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On the Role of VDAC in Apoptosis: Fact and Fiction

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Research on VDAC has accelerated as evidence grows of its importance in mitochondrial function and in apoptosis. New investigators entering the field are often confounded by the VDAC literature and its many apparent conflicts and contradictions. This review is an effort to shed light on the situation and identify reliable information from more questionable claims. Our views on the most important controversial issues are as follows: VDAC is only present in the mitochondrial outer membrane. VDAC functions as a monomer. VDAC functions normally with or without Ca^{2+}. It does not form channels that mediate the flux of proteins through membranes (peptides and unfolded proteins are excluded from this statement). Closure of VDAC, not VDAC opening, leads to mitochondria outer membrane permeabilization and apoptosis.

KEY WORDS: Apoptosis; VDAC; mitochondria; outer membrane; calcium; arsenic trioxide; permeability transition; Bcl-2.

PROPERTIES OF VDAC

Structure

The Functional Unit is a Monomer

VDAC forms 2-dimensional crystals in the outer membrane of mitochondria in *N. crassa* (Mannella, 1982) and some plants (Parsons *et al.*, 1965). These crystalline arrays are composed of roughly cylindrical channels that can be visualized after filling with negative stain or in an unstained frozen–hydrated state in vitreous ice (Mannella and Guo, 1990). The crystal structure is not simply a hexagonal close-packing indicating the presence of asymmetries perhaps arising from surface domains. Indeed, the surface views obtained after freeze-drying and shadowing show elevated regions surrounded by the openings of six channels. The self-association of VDAC from *N. crassa* has a tendency to be organized in a multiple of three channels (Mannella *et al.*, 1983) but, unlike the porins, these are clearly independent channels. Scanning transmission electron microscopy (STEM) mass measurements show definitively that each channel in the array is composed of one single polypeptide chain (Thomas *et al.*, 1991). Evidence that the same monomeric structure exists in channels that are not in an array comes from site-directed mutagenesis. Co-expression of VDAC channels with different functional properties in the yeast, *S. cerevisiae,* followed by isolation of the functional channels showed clearly that no functional hybrids exist (Peng *et al.*, 1992a,b). The probability of missing a hybrid channel by random chance was less than 1 in 10^7.

Self-association of VDAC was a very early observation (Parsons *et al.*, 1965). Arrays were easily detected in mitochondrial outer membranes from plants and these turned out to be VDAC. This self-association can be resistant to denaturing conditions. Indeed, SDS PAGE sometimes indicates the presence of dimers (Mannella and Colombini, 1984). Some early proposed structures assumed that the functional unit was a dimer based, in part, on hydrodynamic analysis of VDAC (Linden and Gellerfors, 1983). The detergent-solubilized form of rat liver VDAC seemed to behave as a dimer even after correction for bound detergent. However, if one includes the large amount of water in the channel's pore as part of the

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mass of the overall structure, then a monomer is indicated. Thus electron microscopic, genetic and sedimentation evidence all agree that the functional unit is a monomer, despite a tendency for monomers to self-assemble into arrays.

Recent speculation that VDAC channels form functional tetramers (Zalk *et al*., 2005) capable of passing proteins through membranes, should be looked at with a great deal of healthy skepticism. The cross-linking technique used to generate these multimers is notorious for generating complexes that either do not exist or are merely transient associations. Care must be taken to distinguish between intramolecular cross-links and the intermolecular ones. In addition, cross-linking VDAC refolded from bacterial inclusion bodies (Shi *et al*., 2003) is prone to even more difficulties.

The Secondary Structure Consists of 1 α Helix and 13 β Strands with a 46° Tilt

Both circular dichroism spectra and a conserved alternating polar/nonpolar pattern in the primary sequence (necessary for β strands to separate a polar channel lumen from the apolar lipid milieu) strongly support the conclusion that VDAC channels are fundamentally β barrels (Mannella *et al*., 1992). A stretch of approximately 20 amino acids at the *N*-terminus on the core VDAC structure (some of the VDAC isoforms have an *N*-terminal extension) has a highly conserved polar/nonpolar pattern consistent with an α helix capable of forming part of the wall of the pore (one side apolar and the other polar or charged). Thus, the wall may not be composed entirely of β structure. Various types of theoretical analyses lead to predictions for the transmembrane strands forming the walls of the channel (Forte *et al*., 1987; De Pinto *et al*., 1991; Rauch and Moran, 1994; Mannella *et al*., 1996; Casadio *et al*., 2002). Some base their proposal in homology to the folding pattern revealed by the crystal structure of an unrelated[4] set of β-barrel proteins, the bacterial porins. Others focus on the biophysical characteristic of the structure: alternating polar/non-polar pattern, location of prolines and aromatic residues, and forbidding charged residues from facing the hydrophobic surface (Song and Colombini, 1996). Theoretical predictions need to survive experimental testing.

Site-directed mutagenesis defined the portions of the protein that face the ion stream and thus form the wall of the channel (Blachly-Dyson *et al*., 1990; Song *et al*., 1998b). This effort was directed by the theoretical analysis. If a charge change at one site altered the selectivity of the channel in the correct direction by an appropriate amount (from theoretical expectations) then that site must face the ion stream and form part of the wall of the channel. No change in selectivity demonstrated conclusively that the site of mutation did not form the wall of the pore. By focusing mutations at the protein–water interface there was less risk of remote effects. This was confirmed by the observation that multiple mutations showed properties that were the linear sum of the changes observed in individual mutations. Some almost perfect alternating polar/nonpolar patterns were found not to be transmembrane strands and were therefore assigned to surface domains. The extra strands in some theoretical models come from these regions.

The use of proteases and peptide-specific antibodies were also used to define the structure of VDAC (Stanley *et al*., 1995). There are wide areas of agreement between the results of these studies and the results deduced from site-directed mutagenesis. Unfortunately, some of the peptides chosen as markers turn out to be transmembrane and have epitopes on both sides. The proteases studies are sometimes conflicting because when the protein is cleaved it allows for structural changes and exposure of previously hidden structures.

Topological studies by double cysteine mutagenesis followed by biotinylation and streptavidin treatment identified a transmembrane strand that was missed in the original studies. The final tally is that the channel wall is composed of 1 α helix and 13 β strands (Song *et al*., 1998b). The inclusion of an α helix into the β barrel may seem to be aesthetically unappealing but it might provide the right amount of structural energy facilitating the large conformation associated with channel gating.

