated to allow the reduction of [K⁺], which is required for activation of apoptotic enzyme activity. Thus we hypothesized that the plasma membrane of the shrunken cells undergoes a significant decrease in water permeability. We investigated this hypothesis by analyzing the cell swelling properties of both normal and shrunken (apoptotic) cells. Granulosa cells, thymocytes, and CHO-AQP-1 cells were induced to undergo apoptosis by growth factor withdrawal for 48 h, and the ability of normal and shrunken (apoptotic) subpopulations to swell in response to a hypotonic insult was measured using the flow cytometer (as described in MATERIALS AND METHODS). Depicted in Fig. 8, A, C, and E are the dot plots from each of these subpopulations of CHO-AQP-1 cells at 48 h and the gates used to differentiate normal (R2) from shrunken (R1) subpopulations. The distribution of each subpopulation was then separately plotted on an overlay (Fig. 8, B, D, and F), and peak heights were normalized for each histogram. The population was then subjected to hypotonic challenge and the results were overlaid on the initial measurements. In all three cell types, the shrunken subpopulation (R1) displayed virtually no swelling in response to the hypotonic insult, whereas the normal cells (R2) displayed a measurable increase in size. This response was exaggerated in the highly permeable CHO-AQP-1 cells (Fig. 8, E and F), where the normal cells increased in size to an amount equivalent to that seen in Fig. 7, whereas the shrunken cells showed no increase in size, which suggests very low plasma membrane permeability in this apoptotic population. Figure 9 displays the dot plots from these cells to illustrate the dramatic disjunction in the permeability rates of these two subpopulations. These results support our hypothesis that water movement through AQPs is vital to the cell death process, and this water
AQP-1 is present on plasma membrane of thymocytes and remains after AVD. We have shown that plasma membrane water permeability is significantly inhibited in cells after the AVD. It is possible that this decrease is mediated by AQP degradation and/or removal from the plasma membrane. Thus we next determined AQP-1 expression, levels, and subcellular localization in thymocytes before and after the AVD. For these studies, thymocytes were used because they are nonadherent and relatively uniform and are therefore easy to differentiate between normal and apoptotic cells based on size.

As shown in Fig. 10A, AQP-1 is expressed in thymocytes, and there is not a significant decrease in the percentage of the population staining for this homolog after 24 h of growth factor withdrawal-induced apoptosis. Thus degradation of AQP-1 seems an unlikely mechanism by which the plasma membrane permeability is decreased after the AVD. We next examined the subcellular location of AQP-1 in shrunken and normal thymocytes exposed to growth factor withdrawal for 24 h to determine whether this protein remained on the plasma membrane. In this population, we were clearly able to distinguish between pre- and post-AVD cells simply by size. The location of the plasma membrane was first determined from Normaski optics. As seen in Fig. 10B, the majority of AQP-1 localized to the plasma membrane of the normal-sized cells (arrowhead) and did not change location after the AVD (open arrows). To further ensure that this AQP was indeed on the membrane, a colocalization experiment was performed with AQP-1 and TNF-R1, a cell surface receptor previously shown to be in the thymocyte plasma membrane (50). In Fig. 10C, AQP-1 localization is indicated by red fluorescence whereas TNF-R1 is indicated by green. By overlaying these images, colocalization is indicated by yellow fluorescence. This experiment clearly
shows that AQP-1 and TNF-R1 colocalize both before and after the AVD, which suggests that AQP-1 remains on the plasma membrane throughout the apoptotic process.