The identified transmembrane strands can form a barrel with a pore size that is consistent with the estimated open-channel diameter. Abrecht and coworkers (2000) demonstrated by infrared spectroscopy that the β strands of VDAC are tilted at an angle of 46°. This tilt means that the normal distance between adjacent β strands in a β sheet would effectively be increased, when measured in the plane of the membrane, from 0.47 to 0.66 nm. Similarly, the effective size of the α helix

[4]The bacterial outer membrane contains a variety of β-barrel proteins, many of which are channel formers. The latter are often referred to as porins. Although diverse, their structure follows a general motif. Most are trimeric in that each protein consists of three monomers each of which forms a channel. Virtually all have a constricted region in the pore arising from loop regions extending into the channel. This constriction is responsible for the selectivity characteristics. Although VDAC is also mainly a β barrel, it has no primary sequence homology with the porins; it is a monomer; it has no constricted region within the pore; and it has an α helix forming part of the wall of the pore. Thus, there is no basis to assume that the structure of any of the porins provides insight into the structure of VDAC.

would increase from 1.4 to 1.9 nm. Thus, the circumference of the channel at the level of the backbone would be 10.5 nm corresponding to a diameter of 3.4 nm. This compares reasonably well with the value of 3.8 nm estimated from electron micrographs of frozen–hydrated VDAC crystals (Mannella *et al*., 1992). It compares better with the minimal center-to-center spacing of adjacent channels of 4.3 nm. After subtracting an estimated wall thickness of 1 nm, the backbone diameter would be 3.3 nm. The size of the aqueous pore is "fuzzy" because it depends on the length of the side chains extending into the pore and the degree to which water and permeating ions move between the side chains. Nevertheless, this dimension is consistent with the estimated diameter of the pore of 2.5–3 nm from electron microscopy. From the size of the largest non-electrolyte able to permeate the pore (Colombini, 1980), PEG 3400 ($R_{se} = 1.6$ nm (Krasilnikov *et al*., 1992)), inulin ($R_{se} = 1.4$ nm), dextran ($M_{av} = 6000$), the estimated size is somewhat larger (2.8 to 3.2 nm in diameter) but that could be accounted for by the flexibility of the size markers.

Despite the lack of direct structural information at atomic resolution, the available constraints define the structure of the open state of VDAC with quite a high level of confidence.

Function

General Properties

The mitochondria of all eukaryotic organisms studied so far have at least one isoform of VDAC[5], which, after reconstitution into planar phospholipid membranes, forms a channel with highly conserved characteristics (Colombini, 1989): a specific single-channel conductance, voltage gating parameters, and ion selectivity. Indeed, the values are far more tightly conserved than one might expect from general functional considerations. For example, conductance in 1.0 M NaCl is 3.3 ± 0.1 nS for VDAC isolated from *N. crassa* (Rostovtseva and Bezrukov, 1998) and 3.4 ± 0.1 nS for rat liver (Rostovtseva *et al*., 2004). Selectivity obtained in 1/0.1 M NaCl gradient for these two VDAC species is -21 and -19 mV, respectively (T.R. unpublished). Many organisms have other VDAC isoforms with rather different properties and these are likely specialized versions of VDAC that perform other, as yet, not clearly defined functions.

[5]Purification of VDAC requires that the protein be solubilized in detergent. The use of mild detergents such as Triton X100 and octyl glucoside does not seem to affect the properties of VDAC. For example, single-channel conductance of VDAC1 isolated from mouse liver mitochondria with Triton X100 is 3.3 ± 0.17 nS and with octyl glucoside is 3.4 ± 0.1 nS in 1 M NaCl solutions (T.R. unpublished).

Selectivity of Permeation Pathway

The pathway formed through the membrane by VDAC has selectivity properties similar to those of narrow channels. The use of the term "general diffusion pore" is not only highly misleading but reflects a naiveté that is no longer justified by the available experimental evidence. Certainly one cannot expect that channels with a large pore will be able to select among alkali metal ions or halide ions. They evolved to generate a large pathway because of the large size of the permeating species, the metabolic anions that must travel between the cytosol, and mitochondrial spaces. Chief among these is ATP, a highly charged anion. Cellular activity sometimes demands a high rate of flux of ATP through the mitochondrial outer membrane (i.e., through VDAC). Thus, it is logical that the permeability pathway may be optimized for ATP flux.

The simplest way to consider the factors influencing the permeation of metabolites through VDAC is to consider the size and charge of the permeants. Clearly size matters but, based on the permeability of non-electrolytes, the size cut-off of VDAC is large enough to allow most metabolites to cross. However, experimental observations show that ions of similar size and charge as ATP (see Table I) can be totally excluded (Rostovtseva *et al*., 2002a). Metabolites larger than ATP, such as NADH, still permeate through VDAC (Rostovtseva *et al*., 2002b). This somewhat surprising finding can be readily understood if one recalls that these ions are not spherically symmetrical. The permeation pathway within VDAC must complement the distribution of charge and partial charge on the permeating ions. Molecules with a different distribution may experience an energy barrier to translocation that limits or precludes flux.

Gating

VDAC channels can exist in a variety of functional states that differ in their ability to pass non-electrolytes and conduct ions (Schein *et al*., 1976; Colombini, 1979; Rostovtseva and Colombini, 1996; Hodge and Colombini, 1997). They also differ in selectivity between cations and anions (Colombini, 1989). The most conductive state is the "open" state. It shows significant preference for anions and especially favors metabolic anions. Elevated voltages favor the closed states. These are states of lower conductance and much reduced permeability to metabolic anions.

Table I. Properties of ATP and Similar Ions in Aqueous Solution

	Bound Na^+	Effective MW	Charge	Diffusion const. ($cm^2\ s^{-1}$)
ATP	2.6	563	−1.4	4.5×10^{-6}
Tetraglutamate	2.0	581	−2.0	4.1×10^{-6}
Hydroxypyrenetrisulphonate	1.6	492	−1.4	4.8×10^{-6}

Therefore, VDAC closure greatly diminishes metabolite flux across the mitochondrial outer membrane. However, when using simple KCl as the permeating ions, the conductance only drops by about 60% (Fig. 1) and this drop varies from trial-to-trial revealing the variable nature of the closed state. Note that channel closure is often accompanied by channel oscillations. Sometimes VDAC channels inserted in a closed state and the lower and rapid conductance fluctuations are clues to that fact. These are sometimes misinterpreted in the literature.

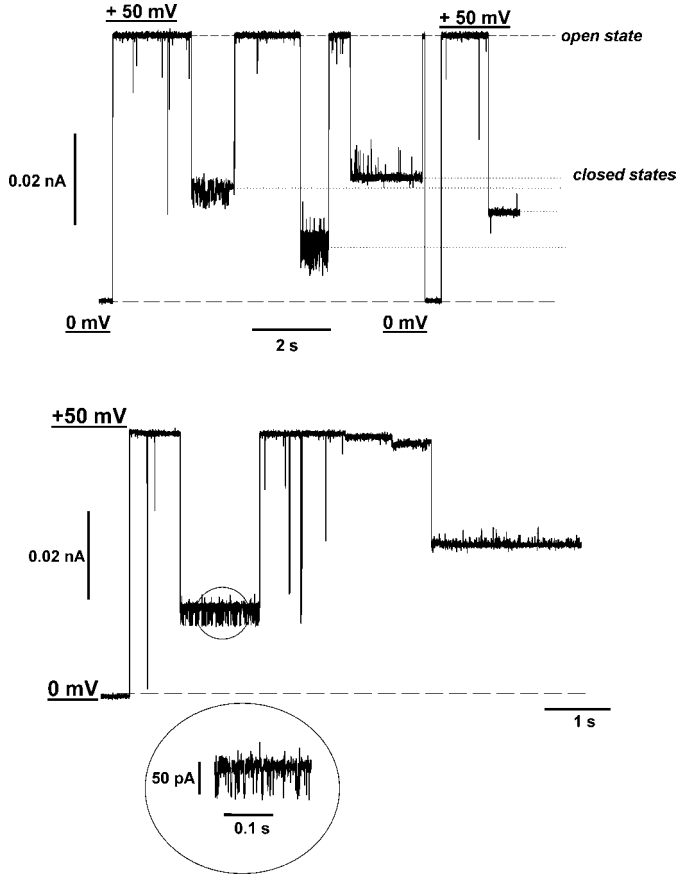

Fig. 1. VDAC channel conductance fluctuates between one high-conductance, "open" state and multiple low-conductance, "closed" states. The inset shows the oscillations of the closed state conductance at a finer scale. VDAC was isolated from *N. crassa*. The medium consisted of 250 mM KCl, 1 mM $CaCl_2$, 5 mM HEPES at pH 7.2. The bilayer membrane was made from 42% (w/w) asolectin, 42% DPhPC, 8% cardiolipin, and 8% cholesterol. Current records were filtered using averaging times of 1 and 0.3 ms (inset). The *dashed line* indicates zero-current level.

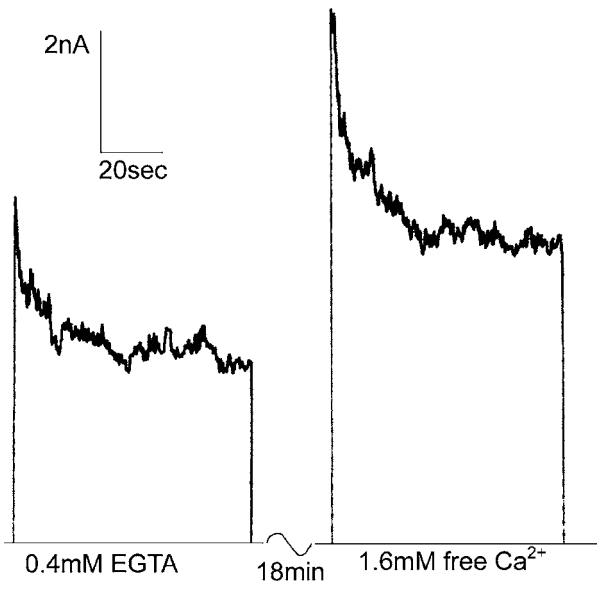

Fig. 2. VDAC's voltage gating is unaffected by Ca^{2+}. VDAC from rat liver mitochondria was reconstituted into planar phospholipid membranes formed from 1:1 mixture of DPhPC and asolectin with 0.05% cholesterol. The medium consisted of 1.0 M KCl buffered with 20 mM HEPES at pH 7.2. To each side of the membrane was added 0.4 mM EGTA to chelate trace amounts of Ca^{2+}. *Right trace* was recorded 3 min after addition of 2 mM Ca^{2+}. The rise of conductance was due to the insertion of the additional VDAC channels. The relative conductance drop and the rate of decay were essentially the same with and without free Ca^{2+}. The applied potential was +50 mV. The *horizontal line* indicates zero current.

The permeability of VDAC to small ions includes Ca^{2+}. This important ion permeates through both the open (Gincel *et al*., 2001) and closed states (T.R. unpublished) of VDAC. The double positive charge does not exclude Ca^{2+} from the open state because the anion selectivity is not very large. The closed state favors cations. The presence or absence of Ca^{2+} does not change the conductance of the open state of VDAC. In 1 M NaCl, the conductance is 3.3 ± 0.3 nS in the presence of 0.1 mM EGTA and 3.4 ± 0.1 nS in the presence of 1 mM $CaCl_2$. Moreover, the voltage gating is unaffected whether Ca^{2+} is present or not (Fig. 2).

A mobile domain in the wall of the channel, called the voltage sensor, makes VDAC voltage gated. VDAC responds to applied fields in either direction by moving the sensor to one surface of the membrane resulting in a pore of diminished diameter (1.8 nm) and inverted selectivity (Peng *et al*., 1992a,b; Thomas *et al*., 1993; Song *et al*., 1998a). The selectivity inversion (from favoring anions to favoring cations) occurs because the sensor has a net positive charge and its translocation to the membrane surface leaves the channel with a net negative charge. The combination of reduction in pore size and inversion in selectivity explains why the closed state of the channel, while still physically large enough to allow the passage of ATP, actually excludes ATP (Rostovtseva and Colombini, 1997).

The inability of the negatively charged ATP to permeate through the closed VDAC pore in spite of the fact that ATP molecule ($R_{se} \sim 0.7$ nm) is smaller than the dimensions of the closed pore (1.8 nm), clearly demonstrates that the electrostatic profile inside the channel plays a more important role in the process of translocation of charged metabolites through the channel than the relative pore/solute molecule sizes. Speculations that cytochrome *c* (3.0 nm × 3.4 nm × 3.4 nm) might somehow squeeze through VDAC (2.5–3 nm in diameter) totally ignore the unfavorable electrostatic interactions. The open state of VDAC has a net positive charge just like cytochrome *c*. The net charge of VDAC does invert in the closed states of VDAC, however, these have even smaller dimensions (1.8 nm in diameter). Thus holocytochrome *c* cannot permeate through VDAC (perhaps the flexible apo form might be able to do this). Certainly, under normal conditions, cytochrome *c* molecules cannot permeate through this channel, otherwise, cytochrome *c* would not be localized within the intermembrane space.