DISCUSSION

The decrease in cell volume during apoptosis is a highly conserved phenotype, and numerous studies have demonstrated the importance of ion efflux, particularly K\(^+\), to this event (7, 8, 12, 14, 19, 28, 42, 60). Although loss of intracellular K\(^+\) is concomitant with the AVD, it also occurs measurably before cytochrome c release, caspase-3 activation, and DNA degradation (19, 37). The loss of K\(^+\) as a cell dies establishes an osmotic gradient that draws water out of the cell and forces the cell to shrink. In the present study, we presented evidence in apoptotic rat granulosa cells that water follows this gradient primarily through proteinaceous water channels known as AQPs. Inhibition of AQP-mediated water movement not only blocked the AVD but also prevented downstream apoptotic characteristics; this suggests that AQP-mediated water loss is not peripheral to the apoptotic cascade but is an essential component in this stepwise process. The effects of AQP inhibition on the AVD and DNA degradation were neither cell type nor signal specific and were not mediated by effects on survival pathways. Importantly, overexpression of AQP-1 in CHO cells increased the amount of apoptosis, which indicates that the water permeability of the plasma membrane during the AVD may be a rate-limiting step in the early stages of cell death.

A reduction in [K\(^+\)], is essential for activation of apoptotic enzymes (7, 8, 14, 19, 28, 42, 60), but a loss of ions does not necessarily imply a reduction in concentration if the loss is...
matched by an efflux in water and a reduction in intracellular size. We have shown within the same population of cells that the subpopulations that have undergone the AVD have very low water permeability, whereas the nonapoptotic cells have normal permeability. This change in water permeability during the AVD would thus allow the loss of K\(^+\) to be unbalanced from the loss of water and facilitate a decrease in [K\(^+\)]. We have also shown that AQP-1 in thymocytes is not proteolytically cleaved or removed from the cell membrane after apoptosis, which suggests that this water channel is inactivated by a posttranslational nondegradative mechanism.

We have shown that thymocytes express AQP-1, which is known to be Hg\(^{2+}\) sensitive (52). In addition, we have shown in previous studies that granulosa cells express AQP-7, -8, and -9 (38). Interestingly, AQP-7 is Hg\(^{2+}\) insensitive because it does not contain the Hg\(^{2+}\)-binding cysteine in its water pore, whereas AQP-8 and -9 are Hg\(^{2+}\) sensitive (52). Moreover, we have shown that granulosa cell apoptosis is in fact inhibited by Hg\(^{2+}\), and therefore we believe that the AVD during granulosa cell apoptosis is predominantly mediated through AQP-8 and -9. It is important to note that the AVD is a critical event during apoptosis. Thus it is reasonable to speculate that most cell types that are highly sensitive to apoptosis would express more than one AQP family member to ensure cell death.

A particularly intriguing finding in this study is that HgCl\(_2\) blockage of water movement inhibits a number of apoptotic effects downstream of the AVD. In this regard, it is interesting to note that several studies have blocked the AVD by a variety of methods including the use of K\(^+\) and Cl\(^-\) channel inhibitors (8, 27, 41, 49, 56, 62), caspase inhibitors (30, 55), and high-K\(^+\) medium (14, 28). In those studies, downstream apoptotic events were also abrogated, further suggesting an essential role for the AVD in the death process. In one study (27), some apoptotic characteristics (such as caspase activity) were detected in the absence of an AVD, although this effect was restricted to nitric oxide-induced apoptosis in a macrophage cell line (RAW 264.7). AQP expression has never been studied in these cells, and a deficiency of water channels may be one explanation for the apparent lack of an AVD and ability to die independent of it. All other inducing agents used with these cells or any other cell types displayed a strict correlation between the AVD and activation of apoptotic enzymes. Because the AVD and the subsequent decrease in [K\(^+\)], are absolutely critical for the activation of apoptotic enzymes, it is reasonable to infer that all cells that express AQPs will use one or more of these water channels to mediate water loss during apoptosis. One interesting scenario is cells that do not appear to express AQPs. As more family members become known, a comprehensive analysis will be needed to ensure that these cells do not express any water channels. Because the AVD is a nearly universal characteristic during apoptosis, it is also possible that in cells that do not normally express AQPs, one or more of the AQP family members may be upregulated in response to an apoptotic stimulus. Conversely, they may rely exclusively on simple diffusion of water across the plasma membranes to facilitate the AVD. However, based on the rapid and regulated nature of the AVD, simple diffusion through the lipid bilayer seems unlikely.