The closure of VDAC would greatly reduce the ability of anionic metabolites to diffuse between the cytosol and the mitochondrial spaces. Restrictions in the flow of adenine nucleotides between the mitochondria and the cytosol has been shown to occur in the initial stages of apoptosis and attributed to the closure of VDAC (see below). The reported mechanisms by which VDAC can undergo a transition from the open to the closed state, are quite numerous. Strong evidence now exists for a potential across the outer membrane sufficient enough to close VDAC (Colombini, 2004; Porcelli *et al*., 2005) and therefore voltage changes through changes in macromolecular charge (e.g., volume changes in the intermembrane space, protein phosphorylation, etc.) are a very viable possibility. This is especially so since the voltage gating of VDAC and the values of the voltage gating parameters are highly conserved among very diverse species, conserved in at least one of the VDAC isoforms in these species (Colombini, 1989). Other factors include: colloidal osmotic pressure (Zimmerberg and Parsegian, 1986), intermembrane space protein(s) referred to as the VDAC modulator (Holden and Colombini, 1988; Liu and Colombini, 1991; Liu *et al*., 1994), polyanions including charged proteins and nucleic acids (Mangan and Colombini, 1987; Colombini *et al*., 1989), NADH and MgNADPH (Lee *et al*., 1994, 1996), tBid (Rostovtseva *et al*., 2004), and phosphorylation by

protein kinase A (Bera and Ghosh, 2001). Some factors favor VDAC closure in only certain species: G-actin (Xu *et al*., 2001), dyenin light chain and mtHSP70 (Schwarzer *et al*., 2002), and La^{3+} (Gincel, 2001). Some factors favor VDAC opening: aluminum trihydroxide and other metal trihydroxides (Dill *et al*., 1987; Zhang and Colombini, 1989) and Bcl-x_L (Vander Heiden *et al*., 2001).

In spite of the diverse list of compounds that favor VDAC channel closure, a specific and potent inhibitor is not yet available. The lack of proven pharmacology is a major problem in the study of VDAC and its role in cellular physiology. One can only have confidence that one is indeed studying VDAC and not some other channel-former by using a combination of characteristic VDAC channel properties and a combination of inhibitors.

HYPOTHESES FOR VDAC's ROLE IN APOPTOSIS

VDAC Channel Regulation by Bcl-2 Family Proteins

There are two major models that associate the Bcl-2 family proteins with the VDAC channel. In a number of studies in yeast and mammalian cells, a direct interaction between Bcl-2 family proteins and VDAC was proposed. The first model postulates that the pro-apoptotic protein Bax directly interacts with VDAC, resulting in cytochrome *c* permeation through membranes (Shimizu *et al*., 1999, 2000a,b,c, 2001). It has been reported that experiments with liposomes and planar lipid membranes show that Bax induces a novel VDAC-containing channel that is larger than the channel observed when the proteins are used alone. It was concluded that although the channels formed by Bax and VDAC alone are unable to allow cytochrome *c* translocation, the new larger pores are permeable to cytochrome *c* (Shimizu *et al*., 2000a) (Fig. 3A).

In complete disagreement with these findings, a detailed analysis of the characteristic properties of VDAC channels isolated from mammalian mitochondria and reconstituted into planar phospholipid membranes has shown that Bax does not change the properties of VDAC channels under a variety of conditions (Rostovtseva *et al*., 2004). In healthy cells, Bax predominantly exists as a monomer in the cytosol (Hsu and Youle, 1998; Antonsson *et al*., 2000). Soluble monomeric Bax is not capable of forming channels in lipid membranes and does not trigger the release of cytochrome *c* from isolated mitochondria (Antonsson *et al*., 2000; Roucou *et al*., 2002; Polster *et al*., 2003; Rostovtseva *et al*., 2004). Channel formation and cytochrome *c* release is associated with Bax oligomerization (Antonsson *et al*., 1997, 2001; Schlesinger *et al*., 1997), which is triggered by the BH3 domain-only protein Bid after cleavage by caspase-8 into its truncated form, tBid (Wei *et al*., 2000; Eskes *et al*., 2000). Extensive testing showed that neither the monomeric, nor the oligomeric form of Bax interacts with VDAC channels (Rostovtseva *et al*., 2004). These results are consistent with the failure to detect any Bax–VDAC interaction by immunoprecipitation (except after ethanol treatment, see below) or cross-linking on cultured rat kidney proximal tubule cells (Mikhailov *et al*., 2001) and the insensitivity of the effect of Bax, expressed in yeast cells, to the knock-out of VDAC (Priault *et al*., 1999; Gross *et al*., 2000; Polcic and Forte, 2003). Indeed, the levels of Bax required for cell killing in VDAC1 knock-out yeast cells were similar to those required in wild-type cells (Polcic and Forte, 2003). The yeast results indicate that, in yeast, VDAC is not involved in any Bax-induced alteration of the permeability of the MOM and doesn't participate in the protective effects of Bcl-x_L (Priault *et al*., 1999; Polcic and Forte, 2003). A similar conclusion was reached in patch-clamp experiments performed on proteoliposomes containing MOM from a yeast strain lacking VDAC1 after expression of human Bax. The Bax-dependent mitochondrial channel, MAC, was detected without the presence of VDAC (Pavlov *et al*., 2001).

In another model (Vander Heiden *et al*., 2000, 2001), VDAC channel closure prevents the efficient exchange of ATP and ADP between the cytosol and the mitochondrial matrix. This leads to permeabilization of the outer membrane and release of proteins from the intermembrane space. This permeabilization could be through defined pathways or merely the result of the accumulation of the products of mitochondrial activity leading to swelling of the matrix space and rupture of the outer membrane. The relevant issue here is that VDAC closure leads to protein release from mitochondria and apoptosis. Experiments show that the removal of growth-factor leads to a deficiency in the exchange of adenine nucleotides that is reversible. The block is at the outer membrane and is almost certainly the result of VDAC closure. The anti-apoptotic protein Bcl-x_L restores the ATP/ADP exchange and rescues the cells (Fig. 3B). Experiments with VDAC channels reconstituted into planar phospholipid membranes demonstrated that Bcl-x_L promotes the open configuration of mammalian VDAC channels and can maintain the permeability of MOM to ATP and ADP (Vander Heiden *et al*., 2001). In accordance with this model, the

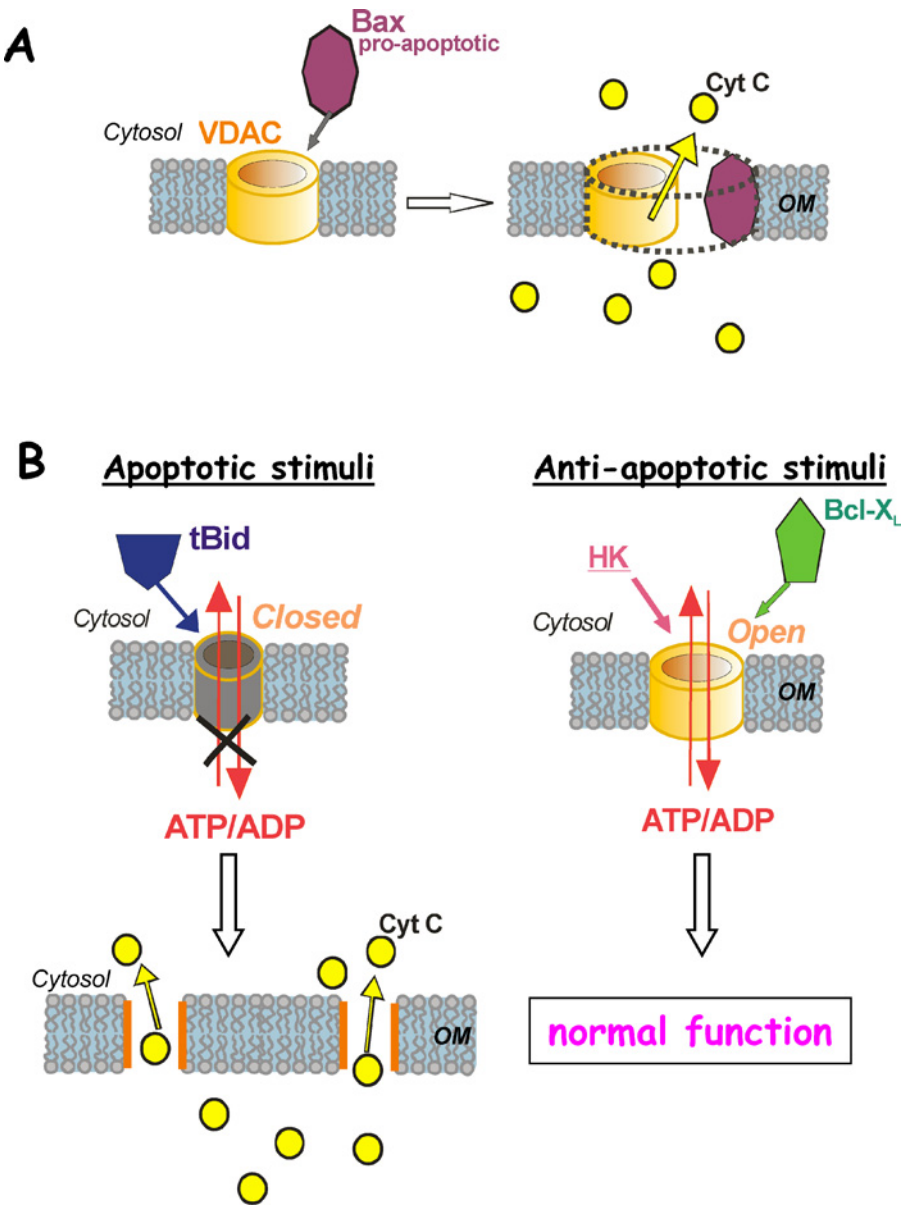

Fig. 3. Models for VDAC's role in the permeabilization of the mitochondrial outer membrane. (A) Pro-apoptotic protein Bax directly interacts with VDAC, resulting in cytochrome *c* permeation through the outer membrane. (B) VDAC closure induced by apoptotic stimuli like tBid prevent the efficient exchange of ATP and ADP between the cytosol and the mitochondrial matrix. This leads to permeabilization of the outer membrane and cytochrome *c* release through unidentified pores in the membrane. Anti-apoptotic stimuli like Bcl-x_L, hexokinase (HKI) promote the open configuration of VDAC and restore ATP/ADP exchange, which restore normal cell function.

pro-apoptotic protein, tBid, induces closure of VDAC channels (Rostovtseva *et al*., 2004). By decreasing the probability of VDAC opening, tBid could reduce the flux of adenine nucleotides across MOM. These studies (Vander Heiden *et al*., 2000, 2001; Rostovtseva *et al*., 2004) support the model in which Bcl-2 family proteins control MOM permeabilization by regulating the opening and closure of VDAC channels.

Both models account for the ability of Bcl-2 proteins to be either anti- or pro-apoptotic. However, the

mechanisms are diametrically opposite. In the first model (Fig. 3A), the pro-apoptotic protein induces the formation of large VDAC-based channels permeable to cytochrome *c*. In the second (Fig. 3B), the pro-apoptotic protein tBid promotes channel closure, whereas the anti-apoptotic protein, Bcl-x_L, helps to maintain VDAC channels in their open, functional state. It would be very difficult to specify a process that would force a β-barrel pore to stretch its entire structure so much that the diameter would increase by almost 40%, from 2.5 nm of the open state, to 3.5 nm, the size of cytochrome *c* (forgetting about the fact that much larger proteins are also released from mitochondria). There are no elecrophysiological or electron microscopical results indicating that VDAC might form a pore that is larger than in its open configuration. In contrast to the lack of support for the "extra large" VDAC channel model, there are results from other systems that support the VDAC closure model. Studies of the staurosporine-induced apoptosis in osteosarcoma cells have revealed a decrease in the permeability of the outer membrane to metabolites such as glutamate, succinate, and ADP, pointing to a closure of VDAC (Duan *et al*., 2003). (However, overexpression of Bcl-2 did not restore the permeability). There is evidence that *N. meningitides* inhibits apoptosis in host cells by inserting its channel (Por B) into the outer membrane, permeabilizing MOM, and thus restoring ATP flux (Tan *et al*., 2004). The proapoptotic oligonucleotide, G3139, favors the closure of rat liver VDAC (W. T. unpublished observations). Thus, while favoring the model in Fig. 3B, it seems clear that if VDAC closure leads to apoptosis, the process likely involves others factors that lead to the permeabilization of the outer membrane to proteins (see other reviews in this volume). The permeabilization of MOM is a very intricate process, in which several proteins and many factors are likely to play a substantial role and unspecific rupture of the outer membrane is just one of the proposed models of MOM permeabilization.