Potential caveats in the literature concerning the use of Hg\(^{2+}\). Whitekus et al. (58) showed that Hg\(^{2+}\) blocked cell death induced by FasL, an effect that is hypothesized to be caused by receptor clustering. Thus in these studies, we have intentionally avoided the use of cell surface death receptors to obviate any concerns with receptors clustering. In addition, there are reports that both inorganic and organic Hg\(^{2+}\) compounds induce apoptosis in lymphoid and nonlymphoid cells (2, 21, 32, 48). Based on the argument put forth by Whitekus et al. (58), this variation may be accounted for by differences in both concentration and distribution of Hg\(^{2+}\). More membrane-permeable organomercurials such as methylmercury display higher toxicity (18), which suggests a mechanism of action independent of the death pathways initiated here. Studies have also shown that HgCl\(_2\) may have direct effects on the MAPK pathway; this signaling pathway has clearly been implicated in survival of apoptotic insult (35, 59). In this study, we addressed the possibility that HgCl\(_2\) may suppress cell death by activating this survival pathway or the well-characterized PI3-K-mediated survival pathway. Inhibition of either pathway had no effect on the ability of HgCl\(_2\) to block apoptosis. Our studies used very low concentrations of HgCl\(_2\), and we have never detected toxicity and/or apoptosis in our controls that contained HgCl\(_2\) alone.

Presently there is debate in the literature concerning the effects of Hg\(^{2+}\) on K\(^+\) channels. For example, Ballatori et al. found that the plasma membrane K\(^+\) permeability was enhanced by treatment with Hg\(^{2+}\) (5, 6), whereas several other studies detected Hg\(^{2+}\)-activated K\(^+\) currents across the membrane (22, 33, 40). In contrast, Gallagher et al. detected a K\(^+\) current in B lymphocytes that was blocked by Hg\(^{2+}\) (23). A single channel mediating K\(^+\) loss during apoptosis in all cells has not been identified, and it is possible that different channels may be involved in a cell or signal-specific manner (8, 9, 41, 57, 61, 62). Using a model of AQP-1 overexpression (CHO-AQP-1 cells), we demonstrated that increased water permeability of the plasma membrane greatly enhanced the rate of cell death, which provides strong evidence independent of this heavy metal that membrane permeability to water can influence the susceptibility of a cell to apoptosis when the cell is appropriately stimulated.

Not only is water movement during the AVD critical to the progression of the cell death cascade, but interestingly, the permeability of the apoptotic cell after the AVD is significantly suppressed. This result can be seen most clearly in the exaggerated response of the CHO-AQP-1 cells. Inhibition of water movement after the AVD would allow [K\(^+\)], to decrease to levels necessary to facilitate the activation of downstream apoptotic enzymes. The results with AQP-1 in thymocytes suggest that this inhibition occurs through a posttranslational nondegradative mechanism while these channels remain on the cell surface. Ongoing studies in our laboratory are focused on the mechanisms of inhibition of these water channels after the AVD.

The results of this study significantly strengthen the importance of the AVD to apoptosis and suggest for the first time that the water loss occurs through specific proteinaceous water channels. In addition, we have shown that AQP inhibition suppresses the appearance of apoptotic characteristics, whereas overexpression of AQPs enhances the rate of intrinsically induced cell death. This suggests that the water permeability of the plasma membrane may alter the ability of the cell to die. Furthermore, although functional AQPs may be necessary for a cell to shrink and die, they are also inactivated after the AVD,
presumably to allow the $[\text{K}^+]_o$ to be reduced to levels conducive to activation of the apoptotic program. This inactivation of AQPs after the AVD seems to be accomplished through means other than degradation of these water channels.

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