A physical interaction of VDAC (wild type, which consists predominantly of the VDAC1 isoform) with tBid or Bcl-x_L (as well as with Bax) has never been demonstrated; therefore the mechanism by which these proteins alter the gating properties of VDAC remains to be understood. It was suggested (Rostovtseva *et al*., 2004) that tBid affects VDAC indirectly through the lipid environment surrounding the protein (see discussion about effect of lipid environment on channel properties by Bezrukov, 2000). Evidence accumulates that lipids can play important roles at different stages of the apoptotic processes driven by Bcl-2 family proteins. For example, tBid was shown to change physical properties of lipid membranes by promoting negative membrane curvature (Epand *et al*., 2002) and increasing lipid transbilayer diffusion (Esposti *et al*., 2001). Recently, it was proposed (Terrones *et al*., 2004) that tBid could act in concert with Bax to form lipidic pore-like non-bilayer structures in the lipid membrane. Indirect protein–protein interactions involving membrane lipids were recently demonstrated for a stretch-activated cation channel and for a model system of gramicidin channels (Suchyna *et al*., 2004).

Role of VDAC Isoforms in Specific Interactions with Bcl-2 Family Proteins

Strong evidence exists for a non-channel mechanism by which VDAC interacts with Bcl-2 family proteins to regulate apoptosis. VDAC inhibits apoptosis by sequestering Bak. (Cheng *et al*., 2003; Chandra *et al*., 2005). However, the effect is isoforms dependent. So far, the most studies on VDAC using defined isoforms have been done on the more abundant isoform, VDAC1. Cheng and coworkers (2003) have found that a less abundant VDAC2 inhibits the activity of Bak and mitochondrial apoptosis. Bak forms complexes with VDAC2, but not with VDAC1. Cells deficient in VDAC2 exhibited enhanced Bak oligomerization and were more sensitive to apoptotic death than cells lacking VDAC1. It is worth noting that VDAC2 did not associate with Bax even in apoptotic cells. Endogenous Bak, but not Bax, was shown to efficiently coprecipitate with VDAC2. Furthermore, another research group has subsequently shown that in Bax-deficient cells there were no significant interaction between VDAC2 and Bak either with or without apoptotic stimulation (Chandra *et al*., 2005). Thus, in this model VDAC2 (but not VDAC1) interacts with Bak and inhibits Bak-activated apoptosis in a Bax-dependent way (Cheng *et al*., 2003; Chandra *et al*., 2005).

VDAC as a Part of Permeability Transition Pore

One of the mechanisms widely regarded as being responsible for MOM permeabilization and cytochrome *c* release is opening of the permeability transition pore (PTP) in the inner membrane, which allows water and solutes up to $\sim$1.5 kDa to diffuse through. Which proteins form the PTP is still an open question (see reviews Green and Kroemer, 2004; Zoratti *et al*., 2005). Most models of this pore postulate the existence of a supramolecular complex spanning both mitochondrial membranes at the contact sites between inner and outer mitochondrial membranes. Major components of this pore are thought to be the adenine nucleotide transporter (ANT) in the inner membrane and VDAC in the outer membrane (see reviews Crompton, 1999; Halestrap and Brenner, 2003;

Vyssokikh and Brdiczka, 2003). Cyclophilin D, the target of cyclosporin A (CsA), in the matrix, creatine kinase in the intermembrane space, and hexokinase (HK) associated with VDAC in the cytosol, are other proposed components of PTP. The proapoptotic protein Bax was also suggested to be a part of the assembly. For each of the putative components of the PTP, there are data published which indicate that their presence is optional and depend on experimental conditions, tissue, and cell type (see the supporting on line text to the review by Green and Kroemer, 2004; and also Halestrap and Brenner, 2003). For example, recent studies have shown that PTP can be formed without ANT (Kokoszka *et al*., 2004), which is usually regarded as a central component of PTP. An alternative model has been proposed (He and Lemasters, 2002), in which neither VDAC nor ANT plays a role but instead the pore is formed by the clustering of misfolded versions of a variety of inner membrane proteins following oxidative damage and other perturbations. The possibility of the participation of the mitochondrial protein import machinery in the permeability transition was also suggested (Zoratti *et al*., 2005). The great abundance of ANT and VDAC in the mitochondrial membranes could explain their frequent (but not exclusive) association with PTP.

Whether VDAC is a permanent component of PTP is still being debated (Halestrap and Brenner, 2003). A number of studies (Cesura *et al*., 2003; Zheng *et al*., 2004; Capano and Crompton, 2002) indicate the involvement of VDAC into PTP-mediated process of MOM permeabilization. Cesura *et al*. (2003) identified the mammalian isoform VDAC1 as a target for the PTP inhibitor, ubiquinone$_0$, using affinity labeling experiments with synthetic compound, Ro 68–3400, which inhibits PTP with potency comparable to that of CsA. In knockout VDAC1 yeast strains, the compound did not bind mitochondria but the labeling was restored after expression of yeast, but not human VDAC1. In addition, this compound failed to alter the electrophysiological properties of VDAC channels reconstituted into phospholipid bilayers (Cesura *et al*., 2003).

Zheng *et al*. (2004) reported that arsenic trioxide, As_2O_3, directly induced cytochrome *c* release from isolated mouse liver mitochondria via the PTP and that VDAC is the target of As_2O_3, responsible for the release of cytochrome *c*. In VDAC-loaded proteoliposomes, As_2O_3 was reported to induce cytochrome *c* release that was inhibited by Bcl-x_L. As_2O_3 induced both the reduction of mitochondrial membrane potential and cytochrome *c* release only in VDAC-expressing, but not in the VDAC1-deficient yeast strains. Yet, others report (Basso *et al*., 2005) that the knock-out of VDAC does not affect the permeability transition. Addition of As_2O_3, or more correctly the sodium salt of arsenious acid, to mammalian VDAC reconstituted into planar membranes causes no increase in conductance that could be construed as generating a permeability pathway for cytochrome *c* (Fig. 4).

Capano and Crompton (2002) have reported that green fluorescent protein-Bax was co-immunoprecipitated along with VDAC and ANT from extracts of cardiomyocytes following the induction of apoptosis with staurosporin. Based on these data, they proposed that Bax might target the VDAC–ANT complex at intermembrane contact sites. One difficulty with these studies is the use of the commercially-available monoclonal antibody to the *N*-terminus of human VDAC1. This antibody generated by Friedrich Thinnes, may not be entirely specific. It has been reported to label the plasma membrane (Dermietzel *et al*., 1994) despite the fact that VDAC is not located there (Yu and Forte, 1996; Linden *et al*., 1984). It has also been shown to block the conductance of a channel found in astrocyte plasma membrane while polyclonal antibodies to VDAC do not block (Dermietzel *et al*., 1994). This result was originally incorrectly interpreted as the discovery of an alternatively-spliced version of VDAC1 targeted to the plasma membrane. In fact, the same channels were observed in cells lacking VDAC1 (D. Spray, personal communication). Thus, the validity of this Bax–VDAC co-immunoprecipitation is in doubt.

If VDAC is part of PTP, then it seems logical that closure of VDAC would close the PTP and protect mitochondria from cytosolic PTP activators, like Ca^{2+}. However, closure of VDAC does not prevent of Ca^{2+} flux and indeed the closed state of VDAC has a similar molecular size cut-off as PTP (see above). Therefore, it is not clear how closure of VDAC protects against PTP opening.

The notion of a supramolecular complex is fashionable but seems unnecessary considering the large number of VDAC channels in the outer membrane and the higher permeability of VDAC than that measured for the PTP. The influx of metabolites that leads to the swelling of the matrix must flow through VDAC whether a macromolecular complex exists or not. Thus at least some of the evidence linking VDAC to the PTP could be accounted for by this requirement.

VDAC and Hexokinases

One of the proteins associated with PTP and VDAC from the cytosolic side is mitochondrial hexokinases I and II (HK). Usually the involvement of HK in apoptosis is interpreted through its ability to bind to or regulate VDAC. It

is presumed that HK binds to mitochondria at the contact sites through VDAC in the form of a HK tetramer. Determination of the molecular mass of the isolated VDAC and HK complexes suggested that HK binds to VDAC and forms tetramers (Beutner *et al*., 1996). The binding of HKII to isolated mitochondria or HKII overexpression in HeLa cells inhibits the ability of Bax to induce the release of cytochrome *c* (Pastorino *et al*., 2002; Pastorino and Hoek, 2003). The converse was also observed. Thus, it was proposed that HKII regulates cytochrome *c* release via direct interaction with VDAC. Authors suggested that HKII could compete with Bax for a VDAC binding site, or alternatively, HKII may act in an allosteric manner.

Majewski and coworkers (2004) have also found that an increased association of HK with mitochondria, enhanced by activated serine/threonine kinase (Akt), inhibits cytochrome *c* release and apoptosis. In disagreement with the interpretations of Pastorino and coworkers (above), the inhibition of apoptosis due to the interaction of HK with mitochondria did not require Bax or Bak, because it was observed in Bax- and Bak-deficient cells. Activated Akt enhanced association of HK with mitochondria. This prevented VDAC closure following a combination of growth factor withdrawal and UV treatment. VDAC closure was monitored by the accumulation of phosphocreatine in mitochondria. Prevention of VDAC closure was associated with the suppression of cytochrome *c* release. In agreement with the work of Vander Heiden *et al*. (2000, 2001), Majewski and coworkers (2004) suggested that HK dissociation from mitochondria could allow VDAC closure, which would lead to mitochondrial swelling and consequently to the cell death. The process of HK dissociation from mitochondria was shown to be independent on Ca^{2+}-induced apoptosis whether or not Bax and Bak were present. Thus, these authors concluded that VDAC closure and PTP formation represent two independent mechanisms for cytochrome *c* release.

A direct inhibitory effect of hexokinase I (HKI) on VDAC channels reconstituted into planar membranes was reported (Azoulay-Zohar *et al*., 2004). HKI induced VDAC channel closure only when it was added to one side of the bilayer from which VDAC was reconstituted indicating that HKI interacts with one surface of VDAC. The channel was reopened after addition of the HKI reaction product, glucose-6-phosphate. In addition, HKI was found to prevent Ca^{2+}-induced PTP opening in isolated mitochondria. This is the opposite of the results reported for HKII (Majewski *et al*., 2004). Overexpression of HKI in the human monocyte cell line or vascular smooth muscle cells suppressed staurosporin-induced cytochrome *c*

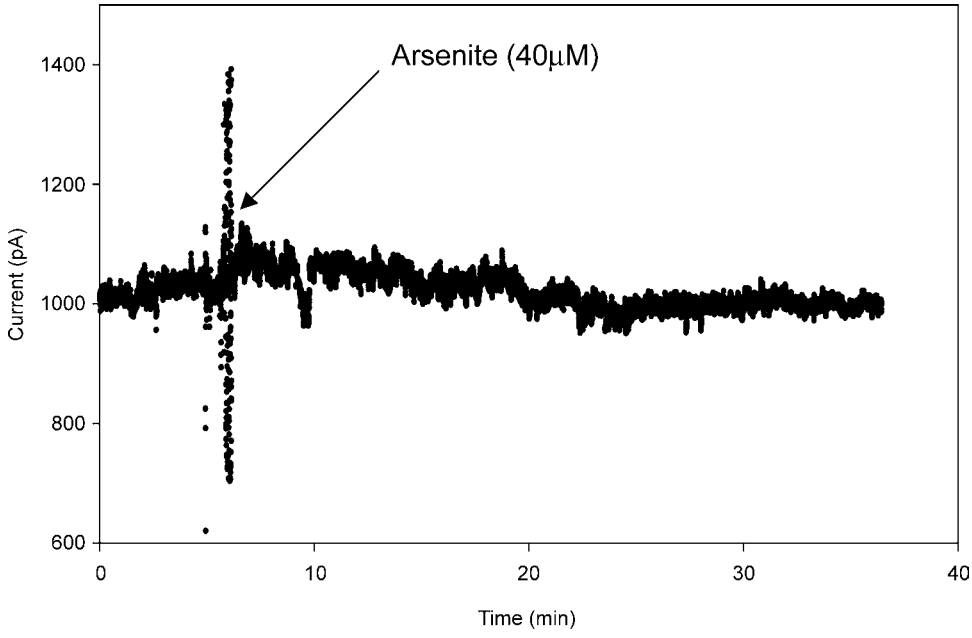

Fig. 4. Arsenic trioxide does not induce closure of VDAC channels. VDAC from rat liver mitochondria was reconstituted into planar phospholipid membranes as in Fig. 2. The *arrow* indicates the addition of arsenic trioxide (40 μM final concentration) as the sodium salt of arsenious acid to the both sides of the membrane. The medium consisted of 1.0 M NaCl buffered with 5 mM HEPES at pH 7.2. The applied potential was 10 mV. The current through a single-channel was 35 pA.

release. HKI as HKII also promoted survival using isolated mitochondria and cell cultures. These authors interpreted their results as a HKI-induced closure of VDAC channels and thus inhibition of PTP.

VDAC and ROS

Another model for MOM permeabilization in the early stages of apoptosis proposes that reactive oxygen species (ROS) convert VDAC into a protein permeation pathway. A number of studies show that ROS, produced by mitochondria, can be involved in cell death (see review Le Bras *et al*., 2005), and that Ca^{2+} accumulation by mitochondria is associated with stimulation of mitochondrial ROS production by a mechanism that is not well understood (Kowaltowski *et al*., 1995, 2001; Starkov *et al*., 2002). However, VDAC has been implicated in this process. The cytochrome *c* release induced by mitochondria-generated ROS or upon addition of superoxide was inhibited by VDAC blockers or anti-VDAC antibody (Madesh and Hajnoczky, 2001; Petrosillo *et al*., 2004), but was not sensitive to PTP inhibitors, like CsA. In isolated rat liver mitochondria, low micromolar concentrations of Ca^{2+} induced both ROS production and cytochrome *c* release. Cardiolipin peroxidation occurred at the same time and this was interpreted as a process by which cytochrome *c* was detached from its cardiolipin-mediated attachment to the inner membrane (Petrosillo *et al*., 2004). More importantly, cytochrome *c* was proposed to cross the outer membrane through VDAC. This notion was supported by experiments on VDAC-incorporated proteoliposomes. These released preloaded FITC-labeled cytochrome *c* after exposure to superoxide (generated by xanthine plus xanthine oxidase), and this release was prevented by DIDS (Madesh and Hajnoczky, 2001), a non-specific reagent reported to block VDAC (Shoshan-Barmatz *et al*., 1996). A weakness in the liposome experiments is the inclusion of detergent in the incorporation of VDAC into liposomes and the lack of detergent in the control liposomes. ROS could be acting on the detergent rather on VDAC. For example, Triton is easily oxidized. It is difficult to see how ROS can somehow make VDAC large enough to allow cytochrome *c* to cross the membrane. On the other hand, it was reported that dextran sulfate decreased superoxide (O_2^-) release from the matrix and the intermembrane space into the cytosol by inhibiting VDAC (Han *et al*., 2003). This inhibition of superoxide production was not complete, just by ~55%. We would expect such a decrease of permeability if VDAC were closed by the added dextran sulfate. Therefore, superoxide seems to be able to translocate through VDAC without dramatically changing the properties of VDAC.

How superoxide and ROS promote cytochrome *c* release and what is the role of VDAC remain an open question. Certainly, the most direct way to test this further is to probe the effect of ROS on the properties of VDAC channels reconstituted into planar phospholipid membranes.

Mitochondrial lipids and particularly cardiolipin (perhaps due to its high content of unsaturated fatty acids) are susceptible to oxidative stress. Addition of low concentrations of Ca^{2+} to mitochondria results in ROS-induced cardiolipin peroxidation (Petrocilli *et al*., 2004). ROS might target VDAC via lipid oxidation by indirectly altering the physicochemical status of the lipid environment of VDAC. The reactive aldehydes derived from mitochondria lipid peroxidation can impair the membrane function indirectly (Chen *et al*., 1995). In support to this hypothesis we have found that the lipid composition of bilayer membranes affects the gating properties of reconstituted VDAC channels (Kazemi and Rostovtseva, 2005). Addition of 4 mol% of cardiolipin to the membrane-forming lipid content was enough to induce a significant asymmetry in the voltage-gating process.

Zalk and coworkers suggested a rather ambiguous hypothetical model in which ROS induce formation of new tetrameric VDAC channel in such a way that a cytochrome *c* permeable pore is formed in between four VDAC β-barrels (Zalk *et al*., 2005) (It has been proposed that VDAC channels can form tetramers capable of allowing the transmembrane flux of cytochrome *c*.) However, it is difficult to see how the association of four cylindrical proteins would result in a pathway that is large enough. Using the shortest center-to-center distance between channels in 2-dimensional crystals, as measured by Mannella (4.3 nm) (Mannella *et al*., 1992), the diameter of the pore formed by the tetramer would only be 1.8 nm. If the center-to-center distance were increased to 6 nm (larger than reasonable from the VDAC crystal structure) then the size of the pore would only be 2.5 nm in diameter. This is far too small to allow cytochrome *c* (3.5 nm) to permeate through the membrane. An additional problem is the apolar nature of the outer wall of the channel and the energy needed to fill the apolar cavity with water. An alternative would be to visualize the wall of the channel opening up so as to form a very large β barrel consisting of the combined walls of all four channels. In principle, this is possible but the large structural change needed seems unlikely. Another aspect to consider is that reconstitution of the cross-linked structures resulted in conducting units that were smaller than the uncross-linked channels (Zalk *et al*., 2005). Thus, functional evidence of the large tetrameric channel is lacking.

A caspase-8-independent pathway of ethanol-induced apoptosis in rat hepatocytes also links VDAC with ROS (Adachi *et al*., 2004). Ethanol intoxication was shown to induce oxidative stress and apoptosis in primary rat hepatocytes accompanied with MOM permeabilization and cytochrome *c* release (Higuchi *et al*., 2001; Kurose *et al*., 1997a,b). This study reports that Bax translocation to mitochondria is oxidative stress dependent, and that the translocated Bax interacts with VDAC. VDAC only coprecipitated with Bax in ethanol-treated hepatocytes. Furthermore, microinjection of anti-VDAC antibody into the cells before ethanol treatment effectively reduced ethanol-induced apoptosis. PTP inhibitor, CsA, effectively prevented ethanol-induced mitochondria cytochrome *c* release, caspase-3 activation, and apoptosis, but failed to decrease ethanol-induced Bax translocation to mitochondria and its interaction with VDAC. The latter was interpreted as that Bax-VDAC interaction is upstream of PTP opening. Indeed, Holmuhamedov *et al*. (2005) reported that ethanol-treatment of hepatocytes leads to VDAC closure. Thus, VDAC closure and BAX translocation may set up conditions leading to PTP and/or other mechanisms of protein release from mitochondria.

CONCLUSIONS

The recognized roles for VDAC are expanding. This small, ancient and highly-conserved protein appears to be involved in many cellular processes. Some are clearly defined, such as the regulation of metabolite flux across the mitochondrial outer membrane, while others await further clarifying experiments. It is becoming clear that one must distinguish among the different VDAC isoforms because they do have specialized functions. It is also likely that some of the proposed functions for VDAC will turn out to be little more than interesting, but incorrect ideas. The location of VDAC at the crossroads of much critical flux of both matter and information makes it an important site of regulation and unraveling all its functions will take time.

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